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Climate, Physiology, and Distributions: The Role of Thermal Physiology in Biological Invasions

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Synopsis

Climate is a primary factor influencing species range dynamics, particularly for ectotherms whose body temperature is closely tied to the surrounding conditions. While range shifts of ectotherms are attributed to climate warming, the mechanism driving these shifts are not well understood. Studies in macrophysiology demonstrate that the interaction of climate with thermal physiology plays a key role in determining large-scale spatial and temporal patterns for many temperature-sensitive physiological traits. This work has revealed a clear relationship between thermal tolerance breadth and range size. However, more recent analyses of this relationship demonstrate that thermal tolerance breadth only provides a partial explanation for patterns in range size. Resting metabolism is a trait closely tied to energy balance, a key determinant of species distribution and abundance. At the whole-organism level, resting metabolism represents the energetic cost to fuel the maintenance of biological processes. Variation in this trait is related to climate and has a significant impact on how energy is allocated towards growth and reproduction. The aim of this dissertation was to understand the impact of climate on thermal physiology and species range dynamics using the Spongy moth (*Lymantria dispar dispar*) as a model system. The chapters of this dissertation addressed three primary questions: (Chapter 1) Do metabolic rate-temperature relationships (MR-T) vary across the invasive Spongy moth range and is that variation related to climate? (Chapter 2) How does MR-T vary with ontogeny? (Chapter 3) How does thermal performance at the cellular level compare to whole-organism performance? In the first chapter, we found that MR-T of third instar Spongy moth larvae showed significant variation among wild populations and this variation followed a latitudinal cline that was significantly related to climate variation. In the second chapter, larval MR-T showed significant variation across the first five instars of the larval stage. However, we found that the third instar was the most temperature-sensitive and showed the largest increase

in metabolic rate after accounting for differences in body size among instars. In the third chapter, we found that whole-organism performance of metabolic rate and growth were mismatched at temperatures beyond the thermal optimum for growth and development of Spongy moth larvae. As temperature increased, metabolic rate continued to increase while growth rapidly declined. However, mitochondrial performance matched growth performance at these supraoptimal temperatures. Furthermore, this response of MR-T, growth, and mitochondrial performance was consistent across five Spongy moth populations. These results produced three primary conclusions. First, the latitudinal variation in whole-organism MR-T among Spongy moth populations was consistent with a pattern of thermal adaptation to divergent climates and suggests that Spongy moth populations have evolved in response to climate as they have spread and expanded their invasive range. Second, the thermal physiology of Spongy moth show significant variation during ontogeny. However, while MR-T does change with instar, our findings suggest it would not alter the conclusions of chapter 1 which is based on the third instar. Finally, the mismatch of MR-T with growth and mitochondrial performance follows the predictions made by the mitochondrial efficiency hypothesis which posits that changes in mitochondrial coupling efficiency is a key mechanism for reducing ectotherm performance. These provide strong evidence for a temperature-dependent link between mitochondria and whole-organism performance. The implications of these findings for the Spongy moth invasion, species range dynamics, and ectotherm performance are discussed further in each chapter.

1 Climate-related variation of metabolic rate across the distribution of a broadly tolerant invasive forest pest

1.1 Abstract

Metabolic rate is a widely-studied physiological species trait related to energetics, climate, and geographic distributions. Hypotheses have been proposed to explain geographic and climate-related variation in metabolic rate, but evidence has been mixed due to limited sampling and scope of intraspecific studies. Successful biological invasions offer a unique opportunity to examine the evolution of intraspecific physiological variation and how it relates to climate, invasive spread, and species range limits. Using the Spongy moth (*Lymantria dispar dispar*) invasion in North America as a model system, we examined if metabolic rate variation among populations was consistent with a pattern of adaptation to local climate. Larvae were sourced from 14 populations across the invasion front and interior of the current *L. dispar* invasive range and reared at constant temperature. Routine metabolic rates (RMR) of larvae were assayed at three ecologically relevant temperatures. RMR showed significant variation among populations across all assay temperatures, but there was no difference in temperature sensitivity (*i.e.*, slope). This variation followed a positive latitudinal cline with RMR decreasing from the north to south across the invasive range, and also showed a significant relationship with local climate. Overall, the results were consistent with a pattern of thermal adaptation in larval metabolic rate by *L. dispar* in response to divergent climates during the invasion process.

1.2 Introduction

The notion that the interaction between climate and physiology is a driver of population dynamics, species distributions and abundance has a long history in ecology (*e.g.*, Grinnell, 1914; Merriam, 1894; Shelford, 1911). Examination of this interaction has revealed large-scale spatiotemporal patterns for several physiological traits across taxa (Addo-Bediako et al., 2002;

Agosta et al., 2013; Bozinovic et al., 2011; Brattstrom, 1970; Deutsch et al., 2008). Recognition of these large-scale patterns has led to the development of macrophysiology as a subdiscipline (Chown et al., 2004; Chown & Gaston, 1999). Since its inception, macrophysiological studies have produced a number of hypotheses to explain physiological variation and its relation to species distributions, climate and geography including the Climatic Variability hypothesis (Ghalambor et al., 2006; Janzen, 1967), Brattstrom hypothesis (Brattstrom, 1970; Calosi, Bilton, & Spicer, 2008; Calosi, Bilton, Spicer, et al., 2008), Metabolic Cold Adaptation hypothesis (Clarke, 1991, 1993; Krogh, 1916), and Greater Flexibility hypothesis (Baker, 1965; Chown et al., 2007).

While macrophysiology has been an important lens for understanding physiological variation across large geographic extents, much of the literature has focused on one physiological trait in particular, the *breadth of thermal tolerance* (difference between upper and lower limits of thermal tolerance). These studies show that thermal tolerance breadth generally increases towards higher latitudes where climates become more variable (Calosi et al., 2010; Deutsch et al., 2008; Sheth & Angert, 2014; Sunday et al., 2011) and that species with a broader thermal tolerance tend to have larger geographic ranges (Bonino et al., 2015; Calosi et al., 2010; Sheth & Angert, 2014; Slatyer et al., 2013). However, these studies also show that thermal tolerance limits are often mismatched with climatic extremes of species' realized latitudinal ranges for terrestrial ectotherms (Sunday et al., 2012). Given that physiological tolerance is a complex multivariate trait, it is likely that other traits, in addition to thermal tolerance breadth play an important role in determining species range dynamics (Agosta et al., 2013; Gaston, 2003; Sexton et al., 2009). Thus, macrophysiological studies that examine other physiological variables, especially those related to energetics, are needed to expand our understanding of the linkages between climate and physiology.

Studies that examine geographic variation in basal or resting metabolic rates can help fill this gap because they represent the energetic cost to fuel the maintenance of biological processes for an organism. These costs can significantly impact the energy available for growth and reproduction which will impact individual fitness and therefore species distributional limits (Dunham et al., 1989; Martinez et al., 2017). Numerous macro-scale comparative studies have shown that variation in metabolic rate can be related to climate and geography (*e.g.*, Addo-Bediako et al., 2002; Chown & Gaston, 1999; DeLong et al., 2018). From these studies, two main hypotheses have been proposed to explain this variation. The metabolic cold adaptation hypothesis (MCA) predicts that species or populations from colder climates have elevated metabolic rates relative to species or populations from warmer climates when assayed at the same temperature (Addo-Bediako et al., 2002; Clarke, 1991, 1993; DeLong et al., 2018; Krogh, 1916). The 'hotter is better' hypothesis (HIB) predicts that performance of physiological traits of warm-adapted species or populations are driven by thermodynamic effects which results in higher metabolic rate (Bennett, 1987; DeLong et al., 2018; Frazier et al., 2006; Kingsolver & Huey, 2008).

Current support for these hypotheses is mixed with studies generally finding weak evidence (*e.g.*, Addo-Bediako et al., 2002) or no evidence (*e.g.*, Gaitán-Espitia et al., 2017; Messamah et al., 2017; Oikawa et al., 2006) for either. However, there are some important limitations to consider: many of these studies (1) are based on meta-analyses using data collected with different methodologies, (2) focus on *inter*specific variation, (3) have small geographic scales, and/or (4) have limited sample sizes of individuals, populations, or taxa (Chown & Gaston, 2016; Gaston et al., 2009; Spicer & Gaston, 1999). To address these limitations, more comprehensive studies of *intra*specific variation that span several degrees of latitude and a sufficient number of populations are needed.

Biological invasions offer an especially unique opportunity to study macro-scale intraspecific physiological variation in response to climate variation and how this relates to geographic distributions. Successful invasive species are often eurythermal with a broad thermal tolerance (Kelley, 2014), a characteristic that is shared with many other wide-spread species (Calosi et al., 2010; Sheth & Angert, 2014; Slatyer et al., 2013; Sunday et al., 2011). Advantages of a eurythermal physiology include the capacity to perform over a broad array of environmental temperatures, which facilitates spread and expansion towards both relatively cold and warm climates (Kelley, 2014; Thompson et al., 2021). In addition, there is evidence that climate-related physiological and life history traits of invasive species are capable of rapid evolution as they encounter increasingly divergent climates (Hellmann et al., 2008; Moran & Alexander, 2014; Sexton et al., 2009), facilitating invasion and more generally species expansion. Together these features make biological invasions good model systems for understanding the linkages between species range dynamics, climate variation, and physiology and providing insight into how species respond to changing climates.

The Spongy moth (*Lymantria dispar dispar*, Lepidoptera: Erebidae) invasion in North America has become an important model system for understanding these linkages. Previous work has shown that a broad thermal tolerance combined with a broad diversity of potential food plants has allowed *L. dispar* to spread rapidly across 11 degrees of latitude in approximately 150 years and provides strong evidence that this spread is related to climate (Thompson et al., 2021). At the northern end of their range, cold temperatures limit expansion via winter mortality and short growing seasons with fewer degree days for development (Denlinger et al., 1992; Gray, 2004; Madrid & Stewart, 1981). At the southern end of their range, growth and development of larvae appears less constrained by temperature, but their spread south may be limited by insufficient cold temperatures to complete diapause (Gray, 2004). More recent work suggests that expansion at the southern range has stalled and/or contracted possibly due to

prolonged exposure of larvae to supraoptimal temperatures (Tobin et al., 2014). This is consistent with additional work that shows supraoptimal temperatures negatively impact *L. dispar* growth, development, and survival (Banahene et al., 2018; Faske et al., 2019; Thompson et al., 2017). Together these studies establish that climate variation and thermal physiology have played a significant role in *L. dispar* range dynamics.

The aim of our study was to explore the influence of divergent climates on physiological trait variation across the geographic range of an eurythermal invasive species and to determine if that variation is consistent with a pattern of adaptation to local climates. In this study, we addressed the following questions: (1) do the energetics (whole-organism metabolic rate) of *L. dispar* vary across its invasive range? (2) Is this variation related to local climate? (3) How do the observed patterns compare to other *intra*specific studies? We accomplished this by conducting a classic comparative experiment using *L. dispar* larvae from 14 populations across its invasive range and assayed their metabolic rate at three ecologically relevant temperatures. We then surveyed the available macrophysiology literature for *intra*specific metabolic rate studies focusing on ectotherms (Table 1.1). We hypothesized that metabolic rate of *L. dispar* has locally adapted to climate as it has expanded its invasive range over the past 150 years. If supported, we predict variation in population-level metabolic rates will be correlated with local climate and geographic variables. To our knowledge, this study represents the most comprehensive macrophysiological analysis of population-level variation in whole-organism metabolism along a latitudinal gradient for a single species (Table 1.1).

1.3 Methods

1.3.1 Study system

L. dispar is a polyphagous, univoltine, defoliating forest pest that feeds on more than 300 tree species (Liebhold et al., 1995). It was introduced from France to Medford, MA in 1869 and has since spread over 900,000 km^2 (Grayson & Johnson, 2018; Tobin et al., 2012). The current

range of *L. dispar* encompasses eastern Canada, south into southern Virginia and northern North Carolina, and westward into Wisconsin and Minnesota (Fig.1.1). Due to efforts over the past 34 years by state and federal agencies to manage the expansion of *L. dispar*, it is likely one of the most well documented biological invasions in the world (Grayson & Johnson, 2018).

1.3.2 Study populations

This study was conducted in parallel with a study on growth and development by Thompson et al. (2021) in 2018 using the same *L. dispar* populations. These populations were sourced from 14 locations across the current range (Fig. 1.1). Eleven populations were located along the invasion front from areas of active expansion and the three remaining populations were collected from interior portions of its range where it has long been established. At each source location, multiple egg masses (n = 11-60) were collected for each population. For invasion front (*i.e.*, range margin) populations, egg masses were collected from inherently low-density populations, but with densities high enough for sampling. Some populations (MA1, MA2, NY, NC1, NC2) were collected two years before the experiment (during fall of 2016) and were reared under common garden conditions for an entire generation (Thompson et al., 2021). The remaining populations were collected a year later (during fall of 2017) and, along with the previously collected populations, were overwintered in the egg stage in the same common garden conditions (see below) prior to the experiment.

1.3.3 Rearing L. dispar larvae

For each population, we used an admixture of eggs made from multiple egg masses in an effort to randomly sample the gene pool. We hatched eggs at 25°C in an environmental chamber (model I-22VL, Percival Scientific, Inc.) over a 6-week period. Once hatched, we took larvae from each population and group reared (n=10) them in 178 mL cups with 20-25 mL of artificial diet (USDA APHIS formulation). We placed these cups in a single environmental

chamber and at a constant 25°C on a 14-hr light, 10-hr dark cycle. We monitored cups daily and until larvae reached the 3rd instar, the midpoint of larval development.

1.3.4 Routine metabolic rate

We assayed routine metabolic rate (RMR) at three ecologically relevant temperatures (15°C, 25°C, and 30°C). Sample sizes ranged from 6-13 individuals per population per temperature treatment. We broadly defined our measurements as routine (as opposed to resting or basal) metabolic rates to account for absorptive processes and diel locomotor activity of larvae during respirometry trials. However, based on previous work (May et al., 2018) we are confident that individuals were minimally active during the trials. RMR of larvae was measured within 24-72 hours after transitioning to the 3rd instar. We measured RMR using a push-mode, stop-flow respirometry system (Field Metabolic System, model 2, Sable Systems International, Las Vegas, NV) connected to two 8-channel multiplexers (model RM-8, Sable Systems International). Each channel was fitted with a 35-mL chamber. We housed the system in a homemade walk-in temperature control chamber (TCC) described by Martinez and Agosta (2016).

Our respirometry protocol was similar to a previous study (May et al., 2018), with minor modifications. We isolated and held larvae without food for at least 1-hour and then weighed (0.001 g) each individual. During each respirometry trial, air inside each chamber was flushed at a flow rate of 100 mL/min for 3 minutes each hour for 4 hours. During hour 1, we allowed larvae to acclimate to the assay temperature and during hours 2-4, respirometry measurements were taken once per hour. Incurrent air was scrubbed for water vapor and CO_2 using three sequential columns (silica gel, soda lime, Drierite) before being pushed through each selected chamber. The excurrent air from the selected chamber was routed through a water vapor sensor and then scrubbed for water vapor using magnesium perchlorate. This dry air was then routed through a CO_2 sensor and subsequently scrubbed for CO_2 using Ascarite ®. Finally, the dry, CO_2 free air

was routed through an O_2 cell to measure the fractional concentration of O_2. A minimum of one chamber was kept empty to use as a baseline for each trial.

We analyzed O_2 consumption traces using ExpeData software (Sable Systems International). After drift, lag and baseline corrections, all O_2 fractional concentration values were transformed to sample volumes of O_2 (Lighton, 2008):

$$M_S O_2 = \frac{O_2 \times FR}{1 - (0.2095 - O_2)}$$

Where FR is the excurrent air flow rate corrected for standard temperature and pressure. We calculated the total sample volume of O_2 consumed by integrating the area under each $M_S O_2$ peak. We then divided the sample volume by the enclosure time to find the O_2 consumption rate ($\dot{V}O_2$ μL/hr) for each individual.

1.3.5 Statistical analysis of RMR

We analyzed RMR data using a linear mixed effects model using the 'lme4' package (Bates et al., 2015, 2021) in R version 4.1.0 (R Core Team, 2021):

$$RMR = Mass + Temperature + Population + Population \times Temp. + (1|Sample\ hour)$$

Population, assay temperature, and body mass were fixed effects. Population was treated as a fixed effect so we could to test for differences among populations and relate these differences to geographic location. Both assay temperature and body mass, which are known to have a strong influence on metabolic rate, were treated as continuous covariates. The population-by-temperature interaction was included to test for differences in the temperature sensitivity of RMR (*i.e.,* slope of RMR-temperature relationship) among populations. Sample hour was included as a random effect. We assessed statistical significance using α = 0.05.

Using this model, we computed the estimated marginal mean for each population using the 'emmeans' package in R (Lenth et al., 2021). This estimate represents the RMR for each population that has been adjusted to the same body mass across all populations (grand mean).

We then checked for significant differences in RMR among populations using multiple comparisons with a Šidák correction at each assay temperature.

1.3.6 Statistical analysis of RMR and climate

Population estimated marginal means for RMR were analyzed as a function of climate and geographical variables. Climate data were retrieved from the PRISM Climate Group (2012) using the geographic coordinates for each population. From PRISM, we obtained 30-year climate normals (1981-2010) for temperature (mean, minimum, maximum, and range) and precipitation for each season. To reduce dimensionality and quantify local climate for each population we performed a principal components analysis (PCA) with these climate and geographic data. The PCA was done using base R (R Core Team, 2021). From these principal components (PC), we used PC regression to determine if larval RMR was significantly related to climate. Finally, we calculated the temperature coefficient (Q_{10}) for larval RMR by plotting log transformed, RMRs as a function of temperature. Using the slope of this relationship we calculated the Q_{10} of RMR (Lighton 2008):

$$Q_{10} = 10^{m \times 10}$$

Where m is the slope of the RMR-assay temperature relationship. We then used PC regression in R (R Core Team, 2021) to determine if the sensitivity of RMR to temperature (Q_{10}) was related to climate.

1.3.7 Comparison of L. dispar RMR variation to other intraspecific studies

Using the Clarivate *Web of Science* database (© Copyright Clarivate 2022, all rights reserved) we surveyed the literature for studies examining population-level intraspecific variation in metabolic rate in ectotherms. Our queries used METABOLIC RATE as our primary search term with combinations of GEOGRAPHIC VARIATION, LATITUDE, CLIMATE, TEMPERATURE, COMPENSATION, METABOLIC COLD ADAPTATION, and HOTTER IS BETTER as secondary terms. Specifically, we searched for empirical studies making

intraspecific comparisons among different populations (≥ 2) that were located at different latitudes and measured standard, routine, or resting metabolic rates. Studies that did not meet these criteria were removed from our search results.

1.4 Results

1.4.1 RMR of L. dispar populations

We found that population, temperature, body mass, and the population by temperature interaction all had significant effects on RMR (Table 1.2). Overall, the linear mixed effects model explained 70% of the total variance in RMR with 49.3% explained by the fixed effects (Supporting info. Table S1.1). Effect sizes for body mass ($\omega^2 = 0.31$) and assay temperature ($\omega^2 = 0.51$) show these predictors had the largest influence on RMR (Table 1.2). Effect sizes for population ($\omega^2 = 0.08$) and its interaction with temperature ($\omega^2 = 0.07$) were relatively small. Estimated marginal means from this model show that RMR increased as assay temperature increased and that population RMR increased from southern to northern latitudes across assay temperatures (Fig. 1.2).

1.4.2 Population RMR and climate

Correlation matrices for each season show that RMR was negatively related to measures of temperature and precipitation and positively related to latitude of the population source location (supporting info. Fig. S1.1). As expected, many of these climate and geographic variables showed moderate to strong correlations with one another. The nature of these correlations was consistent across seasons. Several of these correlations also show moderate to strong relationships between RMR and local climate and geographic variables (Fig. S1.1). A PCA of these variables showed 86.5% of the cumulative variance was explained by PC1 ($\lambda = 15.26$, $\sigma^2 = 66.4\%$) and PC 2 ($\lambda = 4.62$, $\sigma^2 = 20.1\%$). A PCA biplot revealed that populations grouped by climate similarity and geographic location (Fig. 1.3). Specifically, the angle, magnitude, and direction of loadings characterizes a latitudinal gradient with cold and relatively

dry climates at the northern range margin and warm and relatively wetter climates at the southern range margin.

For PC1, regression showed RMR declined from colder to warmer climates (Fig. 1.4). While the relationship with RMR was weaker at 15°C (Adj. $R^2 = 0.18$, $p = 0.076$) (Fig. 1.4), it was strong and significant at the warmer temperatures (25°C: Adj. $R^2 = 0.71$, $p < 0.001$; 30°C: Adj. $R^2 = 0.46$, $p = 0.005$). For PC2 we found no relationship with RMR (15°C: Adj. $R^2 = -0.07$, $p = 0.725$; 25°C: Adj. $R^2 = 0.01$, $p = 0.307$) or the relationship was weak and not significant (30°C: Adj. $R^2 = 0.13$, $p = 0.115$) (Fig. S1.2). Q_{10} of RMR showed the opposite trend and increased from warmer to colder climates after removal of a single outlier (Fig. 1.5). However, this relationship was weak and not significant (Adj. $R^2 = 0.12$, $p = 0.131$).

1.4.3 Comparison of L. dispar RMR variation to other studies

Our literature search queries found 1,454 unique studies. These were mostly studies that either did not focus on ectotherms, made interspecific comparisons, or were reviews. Removal of these studies yielded a total of 63 studies that were intraspecific and measured metabolism of an ectotherm in relation to location and/or climate (Table 1.1). The latitudinal span (terrestrial: $\bar{x}$ = 11.2°, range = 4-25°; aquatic: $\bar{x}$ = 10.7°, range = 2-26°) and number of populations (terrestrial: $\bar{x}$ = 4, range = 2-9; aquatic: $\bar{x}$ = 4, range = 2-10) was similar between terrestrial and aquatic studies. There was a relatively even number of terrestrial (n = 31) and aquatic (n = 32) studies with 14 taxonomic classes (6 terrestrial, 11 aquatic, 3 shared) and 53 species (24 terrestrial, 29 aquatic), including 7 invasive species (5 terrestrial, 2 aquatic). Within each ecosystem, over half of the studies were from a single class (terrestrial: 13 insect studies with 13 species; aquatic: 14 fish studies with 13 species).

Most studies in the survey found no relationship between metabolic rate and latitude (13 terrestrial, 14 aquatic). This was followed closely by studies showing a positive relationship (10 terrestrial, 14 aquatic); studies showing a negative relationship were the least common (8

terrestrial, 4 aquatic). The majority of studies used latitude as a proxy for climate and statistical comparisons were often based on population location. We found few studies (2 terrestrial, 2 aquatic) that made direct comparisons of metabolic rate with local climate and these studies only examined one variable (temperature).

1.5 Discussion

Across the current invasive range of *L. dispar* in North America, larval metabolic rate showed the expected positive relationship with body mass and assay temperature for an ectotherm (Gillooly et al., 2001) which explained the majority of variance in the data (Table S1.1). We did detect a significant population-by-temperature interaction; however, the effect was small with no systematic differences among populations in the sensitivity (slope) of metabolic rate across the assayed temperature range. This was consistent with a non-significant relationship between Q_{10} and climate among these populations (Fig. 1.5). Population also exhibited a small, but significant effect and revealed a clinal pattern of increasing metabolic rate from south-to-north (Figure 1.2). Further examination using PCs of local climate and geographic variables revealed a significant cline in metabolic rate that persisted across assay temperatures (Fig. 1.4). These results are consistent with our hypothesis that metabolic rate variation among *L. dispar* populations follows a pattern of local adaptation in response to divergent climates. Specifically, they indicate that *L. dispar* physiology at the whole-organism level has responded to climate through adjustments in metabolic rate across ecologically-relevant temperatures (although not sensitivity to these temperatures) as their range has expanded across eastern North America in the past 150 years.

To our knowledge, this study is one of the most comprehensive macrophysiological analyses of intraspecific variation in metabolic rate and its relation to climate and invasive spread for an ectotherm to date (Table 1.1). Only a few studies that we found in our literature search (7 out of 63, 15%) focused on invasive species experiencing recent or ongoing range

expansion. Further, while our study covers similar degrees of latitude compared to the average previous study (~11), it uses significantly more populations (14 vs. mean of 4 with 27% of studies comparing only 2 populations and 32% comparing 3), covering the length of the invasion front from north to south. Finally, while all other studies used geographic location as a predictor variable (*i.e.*, a correlate of climate), we included multiple aspects of local climate (different measures of environmental temperature, precipitation, and season) for each population in our analysis. Only four previous studies that we found included similar data on local climates in their analysis (Table 1.1).

A primary objective in examining macroscale climate-related clines is determining the contribution of phenotypic plasticity and genetically-based local adaptation to these patterns. The latitudinal cline in population-level RMR we observed is consistent with predictions of the MCA hypothesis, which posits that cooler climates select for the evolution of elevated metabolic rates. Definitive evidence of MCA requires a trans-generational acclimation treatment and comparative genetic analysis among populations to determine the contribution of phenotypic plasticity to interpopulation variation in thermal physiology (Terblanche et al., 2009). Previous work by our group has tested for the presence of MCA in *L. dispar* by using two pairwise comparisons with populations from divergent climates and different latitudes (May et al., 2018). In these comparisons, there was no evidence that larval metabolic rate was elevated in northern populations (*i.e.*, no MCA). There was some evidence of an acclimation response, but it was not consistent among populations. While the current study did not test for an acclimation response, we did observe that RMRs assayed at 25°C (rearing temperature for larvae) were less variable (Fig. 1.2) and showed a stronger relationship with climate relative to the other assay temperatures (Fig. 1.4). While our results are suggestive of MCA, we cannot rule out the influence of thermal acclimation on larval metabolic rate variation among these *L. dispar* populations in producing the climate-related patterns.

Along with thermal acclimation by larvae, our results could also have been influenced by non-genetic parental effects since we were unable to rear all populations under common conditions for an entire generation prior to the study (but note that all were overwintered for the same period of time in common conditions during the egg stage prior to the experiment). Previous work has demonstrated that maternal provisioning and maternal diet can influence larval development in *L. dispar* (Rossiter, 1991; Rossiter et al., 1993), and possibly their thermal performance. Despite this, we find it unlikely that thermal acclimation of larvae during rearing and/or trans-generational parental effects explain the climate-related latitudinal cline in RMR we observed. A previous genetic analysis of *L. dispar* populations along the same latitudinal gradient has demonstrated that northern and southern populations have experienced spatially divergent selection pressures on temperature-dependent life history traits (larval development time and pupal mass) (Friedline et al., 2019), strengthening the idea that the patterns we observed in metabolic rate are genetically based. Regardless of the mechanism (evolved differences, expressed plasticity, or both) the observed climate-related latitudinal cline in RMR contributes to growing evidence that, after an initial period of rapid geographic expansion fueled by broad thermal tolerance and abundant food availability, divergent selection pressures related to climate variation have played a significant role in physiological and life history adaptation (see below) as *L. dispar* has spread across the landscape of North America from its point of introduction in 1869.

The climate-related differences in larval metabolic rate found among the populations in this study have also been observed for other *L. dispar* traits. For example, thermal reaction norms show survival decreases and pupal mass increases from north-to-south in these same populations (Thompson et al., 2021). Larger body size in the south could be related to season length, with longer growing seasons supporting longer development times (Friedline et al., 2019). However, the opposing trend with survival suggests there is a climate-related

evolutionary trade-off between body size and survival in this system (Thompson et al., 2021). One common explanation for latitudinal or climate-related body size clines in insects is that they arise from varying levels of stress resistance to starvation or desiccation (Chown & Gaston, 1999; Cushman et al., 1993). However, this seems unlikely in this system given that *L. dispar* are generally not food limited except during outbreaks (Wittman & Aukema, 2019) and the water content of leaves is relatively high (45-75%) (Scriber & Slansky, 1981). A more plausible explanation is that larger body size leads to more efficient energy use. These same populations have an inverse relationship between larval metabolic rate and body size, meaning that larger individuals have a proportionally lower metabolic rate (Blanckenhorn et al., 2007; Kleiber, 1932; Reim et al., 2006). In the north, where there are colder climates and shorter growing seasons, higher larval metabolic rates could facilitate faster growth and maturation at a smaller body size (*i.e.*, MCA). Conversely, larger body sizes in the south may help conserve energy by reducing mass-specific larval metabolic rates in response to prolonged exposure to warmer climates with longer growing seasons.

One limitation of this study is that our conclusions about RMR are based on data from a single instar midway through larval development. Because complex life cycles can result in variable responses across developmental stages (Bowler & Terblanche, 2008; Sinclair et al., 2016; Spicer & Gaston, 1999), it's important to note that metabolic rate in *L. dispar* is expected to vary during larval development since their body mass increases by 10-30 fold. Previous work has shown that whole-organism metabolic rate generally increases with body mass and each successive larval instar (Blossman-Myer & Burggren, 2010; Callier & Nijhout, 2012). While the slope of this relationship may change throughout development, any changes in metabolic rate across different instars would likely result in a *proportional* increase or decrease in metabolic rate (Blossman-Myer & Burggren, 2010; Callier & Nijhout, 2012), which would not alter the overall trends observed in our study. It is worth noting that unpublished data from our group with

the OTIS laboratory strain of *L. dispar* confirm these patterns in RMR with instar and assay temperature and also show that temperature sensitivity of RMR is highest during the third instar (Powers *et al.* unpublished).

To obtain a more complete picture of metabolic performance in *L. dispar*, future work should examine metabolic rate variation across different stages of larval ontogeny and/or assay metabolic rate across a wider range of ecologically relevant temperatures. Such work would be very useful for predicting *L. dispar* spread in response to climate change. Additionally, while changes in whole-organism metabolic rate provide a general sense of energetic trade-offs, they offer little information on net energy available for growth, storage, and reproduction. One way to address this is by examining mitochondrial physiology to determine how metabolic substrates are being used to produce cellular energy (*i.e.*, ATP). Previous work from our group revealed that the shape and thermal optimum of mitochondrial performance was similar to the thermal performance of growth but did not match whole-organism MR-T in *Manduca sexta* larvae (Martinez et al., 2017). This mismatch between whole-organism and mitochondrial physiology may be a potential mechanism for producing intraspecific variation in RMR. If this same mismatch exists in *L. dispar*, it could help explain clinal variation in larval RMR observed in this study. Finally, one outstanding question that remains to be addressed in this system is the degree to which the trait variation expressed in the invasive range represents a conserved response from the ancestral range (Huey et al., 2005), or something evolutionarily novel. A phylogeographic study of *L. dispar* that includes an examination of thermal physiology and macro-scale, climate-related patterns in the native range along with their invasive range in Japan is a logical next step and could reveal whether the responses we observed follow a predictable pattern or if they have diverged from their ancestral range. Furthermore, it would help reveal the relative contributions of phenotypic plasticity to thermal adaptation in the rapid evolution of *L. dispar* traits.

Overall, our results support the idea that thermal adaptation of whole-organism physiology plays a key role in facilitating biological invasions and more generally species expansion. Initial success of *L. dispar* upon arrival from its native range in Europe appears to be due to an ancestral thermal physiology that was broad enough to tolerate relatively cold and warm climates, which has been characteristic of many invasive species (Kelley, 2014). This combined with the ability to feed on a broad array of tree species, many which have conspecifics in their native range, facilitated its establishment, spread, and expansion in North America (Liebhold et al., 1995). Despite their ability to perform over a broad range of climates (*i.e.*, eurythermal physiology), we are able to detect fine-scale evolutionary adjustments to their thermal physiology along the invasion front (Fig. 1.4) (and see Friedline et al., 2019; Thompson et al., 2021). This indicates that thermal adaptation along the invasion front (*i.e.*, range margin) in addition to their preexisting phenotypic plasticity has fueled their continued spread and expansion as they have encountered less favorable climates (Faske et al., 2019; Friedline et al., 2019; Thompson et al., 2017, 2021; Walter et al., 2022). However, since we have observed temperature related range contractions along the southern invasion front (Tobin et al., 2014), we may be observing the current limits of climate-related plasticity and evolvability of *L. dispar*.

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1.7 Tables

Table 1.1. Literature survey results for studies (N = 63) examining population-level intraspecific variation in metabolic rate (standard, routine, or resting) among different populations (≥ 2) located at different latitudes in ectotherms. Species in bold are invasive species. References for the studies in the table can be found in the Supporting Information.

Ecosystem	Latitudinal cline	Taxa	Species	Study	Latitudinal range (°)	Populations (n)
Terrestrial	Positive	Amphibian	***Rhinella marina***	Winwood-Smith *et al.* 2015	12	4
		Gastropod	***Cornu aspersum***	Bruning *et al.* 2013	10	3
		Insect	*Drosophila melanogaster*	Berrigan & Partridge 1997	25	6
			Glossina pallidipes	Terblanche *et al.* 2009	14	4
			Culex tarsalis	Vorhees *et al.* 2013	4	3
			Pararge aegeria	Van Dyck & Holveck 2016	5	9
		Reptile	*Sceloporus occidentalis*	Tsuji 1988a	12	2
				Tsuji 1988b	12	2
			Sceloporus undulatus	Angilletta Jr. 2001	6	2
			Agkistrodon piscivorus	Zaidan 2003	7	3
	Negative	Amphibian	*Pleurodema thaul*	Barria & Bacigalupe 2017	13	2
		Insect	*Calathus melanocephalus*	Nylund 1991		3
			Leptinotarsa decemlineata	Lehmann *et al.* 2015	16	3
			Ischnura elegans	Debecker & Stoks 2019	14	6
				Tuzun & Stoks 2022	12	6
		Malacostracan	***Porcellio laevis***	Lardies *et al.* 2004	13	4
				Lardies & Bozinovic 2006	15	5
				Lardies & Bozinovic 2008	10	3
	None	Amphibian	*Rana temporaria*	Lindgren *et al.* 2009	13	8
			Plethodon cinereus	Muñoz *et al.* 2022	5	4
		Arachnid	*Atrax sutherlandi*	Wong *et al.* 2017		6
		Gastropod	***Cornu aspersum***	Naya *et al.* 2011	10	3
				Gaitan-Espitia *et al.* 2013	10	3
				Gaitan-Espitia *et al.* 2013	10	3
				Gaitán-Espitia & Nespolo 2014	10	3
		Insect	*Cicindela longilabris*	Schultz *et al.* 1992	11	4
			Myrmica ruginodis, M. scabrinodis	Nielsen *et al.* 1999	16	5
			Bicyclus anynana	de Jong *et al.* 2010	16	2
			Melanoplus femurrubrum	Parsons & Joern 2014	17	4
			Lymantria dispar	May *et al.* 2018	10	2

		Reptile	***Anolis sagrei***	Kolbe et al. 2014	6	3
Aquatic	Positive	Bivalve	*Mytilus edulis*	Sukhotin *et al.* 2006	15	3
				Thyrring *et al.* 2015	11	3
		Branchiopod	*Daphnia magna*	Chopelet *et al.* 2008	26	3
		Fish	*Girella laevifrons*	Pulgar *et al.* 2006	10	3
			Fundulus heteroclitus	Fangue *et al.* 2009	11	2
			Fundulus notatus, *F. olivaceus*	Schaefer & Walters 2010	7	2
			Acanthochromis polyacanthus	Donelson & Munday 2012	5	2
			Fundulus heteroclitus	Healy & Schulte 2012	8	2
			Leucoraja erinacea	Di Santo 2015	2	2
			Lepomis gibbosus	Rooke *et al.* 2017	2	4
			Cynoscion nebulosus	Song *et al.* 2019	5	2
			Pimephales notatus	Beachum *et al.* 2020	13	10
		Malacostracan	***Faxonius virilis***	Tripp *et al.* 2022	6	8
		Polychaete	*Arenicola marina*	Sommer & Pörtner 2002	13	2
	Negative	Fish	*Salmo trutta*	Lahti *et al.* 2002	6	4
			Stizostedion vitreum	Galarowicz & Wahl 2003	17	8
			Leucoraja erinacea	Di Santo 2016	2	2
		Malacostracan	*Cyclograpsus cinereus*	Lardies *et al.* 2011	18	5
	None	Ascidian	*Herdmania momus*	Hatcher 1991	8	2
		Clitellate	*Enchytraeus albidus*	Fisker *et al.* 2014	25	7
		Copepod	*Tigriopus californicus*	Scheffler *et al.* 2019	12	3
		Fish	*Scartichthys viridis*	Pulgar *et al.* 2007	5	3
			Salvelinus fontinalis	Stitt *et al.* 2014	4	3
		Gastropod	*Littorina saxatilis*	Sokolova & Pörtner 2001	12	4
			Littorina saxatilis	Sokolova & Pörtner 2003	12	2
			Scurria araucana	Rodriguez-Romero *et al.* 2022	5	3
		Insect	*Sigara selecta*	Carbonell *et al.* 2017	12	2
		Malacostracan	*Gammarus pulex*	Foucreau *et al.* 2014	4	5
			Idotea baltica	Wood *et al.* 2014	5	3
			Petrolisthes violaceus	Gaitan-Espitia *et al.* 2017	22	7
			Betaeus emarginatus	Barria *et al.* 2018	20	7
		Thecostracan	*Jehlius cirratus*	Broitman *et al.* 2021	20	7

Table 1.2. ANOVA table for the linear mixed effects model. Denominator degrees of freedom were determined using Satterthwaite's method. Bolded *p* values represent significant effects at α = 0.05. The last column represents the effect size for each model term (computed as omega squared, ω^2). Bolded ω^2 represent large effect sizes for model terms.

Term	**SS**	**MSS**	**DF**	**F**	***p***	**ω^2**
Population	16666.08	1282.01	13, 1260	9.52	**< 0.001**	0.08
Mass	77207.32	77207.32	1, 1260	573.32	**< 0.001**	**0.31**
Temperature	180600.00	180600.00	1, 1260	1340.75	**< 0.001**	**0.51**
Pop. × Temp.	14144.19	1088.01	13, 1260	8.08	**< 0.001**	0.07
Residuals	169700.00	131.43	1291			

1.8 Figures

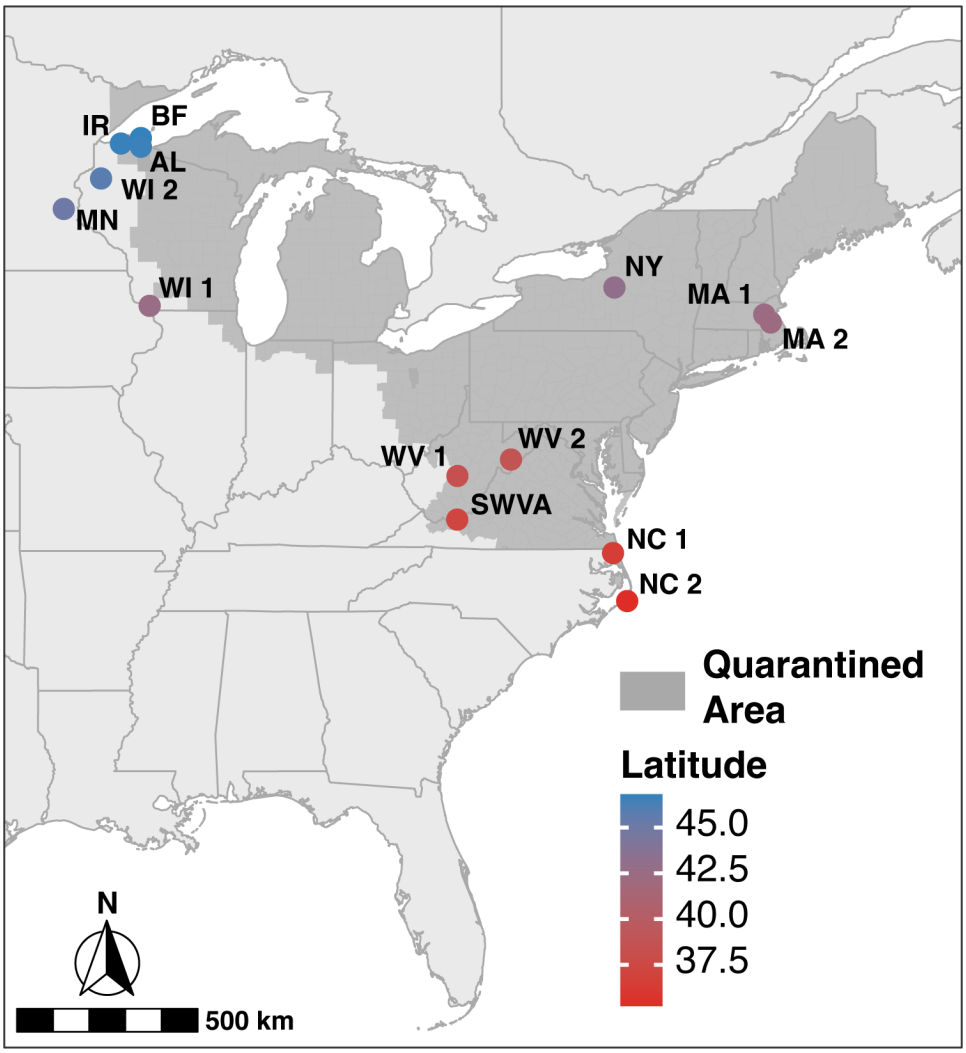

Figure 1.1. Map of *L. dispar* populations that egg masses were collected from. Dark gray shaded region represents the 2018 quarantined areas in the United States. Color scale of points represent the latitude of each population.

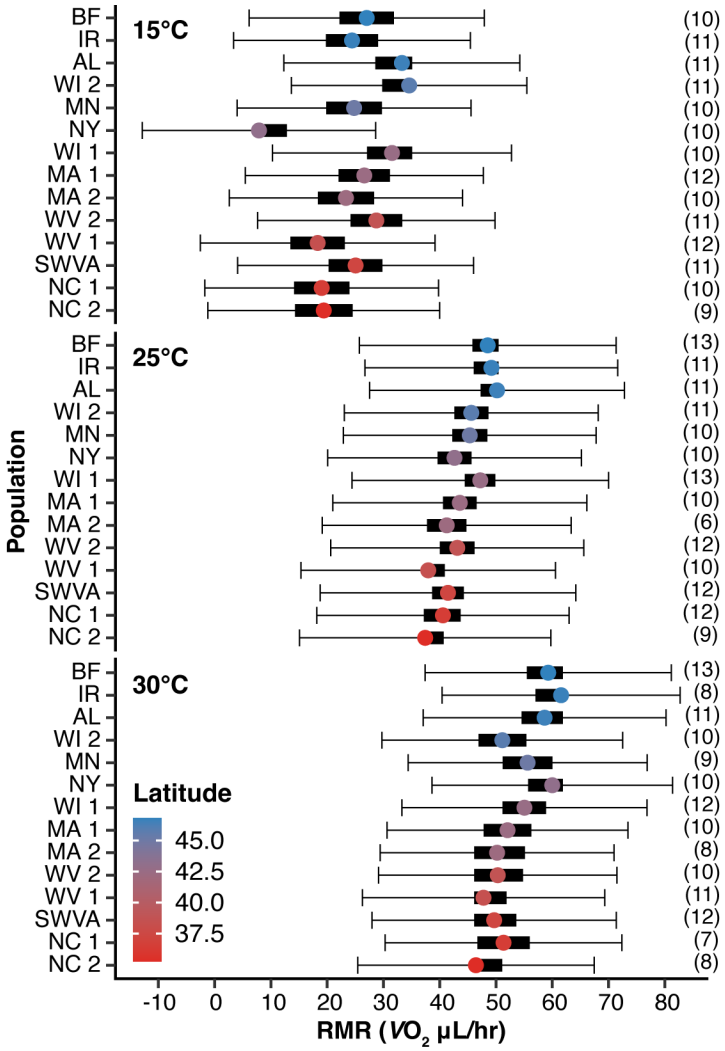

Figure 1.2. Estimated marginal means for population RMRs at each assay temperature. The color scale represents the latitude of each population. Error bars represent the 95% confidence interval. Black bars represent multiple comparisons with a Šidák correction. Estimated marginal means with black bars that do not overlap with other estimated marginal means are significantly different from one another. Numbers in parentheses represent the sample size for each population at each assay temperature.

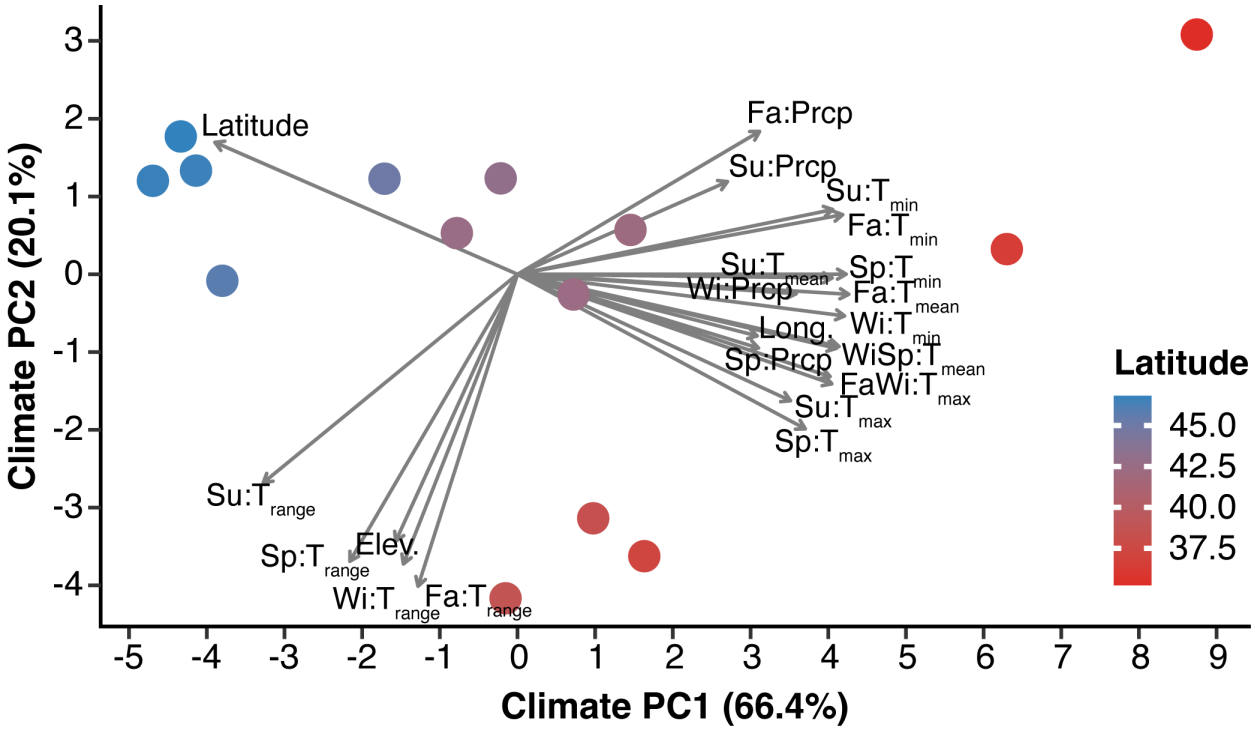

Figure 1.3. PCA biplot with scores for PC1 and PC2 each population and the amount of variance explained by PC1 and PC2. Each point represents a single population, and the color scale represents the latitude of each population. Arrows represent the direction and magnitude of loadings for climate and geographic variables. Arrows for climate variables are labeled by season (Wi = winter, Sp = spring, Su = summer, Fa = fall) and the corresponding variable (temperature [max., min., mean, or range] or precipitation [Prcp]).

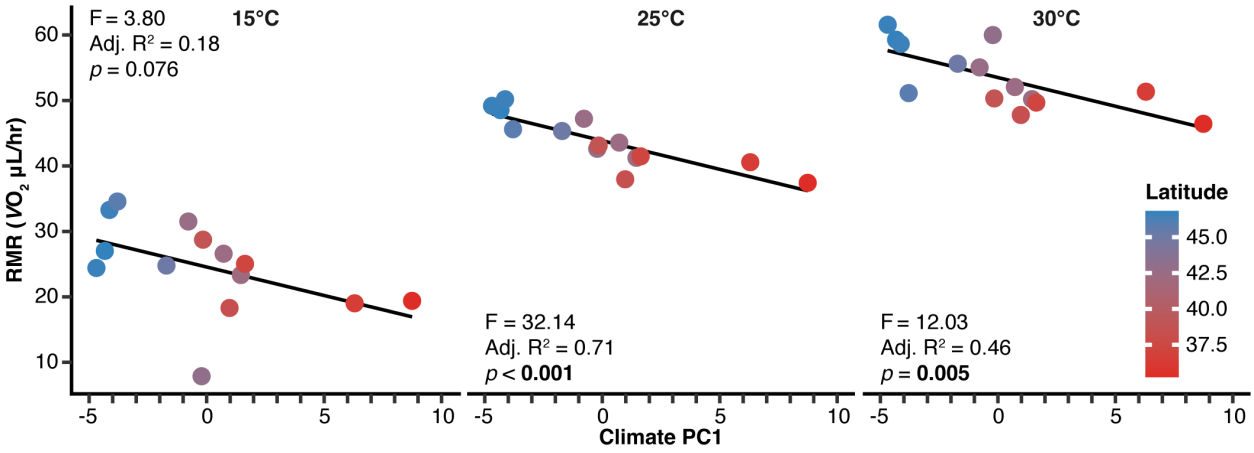

Figure 1.4. PC regression with estimated marginal means of population RMR as a function of PC1 at each assay temperature. Each point represents a single population, and the color scale represents the latitude of each population. Regression lines were fitted to each plot to determine if differences in larval RMR among populations were related to climate. These relationships were considered significant at $\alpha = 0.05$.

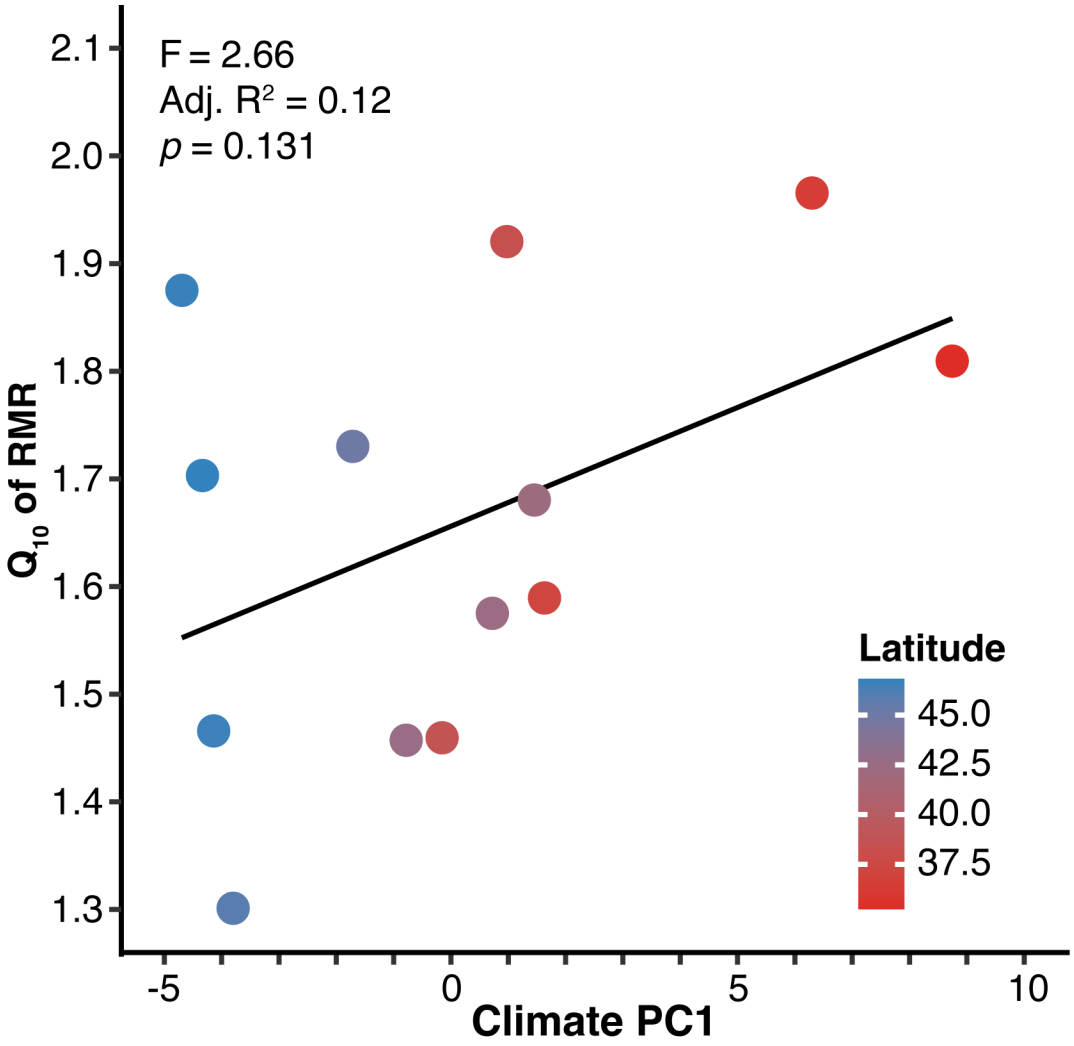

Figure 1.5. Temperature coefficients (Q_{10}) for RMR for each population plotted as a function of PC1. Each point represents a single population, and the color scale represents the latitude of each population. Linear regression was used to determine if temperature sensitivity of larval RMR was related to climate among these *L. dispar* populations.

1.9 Appendix

1.9.1 References from Table 1.1

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1.9.2 Supporting tables

Table S1.1. Summary of the fixed and random effects from the linear mixed effects model. Population terms and Pop. × Temp. terms are arranged by latitude (high to low). Bolded *p* values represent significant effects at $\alpha = 0.05$.

Parameters	**Coefficient**	**95% CI**		**t**	***p***
Fixed effects					
Intercept	-27.03	-41.15,	-12.92	-3.75	**< 0.001**
Temperature	1.70	1.33,	2.07	9.05	**< 0.001**
Mass	569.63	523.00,	616.26	23.94	**< 0.001**
Population					
BF	-6.36	-18.94,	6.22	-0.99	0.322
IR	-13.88	-26.54,	-1.21	-2.15	**0.032**
AL	6.78	-5.77,	19.34	1.06	0.290
WI 2	16.88	4.20,	29.55	2.61	**0.009**
MN	-7.18	-20.10,	5.73	-1.09	0.276
NY	-45.38	-58.37,	-32.39	-6.85	**< 0.001**
WI 1	6.84	-5.36,	19.05	1.1	0.272
MA 1	*Reference*				
MA 2	-4.65	-17.66,	8.35	-0.7	0.483
WV 2	6.02	-6.58,	18.62	0.94	0.349
WV 1	-12.31	-25.01,	0.39	-1.9	0.057
SWVA	-0.72	-13.21,	11.78	-0.11	0.911
NC 1	-14.44	-27.56,	-1.32	-2.16	**0.031**
NC 2	-8.79	-22.03,	4.46	-1.3	0.193
Pop. × Temp.					
BF × Temp.	0.45	-0.07,	0.97	1.71	0.087
IR × Temp.	0.78	0.24,	1.32	2.85	**0.004**
AL × Temp.	-0.01	-0.53,	0.52	-0.02	0.980
WI 2 × Temp.	-0.59	-1.12,	-0.06	-2.19	**0.028**
MN × Temp.	0.36	-0.18,	0.90	1.3	0.194
NY × Temp.	1.78	1.24,	2.32	6.45	**< 0.001**
WI 1 × Temp.	-0.13	-0.64,	0.38	-0.49	0.621
MA 1 × Temp.	*Reference*				
MA 2 × Temp.	0.09	-0.46,	0.64	0.33	0.740
WV 2 × Temp.	-0.26	-0.79,	0.27	-0.95	0.341
WV 1 × Temp.	0.27	-0.26,	0.80	1	0.319
SWVA × Temp.	-0.06	-0.57,	0.46	-0.21	0.834
NC 1 × Temp.	0.46	-0.10,	1.01	1.62	0.105
NC 2 × Temp.	0.11,	-0.45	0.66	0.37	0.709
Random Effects					

Groups	Variance	SD
Sample hour	92.39	9.64
Residual	134.67	11.60
No. of observations	1291	
No. of groups	3	
Marginal R^2	0.493	
Conditional R^2	0.700	

1.9.3 Supporting figures

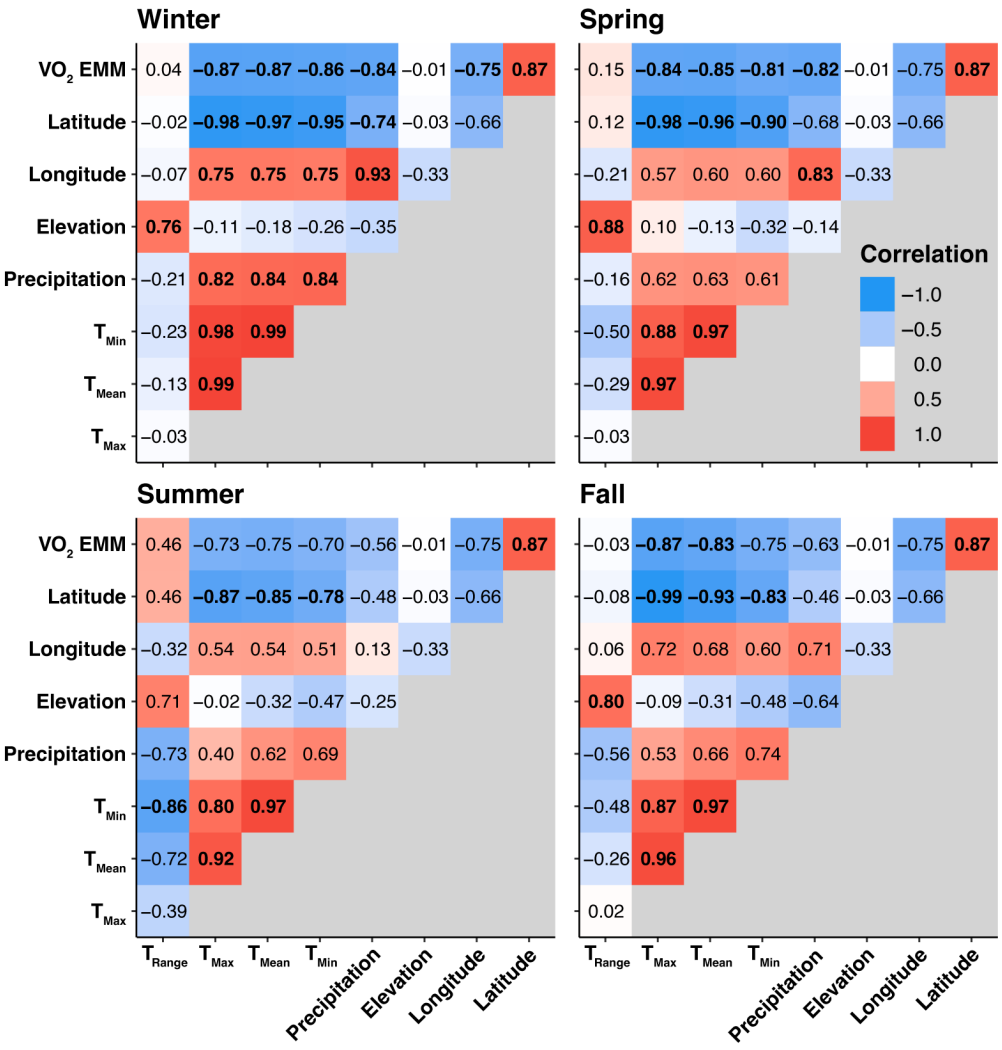

Figure S1.1. Correlation matrices with estimated marginal means for RMR (VO_2 EMM) at 25°C, climate variables, and geographic variables. Matrices are grouped by season. Significant correlations are shown in bold (α = 0.05) and *p* values were adjusted using a Holm correction. The direction and significance of correlations with RMR and predictor variables at 15°C and 30°C were similar to what is shown here.

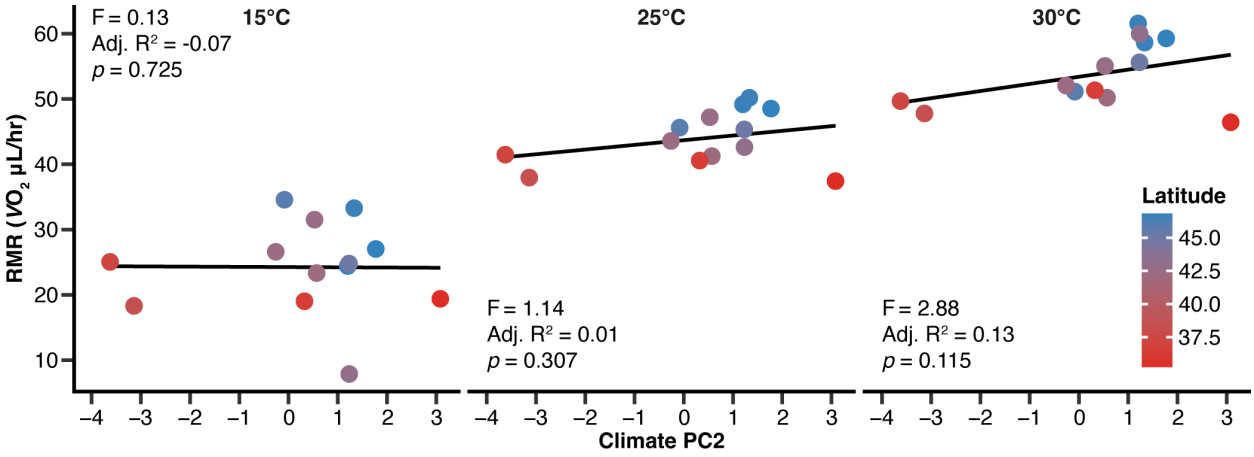

Figure S1.2. Estimated marginal means of population RMR plotted as a function of PC2 at each assay temperature. Each point represents a single population, and the color scale represents the latitude of each population. Regression lines were fitted to each plot to determine if differences in larval RMR among populations were related to climate. These relationships were considered significant at $\alpha = 0.05$.

2 Ontogenetic variation in metabolic rate-temperature relationships during larval development

2.1 Abstract

Predictive models of ectotherm responses to environmental change often rely on thermal performance data from the literature. For insects, the majority of these data focus on two traits, development rate and thermal tolerance limits, and are limited to the adult stage. Consequently, predictions based on these data generally ignore other measures of thermal performance and do not account for the role of ontogenetic variation in thermal physiology across the complex insect life cycle. Theoretical syntheses for predicting metabolic rate also make similar assumptions despite the strong influence of body size as well as temperature on metabolic rate. The aim of this study was to understand the influence of ontogenetic variation on ectotherm physiology and its potential impact on predictive modeling. To do this we examined metabolic rate-temperature (MR-T) relationships across the larval stage in a laboratory strain of the Spongy moth (*Lymantria dispar dispar*). Routine metabolic rates (RMR) of larvae were assayed at nine temperatures across the first five instars of the larval stage. After accounting for differences in body mass, larval instars showed significant variation in MR-T. Both the temperature sensitivity and allometry of RMR increased and peaked during the third instar, then declined in the fourth and fifth instar. Generally, these results show that insect thermal physiology does not remain static during larval ontogeny and suggest that ontogenetic variation should be an important consideration when modeling thermal performance.

2.2 Introduction

Understanding thermal biology has become a vital aspect of predicting the response of ectotherms to novel environments resulting from climate change. This process often involves using data available from the literature for temperature-sensitive traits to generate reaction

norms known as thermal performance curves. These thermal performance curves are then employed to estimate changes in fitness across landscapes in response to current and future climate warming. For example, Deutsch et al. (2008) used available thermal tolerance data for insects and other ectotherms to predict that climate warming would benefit temperate species, but have negative consequences for tropical species. Their global analysis laid the foundation for more studies which made similar global predictions for ectotherms (*e.g.*, Huey et al. 2009, 2012, Dillon et al. 2010, Sheldon et al. 2011, Sunday et al. 2011, Diamond et al. 2012). While this research has advanced our understanding of ectotherm responses to environmental change, the underlying models used for prediction often make extremely simplifying assumptions about ectotherm physiology. One key assumption is that physiology does not change during ontogeny (Sinclair et al. 2016). This is unlikely to be true for most organisms, especially insects with complex life cycles (Kingsolver et al. 2011, Radchuk et al. 2013, Woods 2013, Levy et al. 2015, Kingsolver and Buckley 2020). The idea of invariant physiologies and temperature dependencies through ontogeny is biologically unrealistic (Bowler and Terblanche 2008; Sinclair et al. 2016), suggesting that predictions based on this assumption may at best be overly conservative and at worst entirely erroneous resulting in significant error propagation for climate change research (Woods 2013, Levy et al. 2015, Sinclair et al. 2016).

Part of the challenge in modeling ectotherm responses stems from constraints imposed by the available data. The majority of the data on the thermal biology of insects focuses on two key aspects: development rate and thermal tolerance limits. Furthermore, the available data tends to be heavily skewed towards the adult stage (Bowler and Terblanche 2008, Radchuk et al. 2013, Kingsolver and Buckley 2020, Carter and Sheldon 2020). This bias was underscored in a review by Kingsolver and Buckley (2020) which found that 98% of insect species in data compilations regarding the upper thermal limits focus only on the adult stage. This bias towards adults is problematic for several reasons. First, the duration of the adult stage is generally

shorter than that of the larval stage. Second, a common trend observed in ontogenetic studies is that thermal performance decreases with age and in later life stages (Davison 1969, Petersen et al. 2000, Bowler and Terblanche 2008, Nielsen and Papaj 2015, Zhao et al. 2017, Banahene et al. 2018). Third, the modular nature of the insect life cycle can decouple the thermal environment and physiological responses from one life stage to the next due to exposure from different selection pressures and changes in size and mobility (Spicer and Gaston 1999, Kingsolver et al. 2011, Woods 2013). For these reasons, it is important to explore the role of ontogenetic variation in thermal physiology, including examining a wider array of thermal performance metrics to enhance modeling efforts.

One important metric of organismal performance is metabolic rate, which reflects the costs associated with maintaining biological processes thereby providing insights into an individual's energy demands (Clarke 1993, 2017, Chown and Gaston 1999, Martinez et al. 2017). At an individual level, metabolic rate can significantly influence how organisms interact with the environment affecting how energy is allocated towards growth, survival, and reproduction (Dunham et al. 1989, Brown et al. 2004, Martinez et al. 2017). At the population and landscape levels, metabolic rate can vary with geography and climate (Chown and Gaston 1999, Addo-Bediako et al. 2002, DeLong et al. 2018, Powers et al. *In review*). Furthermore, with increasing temperature variation and rising temperatures, it is expected that insects and other ectotherms will experience increased energy costs and energy demands (Deutsch et al. 2008, Dillon et al. 2010, Williams et al. 2012, Carter and Sheldon 2020).

Similar to thermal tolerance, predictive models based on metabolic rate have generally overlooked the influence of ontogenetic variation. For example, the Metabolic Theory of Ecology (MTE) (Gillooly et al. 2001, Brown et al. 2004) assumes that the scaling exponent for metabolic rate and the slope of the metabolic rate-temperature relationship (MR-T) do not change with development and age. However, intraspecific scaling of metabolic rate often deviates from the

0.75 scaling assumed by the MTE (Glazier 2005, Sears et al. 2012), and the allometry of metabolic rate can also change with ontogeny as the energy cost of growth changes with development (Blossman-Myer and Burggren 2010, Callier and Nijhout 2012). Additionally, an interspecific comparison by Gudowska et al. (2017) found differences in MR-T slopes between smaller and larger carabid beetles. While this relationship has not been tested at the intraspecific level, it is plausible that a similar relationship exists with ontogeny given the significant increase in body mass during the larval stage of insects.

Considering the importance of ontogenetic variation on ectotherm performance and the need to expand on and add to thermal performance metrics for insects, the aim of our study was to explore ontogenetic variation in MR-T. To achieve this, we examined MR-T variation across the larval stage of the Spongy moth (*Lymantria dispar dispar* (L.), Lepidoptera: Erebidae). We used *L. dispar* as our model system for two reasons. First, previous studies have highlighted the importance of thermal physiology in their invasive spread and range expansion (Thompson et al. 2021, Walter et al. 2022). Specifically, these studies show that a broad thermal tolerance has played a critical role in their initial spread and expansion. Furthermore, as range edge populations encountered more extreme climates during their expansion, divergent selection pressures along a latitudinal gradient led to rapid thermal adaptation of their physiology and life history (Friedline et al. 2019, Thompson et al. 2021). Second, recent work examining larval MR-T revealed that northern and southern populations possess a similar acclimation capacity and that MR-T variation across the *L. dispar* range follows a latitudinal cline related to climate variation (May et al. 2018, Powers et al. *In review*). However, these studies focus on a single instar midway through larval development. Consequently, any predictions about *L. dispar* range dynamics derived from this data fail to account for ontogenetic variation, similar to the MTE and other predictive models. Therefore, the utility of our prior research for predicting *L. dispar* range

dynamics will be limited until we have a better understanding of how thermal performance metrics like MR-T vary during ontogeny.

The questions we addressed in this study were: (1) Does MR-T vary during larval ontogeny? (2) What does ontogenetic variation in MR-T suggest about the generality of previous findings relating to *L. dispar* thermal physiology? (3) How do these data compare to the data reported by previous insect MR-T studies? We accomplished this by rearing *L. dispar* to different larval instars (first, second, third, fourth, or fifth). We then assayed larval metabolic rate at eight different temperatures. Using these data, we compared the response of metabolic rate to temperature and body mass among five instars within the larval stage. Given the well-established allometry of metabolic rate (*e.g.*, Kleiber 1932) and studies that have demonstrated ontogenetic variation in thermal physiology (*e.g.*, Bowler and Terblanche 2008, Woods 2013, Kingsolver and Buckley 2020) we hypothesized that the thermal physiology of *L. dispar* at the whole-organism level would change with each successive instar during larval ontogeny. We predicted that temperature sensitivity (slope) of RMR would decline towards the later larval instars and that the scaling of RMR would change with each successive instar.

2.3 Methods

2.3.1 Study system

The Spongy moth (*Lymantria dispar dispar* (L.), Lepidoptera: Erebidae) is an invasive forest pest that was introduced from France to North America (Medford, MA) in 1869. Since its introduction it has spread more than 900,000 km^2. Currently, it has a triangle shaped range that extends from northwestern Michigan, across Canada into Nova Scotia and finally down into southern Virginia and North Carolina. *L. dispar* is a polyphagous univoltine insect species. After hatching, it enters a feeding larval stage with 5 (males) or 6 (females) developmental instars and then transitions to a pupal stage. The duration of the larval stage is approximately seven weeks, and the duration of the pupal stage is approximately two weeks. The length of these

stages varies among individuals and is temperature dependent. Non-feeding adults emerge from pupa to reproduce and live for approximately two weeks. The larvae used for this study were sourced from post-diapause egg masses obtained from the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (AHPIS) Otis laboratory (Buzzards Bay, MA). This laboratory strain (OTIS) originated from egg masses collected near Blairstown, NJ and has been maintained by USDA APHIS laboratory for more than 70 generations (Keena and ODell 1994).

2.3.2 Rearing L. dispar larvae

We used an admixture of eggs made from multiple egg masses in an effort to randomly sample the OTIS strain gene pool. Eggs were warmed and hatched at room temperature. Once hatched, larvae were randomly assigned to 178 mL plastic cups with 20-25 mL of artificial diet (USDA APHIS formulation) and were group reared until individuals reached the desired instar. Smaller instars (first through third) were reared in groups of 10. Once larvae transitioned to the fourth instar they were transferred to new cups and reared in groups of five. These cups were kept at room temperature enclosed with a black tarp on a laboratory cart. Rearing temperature was monitored using a temperature logger (HOBO U23 Pro v2, Onset Computer Corporation). Temperature of the enclosure fluctuated around 22°C (±1.0), but did not go above 24°C or below 21°C (Appendix, Fig. S2.1). These temperatures were similar to the rearing temperatures used by the USDA APHIS Otis laboratory (Keena and ODell 1994). Cups were also monitored daily to check for mold, developmental stage, and to replace diet as needed.

2.3.3 Measurement of routine metabolic rate

We assayed routine metabolic rate (RMR) at eight temperatures (13°C, 18°C, 23°C, 28°C, 30°C, 32°C, 34°C, and 36°C). All instars were assayed at all eight temperatures, except for third and fifth instars which were assayed at seven temperatures, and sample sizes ranged from 3-18 individuals per instar per assay temperature (see Appendix, Table S2.2). We defined

our measurements as routine metabolic rate to account for absorptive processes and diel locomotor activity of the larvae during respirometry trials. However, previous work indicates that individuals are minimally active during respirometry. RMR of larvae was measured within 24-72 hours after transitioning to the desired instar. RMR was measured with stop-flow respirometry in push-mode using a Sable Systems International (Las Vegas, NV USA) Field Metabolic System (FMS, model 2) connected to two 8-channel multiplexers (model RM-8, Sable Systems International). Each channel was fitted with 10-mL chambers for smaller instars (first through third instars) or 50-mL chambers for larger instars (third through fifth instars). This set-up was housed in a custom-made walk-in temperature control chamber (TCC; described in Martinez and Agosta 2016).

The respirometry protocol used in this study was similar to our previous studies (May et al. 2018, Powers et al. *In review*). We isolated and held larvae without food for at least 1-hour and then weighed (0.001 g) each individual. However, the scale used to weigh individuals was not sensitive enough for first instars, so their masses were estimated from RMR-body mass relationships for each assay temperature (see 2.3.5 Data analysis). The enclosure period for individuals during respirometry trials was generally two hours for first through third instars or one hour for fourth and fifth instars with a few exceptions (Table S2.2). During the enclosure period, air inside each chamber was sampled by the FMS and flushed at a flow rate of 100 mL/min for three minutes. Respirometry trials generally lasted 16-20 hours (Table S2.2). During the first enclosure period, larvae were allowed to acclimate to the assay temperature and respirometry measurements from the succeeding enclosure periods were used for analysis. Incurrent air was scrubbed for water vapor and CO_2 using three sequential columns (silica gel, soda lime, Drierite) before being pushed through each selected chamber. The excurrent air from the selected chamber was routed to the FMS where it was pushed through a water vapor sensor and then scrubbed for water vapor using magnesium perchlorate. This dry air was then pushed

through a CO_2 sensor and subsequently scrubbed for CO_2 using Ascarite ®. Finally, the dry, CO_2 free air was pushed through an O_2 cell sensor to measure the fractional concentration of O_2. A minimum of one chamber was kept empty to use as a baseline for each trial. Temperature of the TCC was simultaneously measured during the three-minute sampling of respiratory gasses for each chamber. This was measured by a temperature probe connected to the FMS and was done to ensure that TCC temperature matched the desired assay temperature. O_2 consumption traces were analyzed using ExpeData software (Sable Systems International). After drift, lag and baseline corrections, all O_2 fractional concentration values were transformed to sample volumes of O_2:

$$M_S O_2 = \frac{O_2 \times FR}{1 - (0.2095 - O_2)}$$

Where FR is the excurrent air flow rate corrected for standard temperature and pressure. We calculated the total sample volume of O_2 consumed by integrating the area under each $M_S O_2$ peak. We then divided the sample volume by the enclosure period to find the O_2 consumption rate (VO_2 μL/hr) for each individual.

2.3.4 Literature survey of insect studies investigating metabolic rate variation

We used the Clarivate *Web of Science* database (© Copyright Clarivate 2022, all rights reserved) to survey the literature for insect studies that assayed metabolic rate (routine, resting, or standard) at three or more temperatures. The primary search terms were METABOLIC RATE TEMPERATURE CURVE and METABOLIC RATE TEMPERATURE RELATIONSHIP, and the second search term was INSECT. This search yielded 23 studies that met the search criteria. These 23 studies were then used to seed another literature search using the 'discover' tool from Inciteful.xyz (Weishuhn 2023) to find studies similar to those from the initial Web of Science search. Our combined Web of Science search and Inciteful.xyz search yielded a total of 47 studies that met our search criteria.

2.3.5 Statistical analysis

Variation in MR-T during larval ontogeny was analyzed using a linear mixed effects model (LMM) via the 'lme4' package in R:

$$RMR = Mass + Temp. + Instar + Instar \times Temp. + (1|Sample\ hour) + (1|Days\ at\ Instar)$$

Instar was treated as a fixed effect so we could test for differences in RMR during larval ontogeny. Temperature and body mass were included because of their well-known influence on metabolic rate. We included an instar-by-temperature interaction to test for differences in the temperature sensitivity of RMR (*i.e.*, slope) among instars. The temperature data used in this model was the mean temperature of the TCC while respiratory gasses were sampled during each enclosure period for a chamber. Sample hour and days at instar were included as random effects. Sample represents the repeated sampling for each individual and days at instar represents the number of days an individual was at the desired instar before measuring RMR. RMR data and body mass data were log transformed in order to meet assumptions for normality, linearity, and homogenous variances. Statistical significance for this model was assessed using α = 0.05.

Body mass of first instar larvae were estimated indirectly because our scale was not sensitive enough to measure their individual mass. This was done by fitting linear regression models to $\log_{10}$ transformed data from second through fifth instars with body mass as the response variable for each assay temperature. From these models we were able to predict first instar body masses from the RMR measurements collected at each assay temperature. Differences among instars were assessed using the 'emmeans' package in R version 4.1.0. Using this R package, we computed the estimated marginal mean (EMM) for each instar. This estimate represents the RMR for each instar that has been adjusted to the same body mass across all instars (grand mean). We tested for significant differences among instars at each assay temperature using multiple comparisons with a Šidák correction. We then tested for

differences in the temperature sensitivity of RMR by comparing MR-T slopes among instars. This included calculating the temperature coefficient (Q_{10}) of the MR-T slope for each instar. Q_{10} of RMR was determined using the following equation:

$$Q_{10}=10^{m \times 10}$$

Where m was the MR-T slope for each instar from our LMM.

Scaling of RMR to body mass for the larval stage and each instar was also analyzed using LMM:

$$RMR = Mass + Temp. + Instar + Instar \times Temp. \times Mass + (1|Sample\ hr.) + (1|Days\ at\ Instar)$$

The predictors in this LMM were treated the same as in the previous model and body mass and RMR were $\log_{10}$ transformed. We included the 3-way interaction because we were interested in how RMR-body mass slopes (*i.e.*, allometric scaling exponent) changed with each instar and temperature. We then used the 'emmeans' package in R to compute the scaling slope for each instar and to test for differences among instars. Statistical significance for the scaling of RMR was assessed using α = 0.05.

2.4 Results

2.4.1 RMR during larval ontogeny

The LMM explained 92.4% of the total variance with 87.1% explained by the fixed effects (Appendix, Table S2.3). All model terms were significant including the interaction between temperature and body mass ($p < 0.001$, Table 2.1). Effect sizes show that body mass (η^2 = 0.34) and temperature (η^2 = 0.62) had the largest influence on RMR, while effect sizes for Instar (η^2 = 0.05) and Temp. × Instar (η^2 = 0.09) were relatively small.

RMR increased as assay temperature increased (Fig. 2.1), but the MR-T relationship showed significant variation among instars when accounting for differences in body mass (Fig. 2.1 and 2.2). The MR-T relationship for first, second, and fourth instars followed typical patterns associated with body size (*i.e.*, larger instars having higher RMR) (Fig. 2.1, Table 2.2) with a

slight, but significant increase in the MR-T slope with each successive instar (Fig. 2.3). In contrast, the MR-T slope for fifth instars was significantly lower than all other instars ($p < 0.001$, Fig. 2.3) with significantly lower RMR relative to third and fourth instars at warmer temperatures (> 28°C) (Fig. 2.2, Table 2.2). The MR-T slope for third instars was significantly higher than all other instars ($p < 0.001$, Fig. 2.3) and exhibited higher RMR at warmer temperatures that were also similar to fourth instars (Fig. 2.2, Table 2.2). Q_{10} indicates that the RMR of fifth instars was relatively temperature insensitive while the RMR of third instars was the most temperature sensitive (Fig. 2.3).

2.4.2 Allometry of RMR

The LMM used to test for differences in the allometry of RMR among larval instars explained 88.9% of the total variance with 85.6% explained by the fixed effects (Table S2.4). All fixed effects and interactions were significant except for the body mass-by-temperature interaction (Table 2.3). This means the response of RMR was dependent on the body mass of each instar, and each instar's response to temperature, but the allometry of RMR within each instar did not change with assay temperature. Comparison of mass-scaling exponents showed a clear pattern with ontogeny (Table 2.4). Scaling exponents were lowest in the second instar (0.458) and then increased significantly in the third instar (0.931). After the third instar, scaling exponents decreased again in the fourth instar (0.624) and did not change significantly in the fifth instar (0.661). The scaling exponent across all instars was 0.672.

2.4.3 Literature survey of insect studies investigating metabolic rate variation

The initial literature survey using Web of Science yielded 23 studies. We found an additional 24 studies in our second search using Inciteful.xyz (Weishuhn 2023). In total our literature survey yielded 47 studies that met our search criteria. These studies were published between 1983-2021 (Table 2.5) and included 12 taxonomic orders and 65 species. However,

over half of these studies focused on coleopterans (23.4%) and hymenopterans (31.9%) (Table S2.1). We also found that within these 65 species only two were invasive.

Few studies reported MR-T data for more than one life stage (n = 5, Table 2.5) while the majority only report data for a single stage (n = 42, Table 2.5). When looking at the data reported by stage, we found 40 studies that reported data for the adult stage, 10 studies that reported data for the larval stage, and only two studies that reported data for the pupal stage (Table S2.1). The adult studies included 54 species, while studies with larva included 17 species, and studies with pupa only included two species. With the larval stage, only two studies provided data for more than one instar (Table S2.1). Finally, when reviewing the assay temperatures of these studies, we found that on average the number of assay temperatures used (n = 5-6), the minimum temperature (~12-13ºC), the maximum temperature (~33-35ºC), and the temperature range (~21-23ºC) was similar across life stages (Table S2.1) and similar to the temperatures used in this study.

2.5 Discussion

Larval metabolic rate of the *L. dispar* OTIS laboratory strain followed the expected patterns with size and temperature. RMR increased with assay temperature (Fig. 2.1, 2.2) and the temperature sensitivity of RMR varied significantly with ontogeny (Fig. 2.3). The MR-T slope increased dramatically from the first instar to the third instar where MR-T peaked and then declined rapidly from the third instar to the fifth instar. RMR also increased with larval body mass and the allometry of RMR varied significantly with instar (Table 2.4). Similar to what we observed with MR-T, scaling of RMR increased from the second to third instar where it peaked. After the third instar, the scaling of RMR decreased in the fourth instar and showed no significant change in the fifth instar. Collectively, these results demonstrate that the thermal sensitivity of metabolism and allometric scaling in *L. dispar* varies during larval ontogeny, consistent with previous work with other insect species (Table 2.5).

Our literature review yielded 47 previous studies that have examined insect MR-T for 65 species. These studies further illustrate the limitations of the available data for thermal performance metrics. Similar to data for thermal tolerance and development rate, we found the available MR-T data were highly skewed towards the adult stage with few studies investigating ontogenetic variation (Table 2.5). Also, only two invasive species have been examined including *L. dispar* (Table 2.5). On average, the number of temperatures and temperature range used in these studies were similar to those used in our study. However, it is important to note that our assay range was designed to include temperatures that we knew to be supraoptimal for other fitness-related traits. For example, previous studies from our group demonstrated that growth and survival for *L. dispar* rapidly declines with prolonged exposure to temperatures above 28ºC and is generally fatal at temperatures above 32ºC (Thompson et al. 2017, 2021, Banahene et al. 2018), prompting us to choose two temperatures beyond this point for our MR-T assays. In short, careful attention should be given to the temperature range used to assess thermal performance particularly for investigators that are interested in understanding linkages between growth, metabolism, and fitness (*e.g.*, Martinez et al. 2017).

Much of what is known about *L. dispar* thermal physiology has come from the OTIS laboratory strain. This strain has been reared in a constant temperature environment on an artificial diet for more than 70 generations to select for rapid development and reproductive capacity (Keena and ODell 1994). As a result, OTIS generally outperforms wild populations in growth and development (Grayson et al. 2015, Thompson et al. 2017). Despite this prolonged rearing under laboratory conditions, their thermal physiology still shares similarities with wild populations. Their thermal optimum for growth and development falls between 28-30ºC (Logan et al. 1991, Thompson et al. 2021), rearing temperatures exceeding 31ºC are generally lethal, and prolonged exposure to supraoptimal temperatures results in decreased survival and increased mortality (Thompson et al. 2017). While OTIS does show better survival than wild

populations when exposed to supraoptimal temperatures (Thompson et al. 2017), this may be attributed to their larger size: *L. dispar* from warmer climates tend to be larger than *L. dispar* from colder climates (Friedline et al. 2019, Thompson et al. 2021) and previous work shows larger body sizes can provide more resistance to environmental stressors like high temperatures (Davison 1969, Cushman et al. 1993, Chown and Gaston 1999, Lawhorn and Yanoviak 2022).

Our estimates of MR-T for the OTIS strain were also comparable to those reported for 14 wild populations across the *L. dispar* invasive range (Powers et al. *In review*). For example, RMR of third instar OTIS (EMM = 40.0 $\dot{V}O_2$ µL/hr) falls within the range of RMRs for wild populations (range of EMMs = 37.4-50.2 $\dot{V}O_2$ µL/hr, EMM across populations = 43.9 $\dot{V}O_2$ µL/hr), but is significantly less variable (95% CI = 36.4-43.8) relative to wild populations (95% CI = 19.9-67.8), which is not surprising given the relatively constant conditions (temperature, moisture, lighting, food, etc.) of their rearing environment over the past 70+ generations. Additionally, the Q_{10} of OTIS (2.15) was most similar to more southern populations (1.81-1.97) providing further evidence that OTIS thermal physiology is comparable to wild populations which has important implications for recent and future work. First, the observed patterns in MR-T and body size are likely present to some degree in wild populations, but further work would be helpful to confirm this. Second, after accounting for differences in body mass, the RMR in the third instar is relatively similar to later instars and potentially could be used to approximate RMR for the latter half of larval development. Third, since thermal physiology of OTIS more closely resembles more southern *L. dispar* populations it could be a useful model for understanding the thermal response of southern *L. dispar* to climate warming.

More broadly, the pattern in the temperature sensitivity we observed with RMR fits with what is known about ontogenetic changes in insect thermal physiology. Thermal performance in insects often declines with ontogeny and age (Davison 1969, Petersen et al. 2000, Bowler and Terblanche 2008, Nielsen and Papaj 2015, Zhao et al. 2017, Banahene et al. 2018), and

evidence suggests this is due to a trade-off between size (*i.e.*, mobility) and thermal tolerance (Woods 2013, Nielsen and Papaj 2015). More specifically, earlier instars are less mobile due to their smaller size. Consequently, their body temperature is coupled to the surface temperature of their environment (*e.g.*, the boundary layer on a leaf) (Woods 2013). In contrast, body temperature of later instars may become decoupled from the environments experienced by earlier instars due to their larger size (*e.g.*, if they grow out of leaf surface boundary layers). This may increase their exposure to different conditions, causing them to rely on greater mobility to thermoregulate and avoid extreme temperatures, which may be accompanied by a lower heat tolerance (Woods 2013, Nielsen and Papaj 2015). Prior work by our group has shown some evidence that later instars of the *L. dispar* OTIS strain are less heat tolerant than earlier instars (Banahene et al. 2018) and also found a similar pattern in another caterpillar species *Manduca sexta* (Agosta et al. 2018). Therefore, the ontogenetic variation in the thermal sensitivity of metabolic rate we observed may be related to managing thermal stress as thermal tolerance changes with size.

The allometry of RMR with body mass showed a pattern similar to temperature sensitivity, but with a less dramatic decline in scaling after the third instar. These results share similarities with other studies investigating this scaling relationship across the larval stage. Like other species, scaling across the larval stage differed from the scaling within individual instars, scaling exponents did not follow Kleiber's law (0.75), and mass-specific metabolic rates decreased with increasing body size (Glazier 2005, Greenlee and Harrison 2005, Blossman-Myer and Burggren 2010, Sears et al. 2012, Callier and Nijhout 2012). However, our study differed from some h previous studies that found no significant change in scaling of RMR across instars and higher scaling exponents (larval stage: 0.82-0.98, instar: 0.65-1.31) (Blossman-Myer and Burggren 2010, Sears et al. 2012, Callier and Nijhout 2012) then we measured (Table 2.4).

The differences between our study and some previous studies could be due to differences between species, but also differences in methodology. For example, Greenlee and Harrison (2005) allowed larvae to feed during respirometry trials and Sears et al. (2012) allowed larvae to feed right up to respirometry trials. If metabolic rates were elevated due to specific dynamic action, this could elevate scaling exponents. Trial length with closed-system respirometry can also influence metabolic rates. In our study, on average, the longer trials proceeded (up to 20 hours) the lower estimates of metabolic rate became (*i.e.*, there is a strong acclimation response in the data; Fig. 2.1). In prior studies, trial periods were significantly longer for smaller instars (2-3 hours) than for larger instars (15-20 minutes) (Blossman-Myer and Burggren 2010, Sears et al. 2012, Callier and Nijhout 2012). Consequently, if metabolic rate was averaged over the trial period, means for smaller instars would be artificially lowered relative to larger instars with shortened trial periods, which would influence the estimation of scaling exponents. In our study, the measurement of mass did not account for weight loss over the relatively long trial period (16-20 hours), which also could artificially lower scaling estimates.

While the mechanisms are unknown, the results from this study indicate *L. dispar* undergo qualitative and/or quantitative temperature-dependent changes in whole-organism physiology during the larval stage. Predictive models that assume the effects of body mass and temperature are independent of one another and/or assume metabolic scaling and metabolic rate temperature sensitivity are invariant (*e.g.*, Gillooly et al. 2001) run the risk of oversimplifying ectotherm physiology. To resolve this, studies attempting to model species responses should include fitness-related thermal performance metrics for both juvenile/larval and adult life stages when possible. For the larval and juvenile stage, careful consideration should be given to what age/substage(s) are used. If this is not possible, then investigators should take care to frame their predictions in the context of the focal species' age and life stage. Otherwise, studies that do not account for this ontogenetic variation will likely under or overestimate predictive model

parameters that will ultimately lead to significant error propagation for climate change research (Kingsolver et al. 2011, Radchuk et al. 2013, Woods 2013, Levy et al. 2015, Sinclair et al. 2016, Kingsolver and Buckley 2020).

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2.7 Tables

Table 2.1. ANOVA table for our linear mixed effects model. Denominator degrees of freedom were determined using Satterthwaite's method. Bolded *p* values represent significant effects at α = 0.05. Bolded η^2 represent large effect sizes for model terms.

Term	SS	MSS	DF	F	*p*	η^2
Instar	7.33	1.83	4, 5402.71	65.95	**< 0.001**	0.05
Mass	64.14	64.14	1, 4421.05	2308.11	**< 0.001**	**0.34**
Temperature	239.19	239.19	1, 5354.97	8607.71	**< 0.001**	**0.62**
Temp. × Instar	13.78	3.44	4, 5286.52	123.95	**< 0.001**	0.09

Table 2.2. Comparison of instar RMRs at each assay temperature. Contrasts among instars are ordered from least to greatest and superscript letters represent statistical comparisons. Different superscript letters represent significant differences. A Šidák correction was used to adjust for familywise error.

Assay temp. (°C)	Contrasts
13	$1^a < 2^b < 3^c < 4^d < 5^e$
18	$1^a < 2^b < 3^b < 4^d < 5^e$
23	$1^a < 2^b < 3^c < 4^d < 5^d$
28	$1^a < 2^b < 5^c < 3^c < 4^d$
30	$1^a < 2^b < 5^c < 3^d < 4^d$
32	$1^a < 2^b < 5^c < 3^d < 4^d$
34	$1^a < 2^b < 5^c < 4^d < 3^d$
36	$1^a < 2^b < 5^c < 4^d < 3^d$

Table 2.3. ANOVA table the linear mixed effects model used to test for differences in the allometry of RMR. Denominator degrees of freedom were determined using Satterthwaite's method. The effect size for model terms were computed as eta squared (η^2). Bolded *p* values represent significant effects at $\alpha = 0.05$. Bolded η^2 represent large effect sizes for model terms.

Term	**SS**	**MSS**	**DF**	**F**	***p***	**η^2**
Mass	2.454	2.454	1, 4013.1	110.835	**< 0.001**	0.03
Temperature	3.501	3.501	3, 4013.2	158.106	**< 0.001**	0.04
Instar	0.976	0.326	3, 4012.6	14.698	**< 0.001**	0.01
Mass × Temp	0.058	0.058	1, 4013.5	2.607	0.106	< 0.01
Mass × Instar	0.690	0.230	3, 4012.7	10.382	**< 0.001**	< 0.01
Temp. × Instar	0.726	0.242	3, 4013.3	10.923	**< 0.001**	< 0.01
Mass × Temp. × Instar	0.351	0.117	3, 4013.2	5.284	**0.001**	< 0.01

Table 2.4. Allometric scaling exponents for RMR averaged across assay temperature. Different lowercase letters represent significant differences between scaling exponents of larval instars. Tukey's method was used to adjust p values for familywise error from pairwise contrasts between instars.

Instar	Scaling exp.	95% CI	Contrasts
2	0.458	0.382, 0.534	a
3	0.931	0.875, 0.988	b
4	0.624	0.575, 0.674	c
5	0.661	0.609, 0.713	c
2-5	0.672	0.640, 0.703	

Table 2.5. Results from a literature search for studies that measured MR-T relationships in insects. These are studies that assayed metabolic rate (resting, routine, or standard) at three or more temperatures. There were 47 total studies that were published between 1983-2021 which cover 12 taxonomic orders and 66 different species. Species in boldface are invasive. The life stage column identifies which life stages data were collected from (L = Larva, numbers in parentheses represent the instars that were measured; P = pupa; A = adult).

				Assay temperature (°C)			
Order	***Genus species***	**Study**	**Life stage**	**n**	**Min.**	**Max.**	**Range**
Blattodea	*Aptera fusca*	Groenewald et al. 2013	A	4	15.0	30.0	15.0
	Gromphadorhina portentosa	Streicher et al. 2012	L, A	4	16.0	34.0	18.0
Coleoptera	*Abax ovalis*	Gudowska et al. 2017	A	4	6.0	21.0	15.0
	Alphitobius diaperinus	Bjørge et al. 2018	L	8	15.0	38.0	23.0
	Bothrometopus elongatus	Chown et al. 1997	A	7	2.5	30.0	27.5
	B. parvu	Chown et al. 1997	A	7	2.5	30.0	27.5
	B. randi	Chown et al. 1997	A	6	5.0	30.0	25.0
	Calathus melanoephalus	Nylund 1991	A	4	5.0	20.0	15.0
	Carabus coriaceus	Gudowska et al. 2017	A	4	6.0	21.0	15.0
	C. linnei	Gudowska et al. 2017	A	4	6.0	21.0	15.0
	Cicindela longilabris	Schultz et al. 1992	A	3	15.0	25.0	10.0
	Ectemnorhinus marioni	Chown et al. 1997	A	7	2.5	30.0	27.5
	E. similis	Chown et al. 1997	A	7	2.5	30.0	27.5
	Enochrus jesusarribasi	Pallarés et al. 2021	A	7	5.0	35.0	30.0
	Onthophagus taurus	Carter & Sheldon 2020	A, P	4	15.0	30.0	15.0
	Palirhoeus eaton	Chown et al. 1997	A	6	5.0	30.0	25.0
	Scarabaeus galenus	Davis et al. 2000	A	5	16.0	32.0	16.0
	S. gariepinus	Davis et al. 2000	A	5	16.0	32.0	16.0
	S. hippocrates	Davis et al. 2000	A	5	16.0	32.0	16.0
	S. rusticus	Davis et al. 2000	A	5	16.0	32.0	16.0
	S. spretus	Terblanche et al. 2010	A	3	15.0	25.0	10.0
	S. striatum	Davis et al. 2000	A	5	16.0	32.0	16.0
	S. westwoodi	Davis et al. 2000	A	5	16.0	32.0	16.0
	Tenebrio molitor	Bjørge et al. 2018	L	8	15.0	38.0	23.0

		Bozinovic et al. 2013	L	8	12.0	42.0	30.0
	Xylotrupes gideon	Tomlinson et al. 2014	A	3	20.0	30.0	10.0
Dictyoptera	*Blattella germanica*	Dingha et al. 2009	A	8	5.0	40.0	35.0
Diptera	*Culex pipiens*	Gray 2013	A	3	14.0	32.0	18.0
	Episyrphus balteatus	Tomlinson & Menz 2015	A	5	5.0	40.0	35.0
	Eristalis tenax	Tomlinson & Menz 2015	A	5	5.0	40.0	35.0
	Glossina morsitans centralis	Terblanche & Chown 2007	A	4	20.0	32.0	12.0
	G. morsitans morsitans	Terblanche et al. 2005	A	4	20.0	32.0	12.0
	G. pallidipes	Terblanche et al. 2009	A	4	20.0	32.0	12.0
Ephemeroptera	*Andesiops*	Shah et al. 2021	L	9	5.0	25.0	20.0
	Baetis bicaudatus	Shah et al. 2021	L	9	5.0	25.0	20.0
	B. tricaudatus	Shah et al. 2021	L	9	5.0	25.0	20.0
	Isonychia bicolor	Camp & Buchwalter 2016	L	4	15.0	24.0	9.0
Hemiptera	*Cimex lectularius*	DeVries et al. 2013	L (1-5), A	5	10.0	35.0	25.0
Hymenoptera	*Amegilla chlorocyanea*	Tomlinson et al. 2015	A	4	25.0	35.0	10.0
		Tomlinson et al. 2015	A	4	25.0	42.0	17.0
	Aphaenogaster iberica	Shik et al. 2019	A	5	15.0	32.0	17.0
	Apis mellifera	Tomlinson et al. 2015	A	4	15.0	32.0	17.0
		Tomlinson et al. 2015	A	6	15.0	42.0	27.0
	Apis mellifera carnica	Kovac et al. 2007	A	10	5.0	40.0	35.0
		Stabentheiner et al. 2003	A	7	10.0	40.0	30.0
	Cataglyphis bicolor	Lighton & Wehner 1993	A	7	15.0	45.0	30.0
	Lissopimpla exclesa	Tomlinson & Phillips 2012	A	4	12.0	40.0	28.0
	Pogonomyrmex rugosus	Lighton & Bartholomew 1988	A	8	10.0	45.0	35.0
	Polistes biglumis	Kovac et al. 2020a	A	8	5.0	45.0	40.0
	P. dominula	Kovac et al. 2017	A	3	15.0	35.0	20.0
		Kovac et al. 2020b	A	7	15.0	45.0	30.0
	P. dominulus	Käfer et al. 2015	A	9	2.9	40.3	37.4
	P. gallicus	Kovac et al. 2017	A	3	15.0	35.0	20.0
		Kovac et al. 2020b	A	7	15.0	45.0	30.0
	Solenopsis invicta	Vogt & Appel 1999	L, P, A	7	10.0	40.0	30.0
	Vespula germanica	Käfer et al. 2012	A	12	2.5	45.0	42.5
		Käfer et al. 2013	A	14	2.9	42.4	39.5
	V. vulgaris	Käfer et al. 2012	A	12	2.5	45.0	42.5
		Käfer et al. 2013	A	14	2.9	42.4	39.5
	Zaspilothynnus nigripes	Tomlinson & Phillips 2015	A	5	12.0	32.0	20.0
Isopoda	*Porcellio laevis*	Lardies et al. 2004a	A	4	5.0	25.0	20.0

		Lardies et al. 2004b	A	4	5.0	25.0	20.0
Lepidoptera	***Lymantria dispar***	May et al. 2018	L (2)	4	18.0	32.0	14.0
		May et al. 2018	L (3)	4	20.0	35.0	15.0
	Manduca sexta	Kingsolver & Woods 1997	L (5)	6	14.0	42.0	28.0
	Pringleophaga marioni	Chown et al. 2016	L	4	0.0	15.0	15.0
Orthoptera	*Acheta domesticus*	Lachenicht et al. 2010	A	5	15.0	35.0	20.0
	Hophlosphyrum griseus	Nespolo et al. 2003	A	3	7.0	27.0	20.0
	Melanoplus sanguinipes	Chappell 1983	A	5	13	41	28.0
		Rourke 2000	A	3	25.0	42.0	17.0
	Trimerotropis pallidipennis	Chappell 1983	A	9	23.0	49.0	26.0
Plecoptera	*Anacroneuria guambiana*	Shah et al. 2021	L	9	5.0	25.0	20.0
	A. rawlinsi	Shah et al. 2021	L	9	5.0	25.0	20.0
	Hesperoperla pacifica	Shah et al. 2021	L	9	5.0	25.0	20.0
Zygentoma	*Lepisma saccharina*	DeVries & Appel 2013	L, A	5	10.0	40.0	30.0
	Thermobia domestica	DeVries & Appel 2013	L, A	5	10.0	40.0	30.0

2.8 Figures

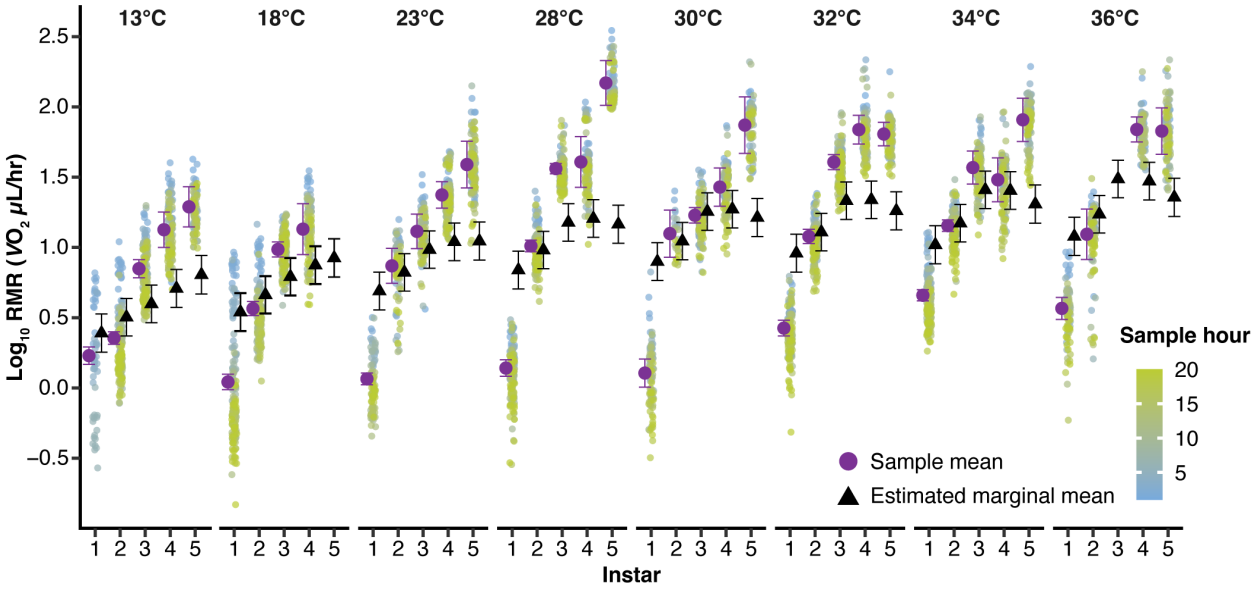

Figure 2.1. RMR measurements taken during each respirometry trial for each instar by assay temperature. Points represent repeated measurements for all individuals for each instar at each assay temperature. Points are colored by the sampling hour of the respirometry trial. Purple dots represent mean RMR over the sampling period. Black triangles represent the estimated marginal mean for RMR from the linear mixed effects model. Error bars represent the 95% confidence interval for each sample mean and estimated marginal mean.

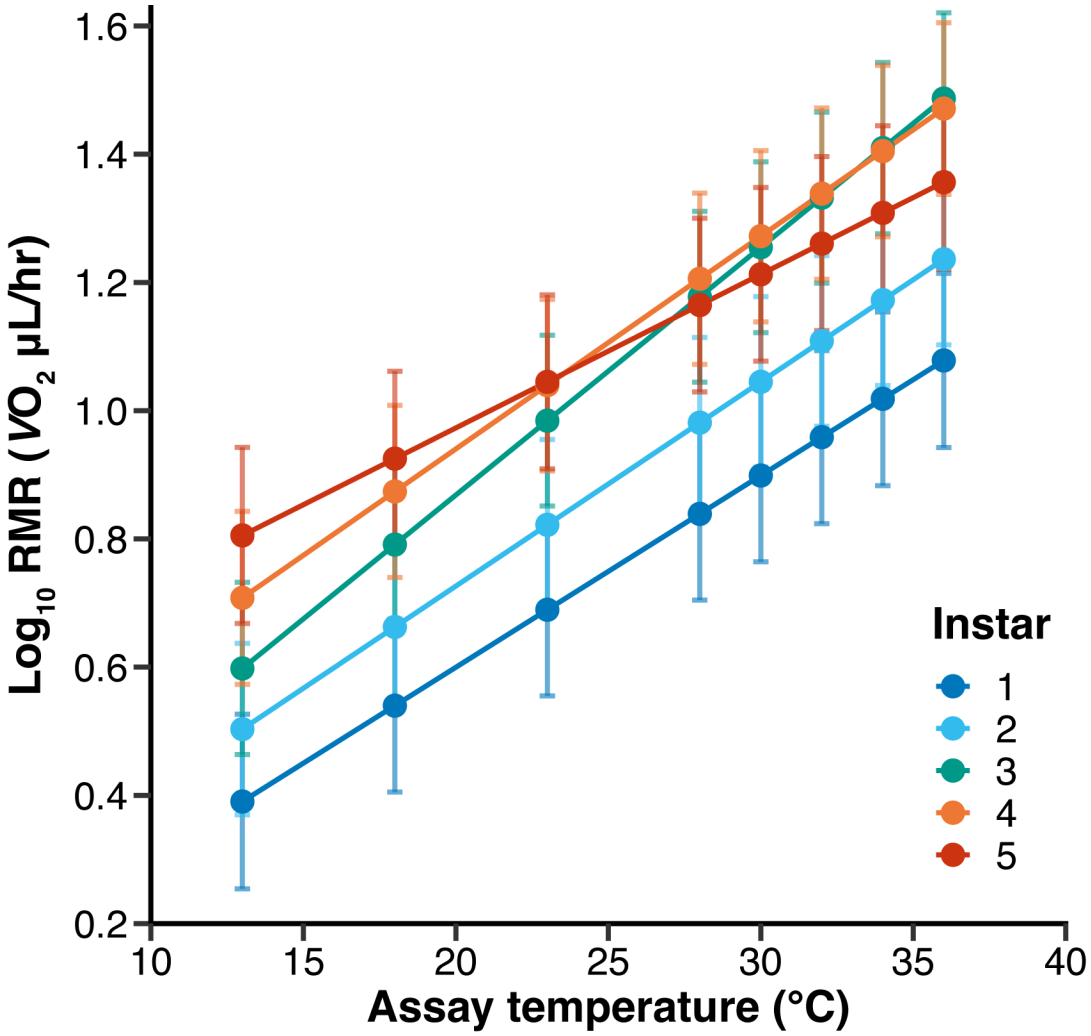

Figure 2.2. Estimated marginal means for RMR plotted as a function of assay temperature for each larval instar. Error bars represent the 95% confidence interval.

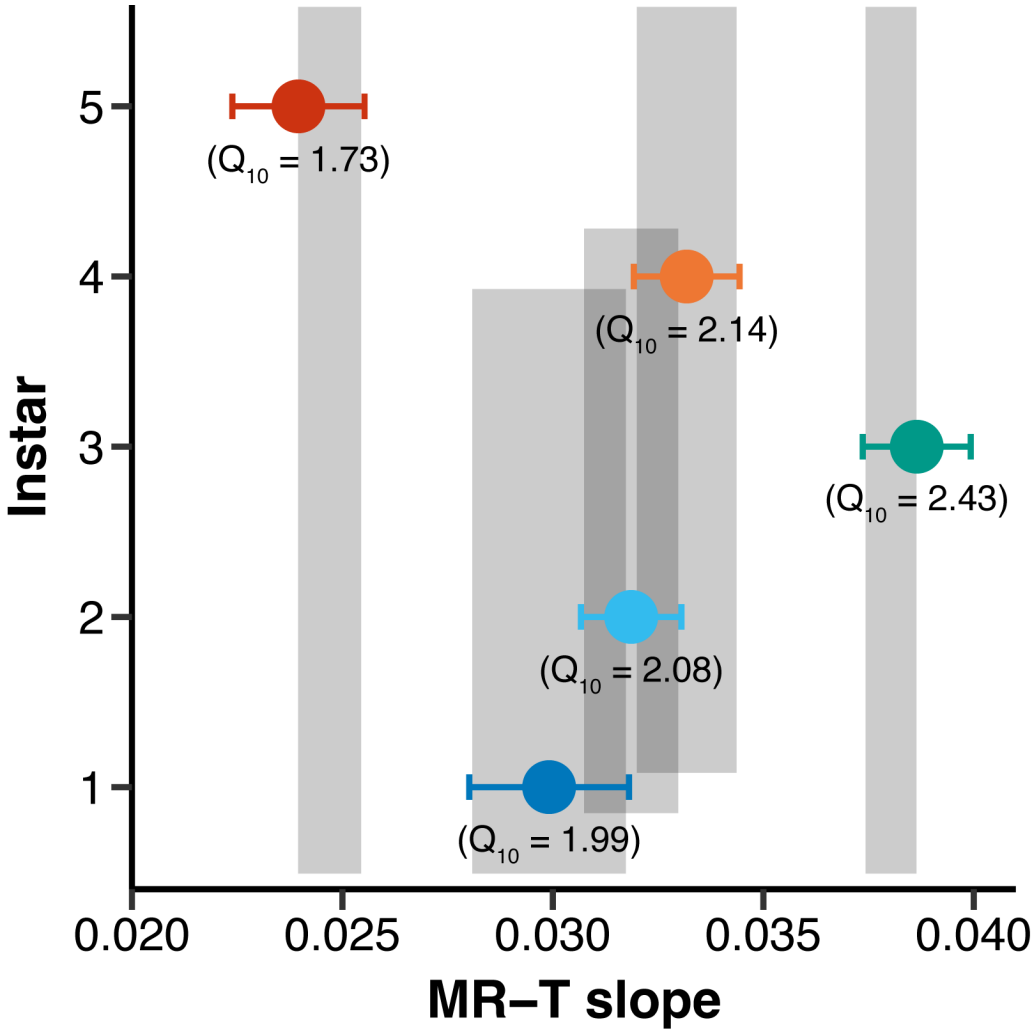

Figure 2.3. Slopes of the RMR-temperature relationship from the linear mixed effects model. Error bars represent the 95% confidence interval. Gray boxes represent multiple comparisons between slopes. Non-overlapping gray boxes along the x-axis represent significant differences between RMR-temperature slopes (α = 0.05). Numbers in parentheses represent the temperature coefficient (Q_{10}) of RMR for each larval instar.

2.9 Appendix

2.9.1 Insect MR-T references for Table 2.5

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2.9.2 Supplemental tables

Table S2.1. Summary of the insect MR-T studies by life stage.

			Summary of assay temperatures (mean ± SD)			
Life Stage	**No. of studies**	**No. of species**	**No. of temp.**	**Min. (°C)**	**Max. (°C)**	**Range (°C)**
Larva	11	17	5.5 ± 1.9	12.1 ± 5.6	33.5 ± 8.3	21.4 ± 7.3
Pupa	2	2	5.5 ± 2.1	12.5 ± 3.5	35.0 ± 7.1	22.5 ± 10.6
Adult	40	54	5.5 ± 2.5	12.1 ± 6.5	35.3 ± 7.1	23.2 ± 9.2

Table S2.2. Summary information on assay temperature, sample size, enclosure time, and trial period of respirometry trials for each larval instar.

Instar	Assay temp. (°C)	n	Enclosure period (hours)	Trial period (hours)
First	13	18	2	6
	18	18	2	20
	23	10	2	20
	28	18	2	20
	30	11	2	20
	32	18	2	20
	34	18	2	20
	36	10	2	20
Second	13	18	2	20
	18	18	2	18
	23	11	2	20
	28	18	2	20
	30	3	2	20
	32	17	2	20
	34	18	2	20
	36	10	2	20
Third	13	18	2	20
	18	18	2	20
	23	7	1	19
	28	18	2	20
	30	12	1	19
	32	18	2	20
	34	7	1	20
Fourth	13	7	1	20
	18	7	1	20
	23	10	1	20
	28	7	1	20
	30	6	1	20
	32	7	1	20
	34	7	1	20
	36	7	1	16
Fifth	13	5	1	20
	23	7	1	20
	28	5	1	20
	30	5	1	20
	32	6	1	20
	34	6	1	20
	36	6	1	20

Table S2.3. Summary of the fixed and random effects from the linear mixed effects model used to compare differences in RMR and the temperature sensitivity of RMR among instars. Bolded *p* values represent significant effects at $\alpha = 0.05$.

Parameter	**Coefficient**	**95% CI**	***t***	***p***
Fixed effects				
Intercept	1.144	0.993, 1.295	14.854	**< 0.001**
Body mass	0.663	0.637, 0.689	50.216	**< 0.001**
Temperature	0.03	0.028, 0.032	30.874	**< 0.001**
Instar				
1	*Reference*			
2	0.088	0.030, 0.145	2.995	**0.003**
3	0.094	0.033, 0.155	3.008	**0.003**
4	0.275	0.210, 0.340	8.319	**< 0.001**
5	0.492	0.420, 0.565	13.25	**< 0.001**
Temp. × Instar				
1	*Reference*			
2	0.002	-0.000, 0.004	1.68	0.093
3	0.009	0.006, 0.011	7.59	**< 0.001**
4	0.003	0.001, 0.005	2.887	**0.004**
5	-0.006	-0.008, -0.003	-4.717	**< 0.001**
Random effects				
Sample hour (n = 20)	0.07			
Days at instar (n = 3)	0.11			
Residual	0.16			
Fit				
Marginal R^2	0.871			
Conditional R^2	0.924			

Table S2.4. Summary of the fixed and random effects from the linear mixed effects model used to compare differences in the allometry of RMR among larval instars. Bolded *p* values represent significant effects at $\alpha = 0.05$.

Parameter	**Coefficient**	**95% CI**		***t***	***p***
Fixed effects					
Intercept	0.615	0.180,	1.050	2.773	**0.006**
Body mass	0.324	0.103,	0.545	2.873	**0.004**
Temperature	0.043	0.026,	0.060	4.888	**< 0.001**
Instar					
2	*Reference*				
3	1.046	0.515,	1.578	3.860	**< 0.001**
4	0.389	-0.085,	0.864	1.609	0.108
5	1.253	0.762,	1.743	5.010	**< 0.001**
Body mass × Temp.	0.005	-0.004,	0.014	1.148	0.251
Body mass × Instar					
2	*Reference*				
3	0.586	0.279,	0.892	3.750	**< 0.001**
4	-0.055	-0.333,	0.223	-0.389	0.697
5	0.517	0.186,	0.847	3.063	**0.002**
Temp. × Instar					
2	*Reference*				
3	-0.002	-0.023,	0.019	-0.215	0.830
4	0.006	-0.013,	0.025	0.634	0.526
5	-0.025	-0.044,	-0.006	-2.549	**0.011**
Temp. × Mass × Instar					
2	*Reference*				
3	-0.005	-0.017,	0.007	-0.771	0.440
4	0.008	-0.003,	0.019	1.408	0.159
5	-0.011	-0.024,	0.001	-1.823	0.068
Random effects					
Sample hour (n = 20)	0.058				
Days at instar (n = 3)	0.054				
Residual	0.149				
Fit					
Marginal R^2	0.858				
Conditional R^2	0.890				

2.9.3 Supplemental figures

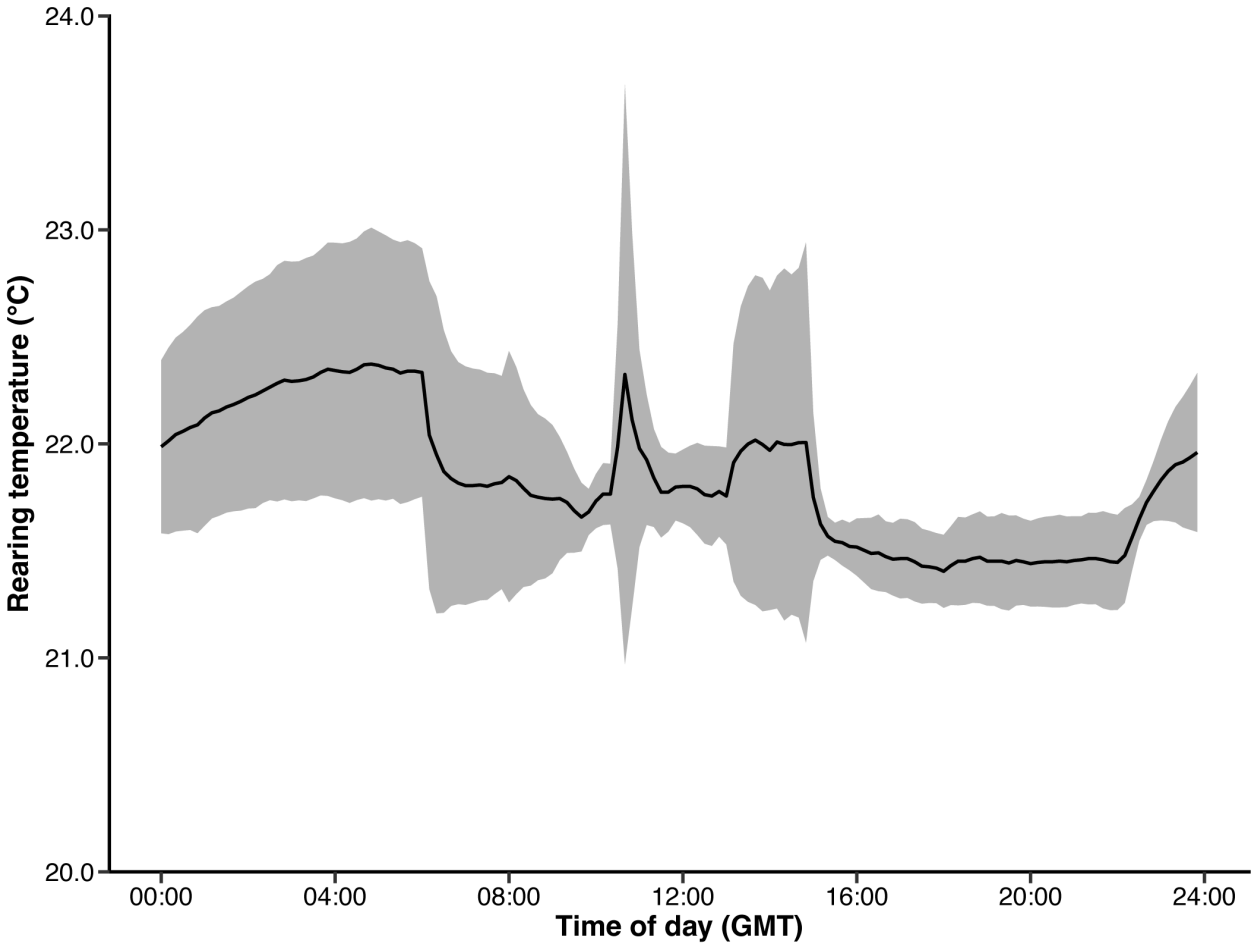

Figure S2.1. Temperature of enclosure that larvae were reared in. These data were collected over an eight-day period with a temperature logger (HOBO U23 Pro v2, Onset Computer Corporation) programmed to log temperature at 10-minute intervals. The black line represents the mean temperature, and the shaded region represents the 95% confidence interval.

3 Linking mitochondria to whole-organism thermal performance: a test of the mitochondrial efficiency hypothesis

3.1 Abstract

Metabolic rate is often considered to be the driving force of ectotherm performance due to the frequent observation that it is strongly positively correlated with whole-organism traits like growth and development. While metabolic rate can estimate overall energy demand, it does not account for how energy is being utilized. For ectotherms like insects, this is ultimately determined by ATP production via oxidative phosphorylation (OXPHOS) by mitochondria. Thus, O_2 consumption measured at the whole-organism level (*i.e.* metabolic rate) is essentially a measure of ATP demand but not all respiration is coupled to ATP production in mitochondria. Some O_2 consumption by mitochondria is uncoupled to ATP production due to proton leak (LEAK) and has been shown to account for a significant fraction of resting metabolism. The mitochondrial efficiency hypothesis (MEH) posits that reduced coupling efficiency by mitochondria (conversion of O_2 to ATP) due to proportional changes in LEAK is a key mechanism for reduced ectotherm performance at supraoptimal temperatures. Currently the generality of the MEH is limited to evidence from a single insect species. The aim of this study was to test if the MEH is operating among wild populations of another insect species, the Spongy moth (*Lymantria dispar dispar*). We measured whole-organism metabolic rate and mitochondrial respiration of *L. dispar* larvae as a function of temperature for six different populations and compared these to growth and development data collected from a previous study for the same six populations. No significant differences in whole-organism or mitochondrial metabolic rates were found among populations. Across all populations, larval growth rate and coupling efficiency of mitochondria at supraoptimal temperatures were mismatched with whole-organism metabolic rate in a manner consistent with the predictions of

the MEH. These findings expand the generality of the MEH and continue to show a direct link between mitochondrial and whole-organism performance.

3.2 Introduction

Ectotherm performance can generally be described using a thermal performance curve (TPC) which summarizes how the rates of biological processes change with temperature (Fig. 3.1) (Angilletta Jr., 2009; Huey & Stevenson, 1979). TPCs are often characterized by an asymmetrical, hump-shaped curve, where rates increase with temperature until they reach a thermal optimum (T_{opt}). At T_{opt}, the rate is maximized (r_{max}), but then rapidly declines as temperature increases above T_{opt} (*i.e.*, supraoptimal temperatures). At the whole-organism level, performance traits like growth rate (*e.g.*, Kingsolver & Woods, 1997), development rate (*e.g.*, Logan et al., 1991; Thompson et al., 2021), and locomotion (*e.g.*, Angilletta Jr. et al., 2002) conform to this shape and characteristics from these curves can be used to make predictions about survival and fitness in response to environmental change (*e.g.*, Deutsch et al., 2008). Indeed, the development and availability of various TPC models has resulted in the characterization of a wide array of performance traits across a vast number of species and taxa. But despite its pervasive use in the fields of thermal biology and ecology, the mechanism driving ectotherm TPCs is not well understood (Martinez et al., 2017; Schulte, 2015; Sinclair et al., 2016).

Theoretical syntheses like the Metabolic Theory of Ecology (MTE) posit that metabolic rate is what drives ectotherm performance because of the temperature sensitive nature of the underlying biochemical reactions that comprise aerobic metabolism and its significant positive relationship to body size (Brown et al., 2004; Glazier, 2015; Schulte, 2015). This hypothesis has also been perpetuated by the frequent observation that whole-organism traits like growth and development are positively correlated with metabolic rate (*e.g.*, Blossman-Myer & Burggren, 2010; Callier & Nijhout, 2012; Sears et al., 2012). However, a review of this hypothesis by

Glazier (2015) found no direct evidence to support this and this relationship tended to disappear after accounting for body size and phylogeny. This was further illustrated by Kingsolver and Woods (1997) who found that at supraoptimal temperatures for larval growth, whole-organism metabolic rate continued to increase while growth rate rapidly declined. Collectively, these findings demonstrate our limited understanding of the linkages between growth and metabolic rate at the whole-organism level (Martinez et al., 2017).

Part of our limited view stems from the focus on whole-organism metabolism because it does not account for how energy is being utilized to do physiological work (Clarke, 1993; Koch et al., 2021; Salin et al., 2015; Sokolova, 2023). This will ultimately be determined by the metabolic pathways responsible for producing cellular energy (*i.e.*, ATP production). For ectotherms like insects, ATP is primarily generated by oxidative phosphorylation (OXPHOS) within mitochondria (Chamberlin, 2004). Specifically, enzymes within the electron transport system (ETS) pump protons from the inner mitochondrial matrix into the mitochondrial intermembrane space as electrons are passed through the ETS. This creates an electrochemical gradient (protonmotive force, PMF) that drives the phosphorylation of ADP to ATP by ATP-synthase. O_2 is then consumed as the final electron acceptor to form water. However, it is important to note that O_2 is also consumed by endogenous proton leak (*i.e.*, uncoupled to ATP production) where protons move back across into the inner mitochondrial matrix which can account for a substantial proportion (10-40%) of resting metabolism (Brand, 2000; Sokolova, 2021). Therefore, while whole-organism metabolic rate can approximate overall energetic costs incurred by an organism, it cannot differentiate between the energy used to generate ATP and the energy consumed by proton leak (LEAK) (Clarke, 1993; Salin et al., 2015).

Changes in LEAK alter the coupling efficiency of mitochondria (*i.e.*, conversion of oxygen consumed into ATP) and are likely to be a significant source of variation at the whole-

organism level (Clarke, 1993; Koch et al., 2021; Sokolova, 2023). Given that coupling efficiency is temperature-dependent in ectotherms (Hardewig et al., 1999; Martinez et al., 2013, 2016, 2017; Weinstein & Somero, 1998) and that LEAK has been shown to significantly increase at supraoptimal temperatures (Abele et al., 2002; Chamberlin, 2004; Hardewig et al., 1999; Martinez et al., 2013, 2017), previous work by our group proposed that reduced mitochondrial efficiency, due to increased LEAK, is a key mechanism in reducing whole-organism performance for ectotherms (mitochondrial efficiency hypothesis, MEH) (Martinez et al., 2017). In that study, we compared the thermal performance of whole-organism metabolic rate, growth, and mitochondrial efficiency in *Manduca sexta* caterpillars. We found that the TPCs for mitochondrial efficiency and growth had a similar shape and T_{opt}. At supraoptimal temperatures, LEAK continued to increase while OXPHOS showed no change caused a rapid decline in coupling efficiency. Collectively, the decline in coupling efficiency along with the continued increase in metabolic rate and correlated decrease in growth rates at supraoptimal temperatures demonstrate that mitochondria had to work harder in order to keep up with ATP demand at supraoptimal temperatures. While this study provided a mechanistic link between mitochondrial level and whole-organism level performance, it is important to note that the generality of these results are very limited given that they were derived from a laboratory strain of a single species.

The aim of the current study is to test the generality of the MEH. To do this, we examined wild populations of another caterpillar species, the Spongy moth, *Lymantria dispar dispar* (L.) (Lepidoptera: Erebidae). We used this system because thermal performance of growth, development, and metabolic rate has been well characterized for the larval stage. Furthermore, studies with wild populations have found significant differences between northern and southern populations in these traits. For example, northern populations tend to have smaller pupal size, shorter development times, and higher metabolic rates (Friedline et al., 2019;

Powers et al., *In review*; Thompson et al., 2021). These differences have resulted in a latitudinal cline that is related to climate. However, the mechanism that underlies these types of clines is not well understood. Given the link between mitochondrial efficiency and whole-organism performance observed in Martinez et al. (2017) it is possible that this climate-related variation in these whole-organism traits may arise from variation in mitochondrial performance.

Specifically, we addressed the following questions. (1) Is larval metabolic rate mismatched with growth/larval development and mitochondrial efficiency like we previously observed in the lab strain of *M. sexta*? We hypothesize that MEH is present in wild *L. dispar* populations and predict that at supraoptimal temperatures increased LEAK will reduce mitochondrial efficiency resulting in similarly shaped TPCs with similar T_{opt} between larval growth/development and mitochondrial efficiency. At supraoptimal temperatures we also predict that metabolic rate will be mismatched with larval growth/development and mitochondrial efficiency. (2) Does thermal physiology at the mitochondrial level vary with thermal physiology at the whole-organism level and if so, is variation in mitochondrial thermal physiology among wild *L. dispar* populations related to climate? Given that previous work has found climate-related patterns in larval development, pupal size, and metabolic rate, we hypothesize that mitochondrial physiology will show similar variation to whole-organism physiology and that mitochondrial physiology will vary will climate. We predict that LEAK will increase and OXPHOS will decrease for populations towards warmer climates where *L. dispar* are more exposed to supraoptimal temperatures resulting in reduced mitochondrial efficiency for these more southern populations.

3.3 Methods

3.3.1 Study system

The Spongy moth (*L. dispar*) is a polyphagous, univoltine, defoliating forest pest that was first introduced to Medford, MA in 1869 (Grayson & Johnson, 2018; Tobin et al., 2012).

Since their introduction, *L. dispar* has expanded over 900,000 km^2 across eleven degrees of latitude. Its current range extends from northwestern Michigan, across Canada into Nova Scotia and finally down into southern Virginia and North Carolina. Evidence indicates that their initial expansion was fueled by an inherently broad thermal tolerance and the ability to feed on more than 300 tree species (Thompson et al., 2021). Currently, its continued expansion along the northern range edge is limited by cold temperatures that cause mortality during the winter and hinder larval development with short growing seasons that have fewer degree days (Denlinger et al., 1992; Gray, 2004; Madrid & Stewart, 1981). In the south, expansion is constrained by winter temperatures that are too warm for *L. dispar* to complete diapause (Gray, 2004). Evidence has also demonstrated that increased exposure to supraoptimal temperatures for larval growth and development has resulted in range contractions along the southernmost extremes of their distribution. Together these studies provide strong evidence that exposure to divergent climates combined with a broadly tolerant physiology has played a significant role in shaping *L. dispar* range dynamics.

3.3.2 Study populations

This study was conducted during the spring and summer of 2021 and used egg masses derived from a subset of the same *L. dispar* populations used by Thompson et al. (2021). We used a total of six populations: two northern populations sourced from Wisconsin (IR and AL), two southern populations sourced from North Carolina (NC1 and NC2), and two populations from the core of the *L. dispar* range located in Massachusetts (MA1 and MA2) which is the region where *L dispar* was originally introduced. Egg masses from the core and southern range were collected in 2016-2017 and egg masses for the northern populations were collected in 2017. At each source location, multiple egg masses (n = 11-60) were collected for each population. For invasion front (*i.e.*, range margin) populations, egg masses were collected from inherently low-density populations, but with densities high enough for sampling. Since their

collection in 2016-2017, these populations have been reared and maintained under the same common garden conditions.

3.3.3 Rearing L. dispar larvae

In order to randomly sample the gene pool, we created an admixture of eggs from multiple egg masses for each population. These admixtures of eggs were hatched at 25ºC in an environmental chamber. Once hatched, larvae from each population were transferred into 178 mL plastic cups with 20-25 mL of artificial diet (USDA APHIS formulation) and group reared (n=10). These cups were placed in a single environmental chamber and at a constant 25°C on a 14-hr light, 10-hr dark cycle. These cups were monitored daily to check for mold, developmental stage, and to replace diet as needed. Larvae for whole-organism respirometry were reared separately from the larvae used to measure mitochondrial respiration. Larvae for whole-organism respirometry were reared to the third instar and larvae for mitochondrial respiration were reared to the fourth or fifth instar.

3.3.4 Measuring routine metabolic rate

We assayed routine metabolic rate (RMR) at six temperatures (15°C, 25°C, 28ºC, 30°C, 34ºC, and 36ºC). This temperature range includes ecologically-relevant temperatures as well as sub- and supraoptimal temperatures for larval growth and development. We defined our measurements as routine metabolic rates to account for absorptive processes and diel locomotor activity of larvae during respirometry trials. Once larvae transitioned to the 3rd instar their RMR was assayed within 24-72 hours. Our measurements were made with a push-mode, stop-flow respirometry system (Field Metabolic System, model 2, Sable Systems International, Las Vegas, NV) connected to two 8-channel multiplexers (model RM-8, Sable Systems International). Each channel was fitted with a 35-mL chamber and this system was housed in a custom walk-in temperature control chamber (TCC) described by Martinez and Agosta (2016).

Prior to the respirometry trial, each larva was isolated and held without food for at least 1-hour and then weighed (0.001 g). During each trial, air inside each chamber was flushed at a flow rate of 100 mL/min for 3 minutes each hour for 4 hours. During hour 1, we allowed larvae to acclimate to the assay temperature and during hours 2-4, respirometry measurements were taken once per hour. Incurrent air was scrubbed for water vapor and CO_2 using three sequential columns (silica gel, soda lime, Drierite) before being pushed through each selected chamber. The excurrent air from the selected chamber was routed through a water vapor sensor and then scrubbed for water vapor using magnesium perchlorate. This dry air was then routed through a CO_2 sensor and subsequently scrubbed for CO_2 using Ascarite ®. The dry, CO_2 free air was then routed through an O_2 cell to measure the fractional concentration of O_2. A minimum of one chamber was kept empty to use as a baseline for each trial.

O_2 consumption traces were analyzed using ExpeData software (Sable Systems International). After drift, lag, and baseline corrections, all O_2 fractional concentration values were transformed to sample volumes of O_2 (Lighton, 2008):

$$M_S O_2 = \frac{O_2 \times FR}{1 - (0.2095 - O_2)}$$

FR represents the excurrent air flow rate corrected for standard temperature and pressure. To calculate the total sample volume of O_2 consumed we integrated the area under each $M_S O_2$ peak and then divided the sample volume by the enclosure time to find the O_2 consumption rate ($\dot{V}O_2$ μL/hr) for each individual.

3.3.5 Tissue extraction and mitochondrial isolation

We used 15-20 fourth or fifth instar larvae for each mitochondrial isolation. Each larva was dissected ventrally to remove the digestive tract. Once removed, the peritrophic membrane of each tube was removed, and the clean digestive tubes were place in 1mL of ice-cold Isolation Medium (IM; 250mM sucrose, 2mM ethyleneglycol-bis (β-aminoethyl ether) N, N'-tetraacetic

acid [EGTA], morpholinopropane sulfonic acid [MOPS], 0.5% fattyacid-free bovine serum albumin [BSA], pH = 7.4, 20C, 272 mmol kg-1). This pool of digestive tubes was first minced with a pair of scissors and then homogenized in 5mL of IM with a 7mL Potter Elehjem homogenizer coupled with a variable speed drill. It generally took five passes at low speed (~400 rpm) to adequately homogenize the tissue. Homogenate was then centrifuged at 700g for 10 minutes at 4ºC to remove cellular debris and undisrupted tissue. The supernatant was collected and centrifuged at 9,600g for 15 minutes at 4°C to sediment the mitochondrial fraction. Pellets were washed with IM, resuspended, and recovered by centrifugation at 9,600g for 15 minutes at 4°C two consecutive times. The final pellet was resuspended in 250uL of IM and stored on ice for 1h before trials.

3.3.6 Measuring mitochondrial respiration and coupling efficiency

We assayed mitochondrial respiration at five temperatures (15°C, 25°C, 28ºC, 30°C, and 34ºC) using a high-resolution respirometry system. This system consisted of two 2.0 mL thermo-controlled respirometric chambers and Clark-type polarographic oxygen electrodes (Oroboros Instruments; O2k Oxygraph). The polarographic O_2 electrodes were calibrated for each assay temperature across a range of O_2 tensions, in the presence of 2.0 mL of respiration medium (RM; respiration medium prepared according to Keeley (1973), modified by Martinez et. al. (2017) consisting of 250 mM sucrose, 0.3% w/v BSA, 15 mM KCl, 5.0 mM $MgCl_2$, 0.1 mM EGTA, 25 mM K_2PO_4, and 50 mM MOPS (pH = 7.4, 20°C), to account for temperature effects and background O_2 consumption by the probes. Additionally, probes were zeroed at each assay temperature by injecting 20 µL of a freshly prepared 40 mg/mL sodium dithionite solution into each chamber. The background flux was recorded before mitochondrial injection and subtracted from each run. Oxygen concentration was maintained between 350 and 200 nmol/mL by gently opening each respirometric chamber to gradually fill the gas phase above the respiration medium if needed.

The titration for each trial began by injecting 25 µL of purified mitochondria (246-520 µg of mitochondrial protein) into the respirometer chamber containing 2.0 mL of RM. Electron transport via complexes I, II, and glycerol-3-phosphate dehydrogenase (GPDH) to the ETS was assayed concurrently in each trial. The respiration not coupled to ATP production (*i.e.*, oxygen consumption due to proton conductance, proton slip, and cation cycling at saturating substrate concentrations or LEAK) was initiated by adding 2 µL mM malate (M), 10 µL mM glutamate (G), 5 µL mM pyruvate (P), and 10 µL mM proline, which supplies electrons to complex I via production of NADH by mitochondrial dehydrogenases. Convergent electron entry to the ubiquinone pool via $FADH_2$ was initiated by the addition of 10 µL mM succinate, and electron entry via GPDH was initiated by adding 10 µL mM sn-glycerol-3-phosphate. To engage OXPHOS, 10 µL mM ADP was added to the chamber. Mitochondrial coupling efficiency was determined using the respiratory control ratio (RCR; proportion of the respiration coupled to OXPHOS). We calculated RCR by dividing the OXPHOS rate by the LEAK rate at each assay temperature for each isolation. Mitochondrial protein was quantified using a Coomassie Plus Reagent Assay (Thermo Scientific, Rock-ford, IL).

3.3.7 Statistical analysis

Variation in MR-T was analyzed using a linear mixed effects model (LMM) with the 'lme4' package (Bates et al., 2015, 2021) in R version 4.1.0 (R Core Team, 2021):

$$RMR = Mass + Temperature + Population + Population \times Temp. + (1|Sample\ hour)$$

Population, assay temperature, and body mass were fixed effects. Population was treated as a fixed effect so we could test for differences among populations. Both assay temperature and body mass were treated as continuous covariates because of their strong influence on metabolic rate. The population-by-temperature interaction was included to test for differences in the temperature sensitivity of RMR (*i.e.,* slope of RMR-temperature relationship) among populations. We also used this slope to calculate the Q_{10} of RMR (Lighton 2008):

$$Q_{10} = 10^{m \times 10}$$

Sample hour was included as a random effect. We assessed statistical significance using α = 0.05. Data for RMR and body mass were also $\log_{10}$ transformed to meet model assumptions for normality and linearity. With this model we computed the estimated marginal mean (EMM) for each population using the 'emmeans' package in R (Lenth et al., 2021). This estimate represents the RMR for each population that has been adjusted to the same body mass across all populations (grand mean). We then checked for significant differences in RMR among populations using multiple comparisons with a Tukey correction at each assay temperature.

Variation in O_2 flux associated with OXPHOS and LEAK was also analyzed using a LMM:

$$O_2\ flux = Temperature + Population + Protein + (1|Isolation)$$

Population, assay temperature, and protein content were fixed effects. Population was treated as a fixed effect to test for differences among populations. Temperature and protein content were treated as continuous covariates and mitochondrial isolation was included as a random effect. We assessed statistical significance using α = 0.05. LEAK rates were $\log_{10}$ transformed in order to meet model assumptions for linearity. Rates for OXPHOS did not require transformation. EMMs for OXPHOS and LEAK were computed for each population by adjusting O_2 flux rates to the same protein content across all populations (grand mean). We then checked for significant differences in OXPHOS and LEAK among populations using multiple comparisons with a Tukey correction at each assay temperature.

Mitochondrial efficiency (RCR), larval development time, and relative growth rate were analyzed by fitting TPC models to these data. We did this using the 'rTPC' and 'nls.multstart' packages in R and the pipeline described in Padfield et al. (2021). We fit TPC models that included T_{opt} as a model parameter and chose the best fit model based on the lowest AICc and whether or not 95% confidence intervals for model parameters could be estimated. Confidence

intervals for TPC model parameters were estimated using the methods described in 'Bootstrapping with rTPC' vignette from Padfield and O'Sullivan (2023) along with the 'car' and 'boot' packages in R. TPC models were fit to raw data for RCR, larval development rate, and growth rate. Data for larval development time and growth came from Thompson et al. (2021) and only included data up to the third instar to avoid potential confounding effects of sex. Larval development rate was calculated using the inverse of the larval development time (1/days) and growth rate was calculated based on larval mass at third instar and larval development time to the third instar (mass/days). We also fit TPC models to the *M. sexta* data from Kingsolver and Woods (1997) for growth rate and to the RCR data from Martinez et al. (2017). TPCs were fit in the same manner as above, except we fit weighted TPCs because we were only able to obtain the mean and standard error for these data.

To determine if variation in RMR or mitochondrial physiology was related to climate we used principal components from the principal component analysis of climate and geographic variables from Powers et al. (*In review*) to quantify local climate for each *L. dispar* population. Using linear regression, we plotted EMMs for RMR, OXPHOS, and LEAK as a function of these climate PCs to determine if there were significant relationships. For mitochondrial efficiency we plotted parameter estimates from RCR TPCs as a function of climate PCs to determine if population RCR showed a significant relationship with climate.

3.4 Results

3.4.1 Routine metabolic rate

Results from our LMM were comparable to what we have found previously (Powers et al., *In review*). Overall, this model explained 79.4% of the total variance with 67.8% being explained by the fixed effects (Appendix, Table S3.1). Body mass, assay temperature, population, and the temperature-by-population interaction were all significant effects (Table 3.1). Similar to Powers et al. (*In review*), the effects of body mass ($\eta^2 = 0.38$) and assay temperature

(η^2 = 0.68) on RMR were strong while and the effects of population (η^2 = 0.03) and the temperature-by-population interaction (η^2 = 0.03) were relatively weak. EMMs for RMR increased exponentially with assay temperature. Population RMR was significantly higher for IR and MA, but was relatively similar among the remaining populations (Table 3.2). The MR-T slope was relatively similar among most populations (Q_{10} = 1.72-1.80), except for the IR (Q_{10} = 1.58) and MA2 (Q_{10} = 1.56) populations which had a significantly lower MR-T slope.

3.4.2 Mitochondrial respiration

Results for OXPHOS and LEAK LMMs were comparatively similar. The OXPHOS LMM explained 79.3% of the total variance with 75.9% being explained by the fixed effects. The LEAK LMM explained 91.2% of the total variance with 87.8% being explained by the fixed effects. Assay temperature, population, and protein content were significant for both models (Table 3.3). The effect of temperature on mitochondrial respiration was very strong (η^2 = 0.77 [OXPHOS], 0.91[LEAK]) while the effects of protein content (η^2 = 0.25 [OXPHOS], 0.20[LEAK]) and population (η^2 = 0.14 [OXPHOS], 0.24[LEAK]) were more moderate (Table 3.3). Population EMMs for OXPHOS and LEAK increased as assay temperature increased (Fig. 3.4). However, the response of OXPHOS was linear, while the LEAK response was exponential. Paired contrasts show respiration rates are relatively similar among populations across temperature, except for the AL population which was significantly higher (Table 3.4). Finally, we re-analyzed the OXPHOS LMM with log transformed data to look for differences in temperature sensitivity. We found that LEAK was more temperature-sensitive (slope = 0.036, 95% CI = 0.034-0.039; Q_{10} = 2.31) than OXPHOS (slope = 0.031, 95% CI = 0.028-0.035; Q_{10} = 2.06).

3.4.3 Mitochondrial coupling efficiency

Thermal performance of mitochondrial coupling efficiency (RCR) followed the general shape of a TPC (Fig. 3.5). The Gaussian model (Appendix 3.3.8.; Lynch & Gabriel, 1987; Padfield & O'Sullivan, 2023) was the best fit TPC model for the RCR data. Parameter estimates

from this model show that T_{opt} for most populations was between 22-23ºC, except for the NC1 population (T_{opt} = 21.5ºC). The corresponding maximum RCR at T_{opt} was relatively similar among populations (r_{max} = 6.3-6.6) (Fig. 6). Thermal performance breadth showed the most variation with the NC1 population having a relatively broad TPC (a = 15.4ºC) compared to the rest of the populations (a = 10.8-12.6). While we did observe some variability in TPC shape (Fig. 3.5), there was substantial overlap in confidence intervals among population estimates for all TPC parameters (Fig. 3.6). In addition, we observed substantial overlap of TPC parameter confidence intervals when comparing population estimates to parameter estimates from a single TPC where population data were pooled together (Fig. 3.6).

3.4.4 Relationship of RMR and mitochondrial respiration with climate variation

Adjusted R^2 values show moderate negative relationships for RMR and OXPHOS with climate, but these relationships were not significant (Fig. 3.7). For LEAK and RCR, adjusted R^2 values show no relationship with climate and these regressions were also not significant (Fig. 3.7 and 3.8).

3.4.5 Comparison of thermal performance traits among L. dispar populations

Thermal performance of larval development and growth also followed the general shape of a TPC (Fig. 3.9). For larval development, the best fit TPC was the Lobry-Rosso-Flandros model (Padfield & O'Sullivan, 2023; Rosso et al., 1993) and the Gaussian model was the best fit TPC for larval growth rate (Appendix 3.8.2.). The range of T_{opt} for larval development rate (29.2-30.3ºC) and growth rate (28.1-31.2ºC) was relatively similar among populations. Comparatively, T_{opt} of larval development rate (mean = 29.9ºC, 95% CI = 29.1-30.7) and growth rate were also similar (mean = 28.9ºC, 95% CI = 27.2-30.5) (Fig. 3.9) and these estimates were comparable to what was previously estimated from the OTIS laboratory strain of *L. dispar* (28.2-30.0ºC) (Logan et al., 1991).

Thermal performance of larval development, growth, and coupling efficiency all showed a hump-shaped response to temperature while RMR showed an exponential increase with temperature (Fig. 3.9). TPC shape differed between RCR and development/growth. RCR TPCs were left skewed with a lower T_{opt} relative to development/growth TPCs which were left skewed. On average, T_{opt} for RCR was 7.5ºC lower than development TPCs and 6.7ºC lower than growth TPCs.

For the *M. sexta* data, the best fit TPC for the growth data was the Jöhnk model (Jöhnk et al., 2008; Padfield & O'Sullivan, 2023) and the best fit model for the RCR data was the Gaussian model (Appendix 3.8.2.). We found the same pattern with the *M. sexta* data after fitting TPC models to the data (Fig. 3.10). The RCR TPC was left skewed with a lower T_{opt} relative to the growth TPC which was right skewed. The RCR T_{opt} was 6.3ºC lower relative to the T_{opt} for growth. However, the shape of the RCR TPCs between these species had some notable differences. The RCR TPC for *M. sexta* was relatively flat with a broader thermal tolerance and the coupling efficiency for *L. dispar* (RCR range = 4.2-6.5) was substantially higher relative to *M. sexta* (RCR range = 2.1-3.1).

3.5 Discussion

The results from this study provide strong evidence for the MEH operating in *L. dispar* during the larval stage. Similar to Kingsolver and Woods (1997) for *M. sexta* we found that whole-organism metabolic rate was mismatched with larval growth/development (Fig. 3.10). At the mitochondrial level, we found that LEAK respiration was more temperature sensitive than OXPHOS. This difference in temperature sensitivity resulted in a decline in mitochondrial coupling efficiency at temperatures above 25ºC (Fig. 3.5). Mitochondrial coupling efficiency showed the expected hump-shaped pattern of a TPC, but the T_{opt} for RCR was 6-8ºC lower than the T_{opt} for growth/development (Fig. 3.9). Finally, there were significant differences among a subset of the same *L. dispar* populations that were used in Powers et al. (*in review*) in metabolic

rate at both the whole-organism and mitochondrial level. However, these differences did not result in any systemic patterns with climate or latitude (Fig. 3.2, 3.4; Table 3.1-3.4). Linear regression with climate PCs suggested a latitudinal cline in RMR and OXPHOS, but the patterns were not significant (Fig. 3.7). Compared to our previous studies on RMR using twice as many populations (Powers et al., *In review*), these results provide only weak support for the hypothesis that variation in cellular level metabolism is related to climate.

Our analysis of MEH with *L. dispar* highlights two fundamental observations. First, these results continue to support a direct link between cellular and whole-organism performance. Specifically, our data support the notion that as temperature increases, LEAK respiration increases consuming more and more substrates that would otherwise be used to produce ATP (Fig. 3.11). Thus, as LEAK increases it constitutes a larger fraction of temperature-dependent maintenance costs. At supraoptimal temperatures, LEAK outpaces OXPHOS reducing efficiency of ATP production. To keep up with maintenance related ATP demands, whole-organism metabolic rate continues to increase (Fig. 3.11). Eventually, maintenance related ATP demands become too high to sustain growth resulting in a rapid decline in whole-organism performance (Fig. 3.11). While our findings were similar to Martinez et al. (2017) for *M. sexta, our* analysis found no overlap in T_{opt} between growth rate and RCR (Fig. 3.9, 3.10). Instead, we found that T_{opt} for growth corresponded with supraoptimal temperatures for coupling efficiency. However, it is important to consider that *L. dispar* displayed relatively high coupling efficiencies across the temperatures assayed compared to *M. sexta*. At T_{opt} for coupling efficiency, RCR was 6.34 and was still relatively high at the T_{opt} for growth (RCR = 5.63). Additionally for *M. sexta*, the difference in RCR between T_{opt} for coupling efficiency (RCR = 3.09) and T_{opt} for growth (RCR = 2.92) was small (0.17). Therefore, ATP production at the T_{opt} for growth was likely adequate to sustain optimal growth for both species. Future studies that assess ATP

production along with RCR may help resolve the significance of the difference in T_{opt} between RCR and growth.

Second, these findings significantly extend the domain of generality of the MEH to the larval stage of a second Lepidoptera species in a different family (Erebidae vs. Sphingidae). The mismatched MR-T with larval growth/development and mitochondrial coupling efficiency was consistent across five *L. dispar* populations representing the latitudinal extremes (two northern and two southern populations) and the established interior of its invasive range (Fig. 3.9). Furthermore, after fitting TPCs to the data from Kingsolver and Woods (1997) and Martinez et al. (2017), the same pattern emerged with *M. sexta* (Fig. 3.10). The difference in temperature sensitivity between OXPHOS and LEAK also match what has been observed with the OTIS laboratory strain of *L. dispar* (Alrashdi, 2022) and more generally what has been observed in other ectotherms (Abele et al., 2002; Chung & Schulte, 2015; Hardewig et al., 1999; Martinez et al., 2013, 2016, 2017). There are two non-mutually exclusive explanations for the higher temperature sensitivity of LEAK than OXPHOS. First, when PMF is high O_2 flux associated with LEAK respiration increases exponentially in order to maintain membrane potential of the inner mitochondrial membrane (Jastroch et al., 2010). Second, LEAK is considered to be an important mechanism for regulating the formation of reactive oxygen species (ROS). Since high PMF also promotes ROS formation, increased LEAK could be a response to increased ROS (Brand, 2000; Brookes, 2005; Koch et al., 2021).

The data we present for mitochondrial respiration were conducted *in vitro*, which is a standard approach for studying metabolism of mitochondria. However, there are some caveats with this approach that need to be addressed. Under *in vitro* conditions, our measurements for coupling efficiency represent an extreme when the impact of LEAK on ATP production is at its maximum. *In vivo*, mitochondrial respiration is subject to complex regulatory processes and normal O_2 consumption by mitochondria will occur at more intermediate levels. However, like

Martinez et al. (2017), this does not affect our observation that coupling efficiency declined at supraoptimal temperatures which resulted from an increase in LEAK respiration. Even at more intermediate levels *in vivo*, increased LEAK respiration will still consume substrates needed for ATP production reducing coupling efficiency and increasing maintenance costs.

The second main focus of this study was to determine if physiological variation at the mitochondrial level was similar to variation at the whole-organism level. We accomplished this by using a subset of *L. dispar* populations from Powers et al. (*in review*). At the whole-organism level, we found that our analysis of MR-T was comparable with previous *L. dispar* metabolic rate studies. Parameter effect sizes from our LMM were similar to the analysis from Powers et al. (*In review*) which used the same model parameters. The effects of temperature and body mass were also similar to the analysis in Chapter 2 for the OTIS laboratory strain. The range of population EMMs for RMR from this study (33.8-38.8 $\dot{V}O_2$/hr) fall within the range of EMMs for the same populations from Powers et al. (*In review*) (34.5-47.5 $\dot{V}O_2$/hr). Finally, the range of Q_{10} for MR-T from populations in the present study (1.56-1.80) were comparable to Powers et al. (*In review*) (1.30-2.00) as well as May et al. (2018). Despite these similarities with prior studies, we did not find strong support that physiological variation at the mitochondrial level was related to latitude or local climate as we have observed at the whole-organism level. With the LMM analysis we were unable to detect the latitudinal cline that was observed in Powers et al. (*In review*) with either MR-T or mitochondrial respiration. The post-hoc analysis indicates that the significant effect of population was only driven by one or two populations and these populations differed between MR-T (IR and MA1) and mitochondrial respiration (AL). We also did not detect the significant relationship with climate PCs that was observed with Powers et al. (*In review*) at either level.

We believe that our inability to detect these relationships was largely due to the small number of populations used in our analysis. For example, an early analysis of larval metabolic rate, which only included four populations (2 northern and 2 southern) failed to detect significant differences, while Powers et al. (*In review*), which included 14 populations, detected a latitudinal cline that was significantly related to climate. Despite the lack of significance, it is still worth noting that the PC regression from the current study did detect moderate relationships with climate PCs for both whole-organism and mitochondrial O_2 consumption (Fig. 3.7) that trended in the same direction as Powers et al. (*In review*). Finally, it is also possible that our inability to detect a relationship is related to the eurythermal nature of *L. dispar* physiology (May et al., 2018; Thompson et al., 2021; Walter et al., 2022). For example, a study with killifish (*Fundulus heteroclitus*) examined whether changes in mitochondrial function in response to thermal acclimation would explain changes in whole-organism thermal tolerance (Chung & Schulte, 2015). However, they found no evidence of mitochondrial dysfunction in response to supraoptimal temperatures after thermal acclimation. Therefore, it is possible that we did not detect any population variation in mitochondrial physiology because larval *L. dispar* have a similar ability to maintain mitochondrial function across a broad range of temperatures.

Other models of thermal tolerance have also proposed a link between mitochondrial respiration, aerobic metabolism, and whole-organism performance, most notably the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis (Pörtner, 2001, 2002; Pörtner et al., 2005). The underlying principle of the OCLTT is that insufficient oxygen supply at suboptimal and supraoptimal temperatures determines the thermal tolerance of aerobic scope which then sets the thermal tolerance of whole-organism performance (Pörtner, 2010). One limitation of this research is the use of aerobic scope as a proxy for whole-organism performance. For example, maximal metabolic rates are not always mismatched with resting metabolic rates which can result in little or no change in aerobic scope with temperature (Schulte, 2015). TPCs for different

performance metrics have different shapes with different T_{opt}'s relative to one another and to aerobic scope (Clark et al., 2013; Sinclair et al., 2016). Also, methodologies for measuring aerobic scope vary among studies which result in different conclusions about performance (Clark et al., 2013). Consequently, empirical support for the OCLTT has been mixed and is generally only supported in aquatic environments where hypoxic conditions are more likely to occur (Verberk et al., 2016). For terrestrial ectotherms under normoxia, like *L. dispar* and *M. sexta*, the OCLTT does not appear to operate suggesting that aerobic scope may not be strongly linked to whole-organism performance in air-breathers (Verberk et al., 2016). Alternatively, our results, in conjunction with Martinez et al. (2017), suggest that thermal sensitivity of LEAK respiration and its relationship to mitochondrial coupling efficiency are responsible for determining the thermal tolerance of whole-organism performance for ectotherms, particularly during ontogenetic stages which are metabolically active with significant changes in growth and development. Empirical tests or even meta-analyses, depending on data availability, in other taxa/species are needed to further determine the generality of the MEH model. Given that we were able to detect the presence of MEH in another Lepidopteran species and found a consistent pattern across multiple populations, this model of the limits to thermal tolerance may be broadly applicable to other Lepidopterans and holometabolous insects in general.

Finally, the MEH may also have important implications for *L. dispar* range dynamics in response to climate warming. Along their northern range where cold temperatures are limiting, climate warming may optimize mitochondrial coupling efficiency which would push larvae into positive energy balance and improve performance from growth and development. In the south where populations experience prolonged exposure to supraoptimal temperatures, maintenance costs would increase due to LEAK-related declines in mitochondrial coupling efficiency. This would push larvae towards negative energy balance and result in decreased survival. Indeed,

this mechanism may explain the observed range retraction in 2015 along the piedmont and coastal plain regions in southern Virginia (Faske et al., 2019). If this is the case, this may apply more broadly to other ectotherm distributions where populations are also experiencing prolonged exposure to supraoptimal temperatures. However, understanding the generality of this mechanism in the context of climate change will require a better understanding of the generality of the MEH as a mechanism limiting thermal performance in ectotherms.

3.6 References

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3.7 Tables

Table 3.1. ANOVA table for the RMR linear mixed effects model. Denominator degrees of freedom were determined using Satterthwaite's method. Bolded p values represent significant effects at α = 0.05. The last column represents the effect size for each model term (computed as eta squared, η^2). Bolded η^2 represent large effect sizes for model terms.

Parameter	SS	MSS	DF	F	p	η^2
Mass	7.784	7.784	1, 1056	641.378	**<0.001**	**0.38**
Temperature	26.903	26.903	1, 1056	2216.612	**<0.001**	**0.68**
Population	0.371	0.074	5, 1056	6.113	**<0.001**	0.03
Temp. × Pop.	0.331	0.066	5, 1056	5.455	**<0.001**	0.03

Table 3.2. Comparison of RMR estimated marginal means for each assay temperature. Comparisons among populations are ordered from least to greatest and superscript letters represent statistical comparisons. The Tukey method was used to adjust for familywise error at $\alpha = 0.05$.

Assay temperature (°C)	Contrasts
15	$NC2^{a} < NC1^{a} < AL^{ab} < MA1^{ab} < MA2^{b} < IR^{b}$
25	$NC1^{a} < NC2^{ab} < AL^{abc} < MA2^{abc} < MA1^{bc} < IR^{c}$
28	$NC1^{a} < NC2^{ab} < AL^{ab} < MA2^{ab} < IR^{b} < MA1^{b}$
30	$NC1^{a} < MA2^{ab} < NC2^{ab} < AL^{ab} < IR^{ab} < MA1^{b}$
34	$MA2^{a} < NC1^{ab} < AL^{ab} < IR^{ab} < NC2^{ab} < MA1^{b}$
36	$MA2^{a} < IR^{ab} < AL^{ab} < NC1^{ab} < NC2^{ab} < MA1^{b}$

Table 3.3. ANOVA table for the OXPHOS and LEAK LMMs. Denominator degrees of freedom were determined using Satterthwaite's method. Bolded p values represent significant effects at α = 0.05. The last column represents the effect size for each model term (computed as eta squared, η^2). Bolded η^2 represent large effect sizes for model terms.

Parameter	**SS**	**MSS**	**DF**	**F**	**p**	**η^2**
OXPHOS						
Temperature	4540219	4540219	1, 90	299.829	**<0.001**	**0.77**
Population	217361	54340	4, 90	3.589	**0.009**	0.14
Protein	434794	434794	1, 90	28.713	**<0.001**	**0.25**
LEAK						
Temperature	5.420	5.420	1, 90	912.070	**<0.001**	**0.91**
Population	0.170	0.043	4, 90	7.151	**<0.001**	**0.24**
Protein	0.136	0.136	1, 90	22.796	**<0.001**	**0.20**

Table 3.4. Comparison of estimated marginal means for OXPHOS and LEAK O2 flux among populations. Comparisons among populations are ordered from least to greatest and superscript letters represent statistical comparisons. The Tukey method was used to adjust for familywise error at = 0.05.

O_2 flux	**Contrasts**
OXPHOS	$MA2^{a} < NC2^{a} < NC1^{ab} < IR^{ab} < AL^{b}$
LEAK	$MA2^{a} < NC1^{a} < IR^{a} < NC2^{ab} < AL^{b}$

3.8 Figures

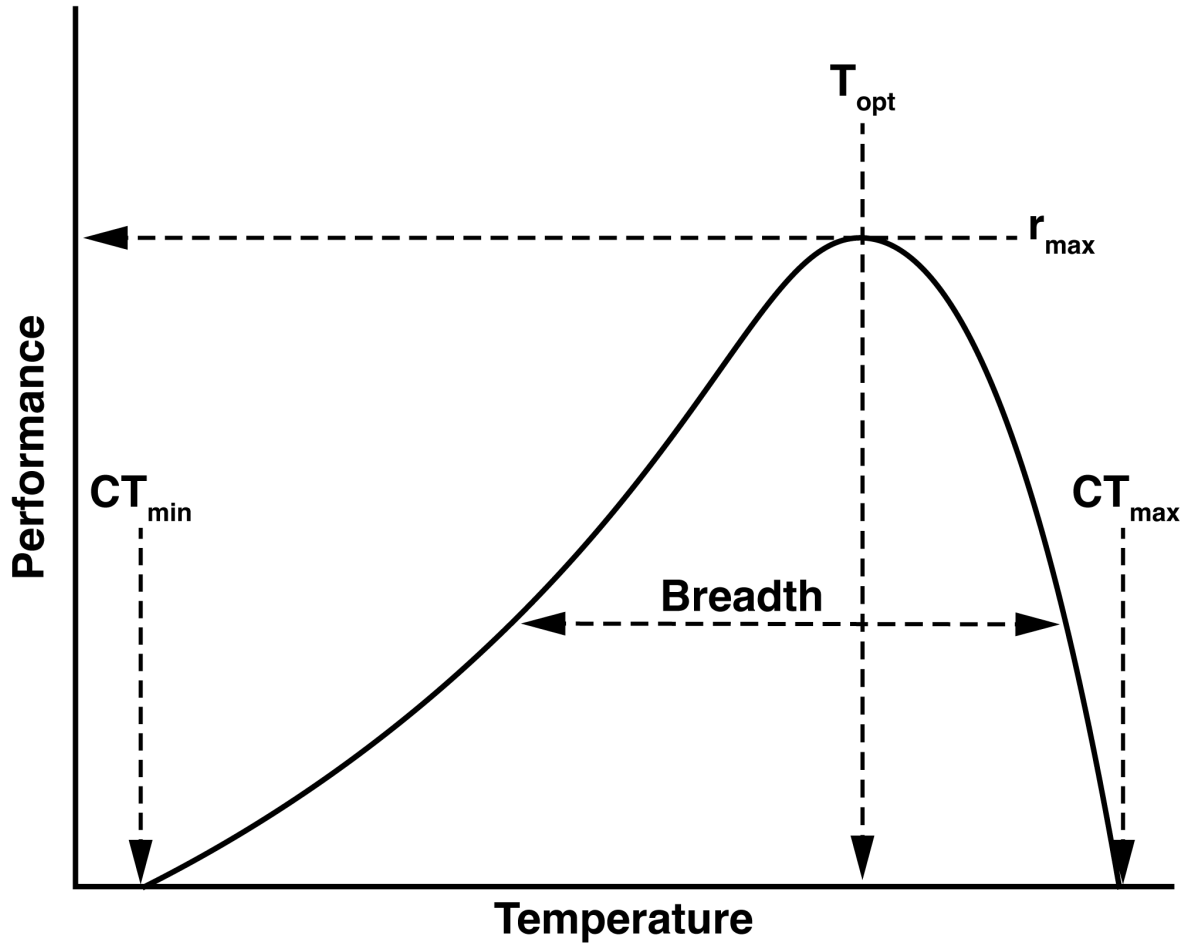

Figure 3.1. Characteristics of a thermal performance curve. The thermal optimum (T_{opt}) is the temperature at which the rate of performance is maximized (r_{max}). Breadth represents the range of temperatures at which an organism can perform well. CT_{min} and CT_{max} represent the limits of thermal tolerance for an organism.

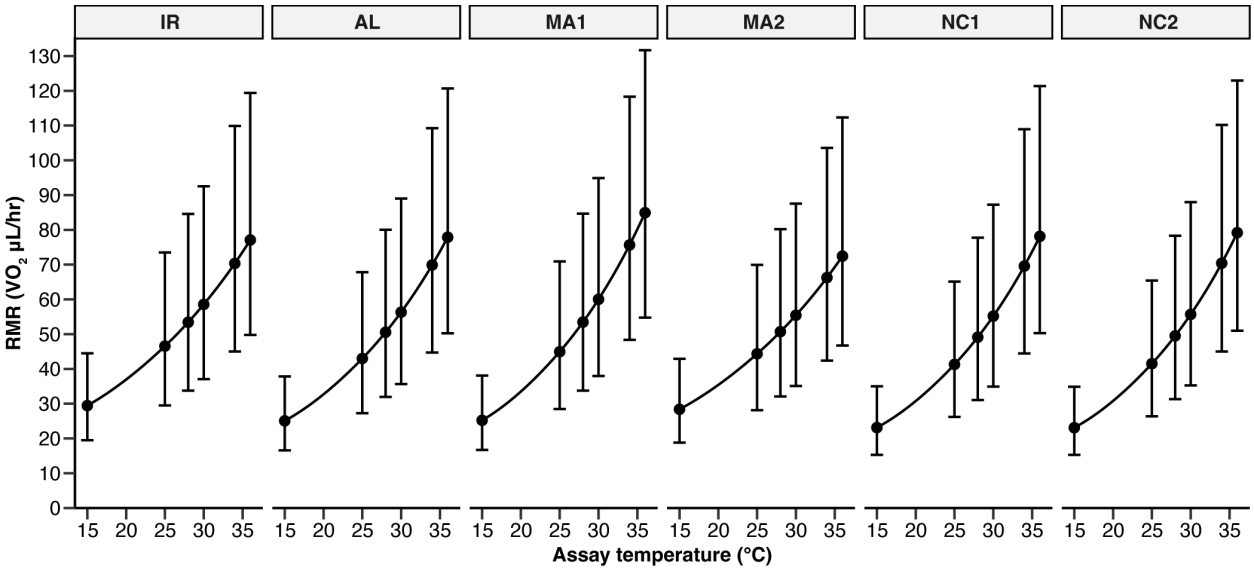

Figure 3.2. Untransformed estimated marginal means for population RMR (± 95% confidence interval) at each assay temperature. Populations are arranged by latitude (north to south).

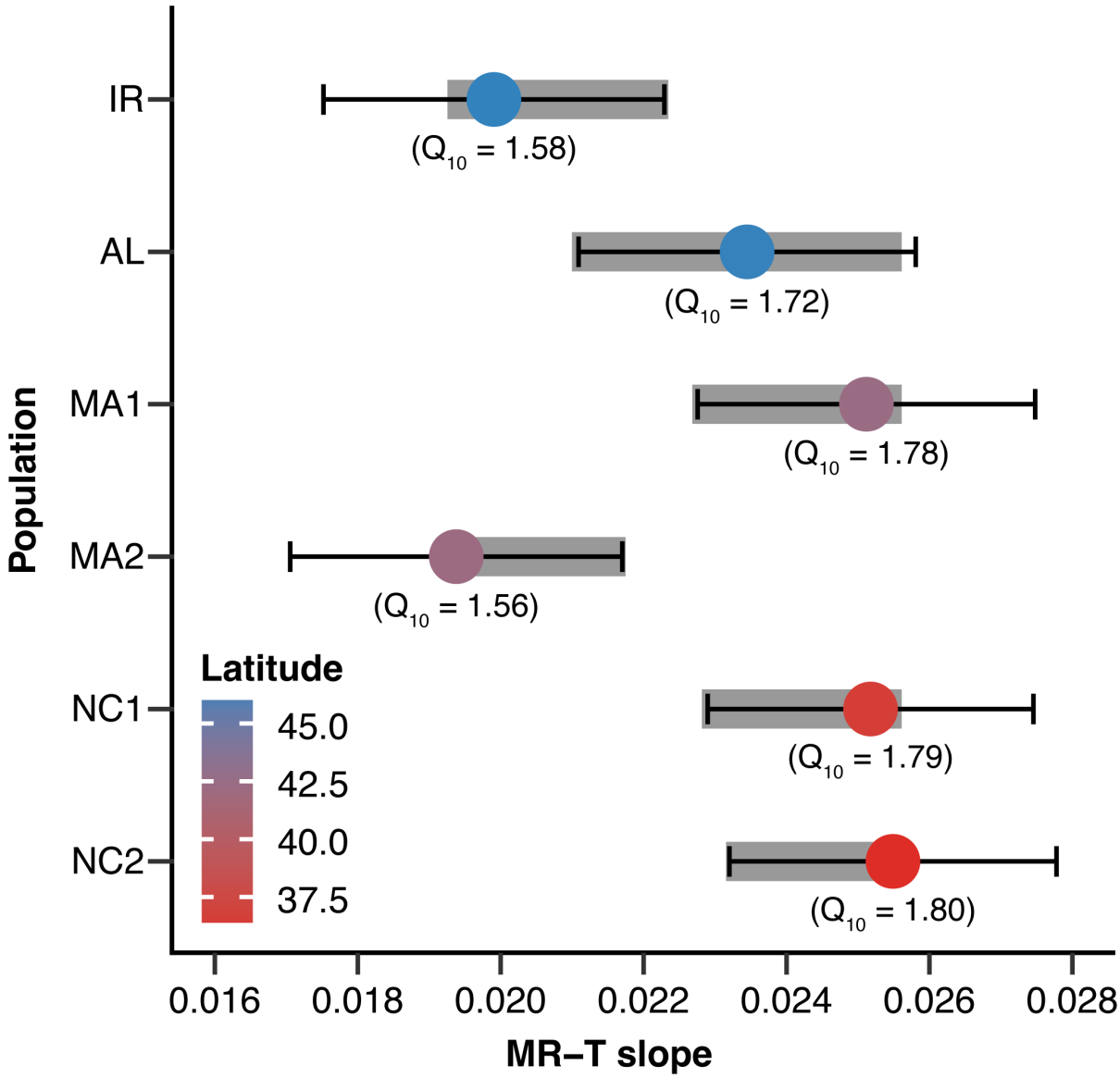

Figure 3.3. Comparison of MR-T slopes. Error bars represent the 95% confidence interval and gray bars represent multiple comparisons with non-overlapping bars being significantly different from one another. Dots are colored by the latitude of each population.

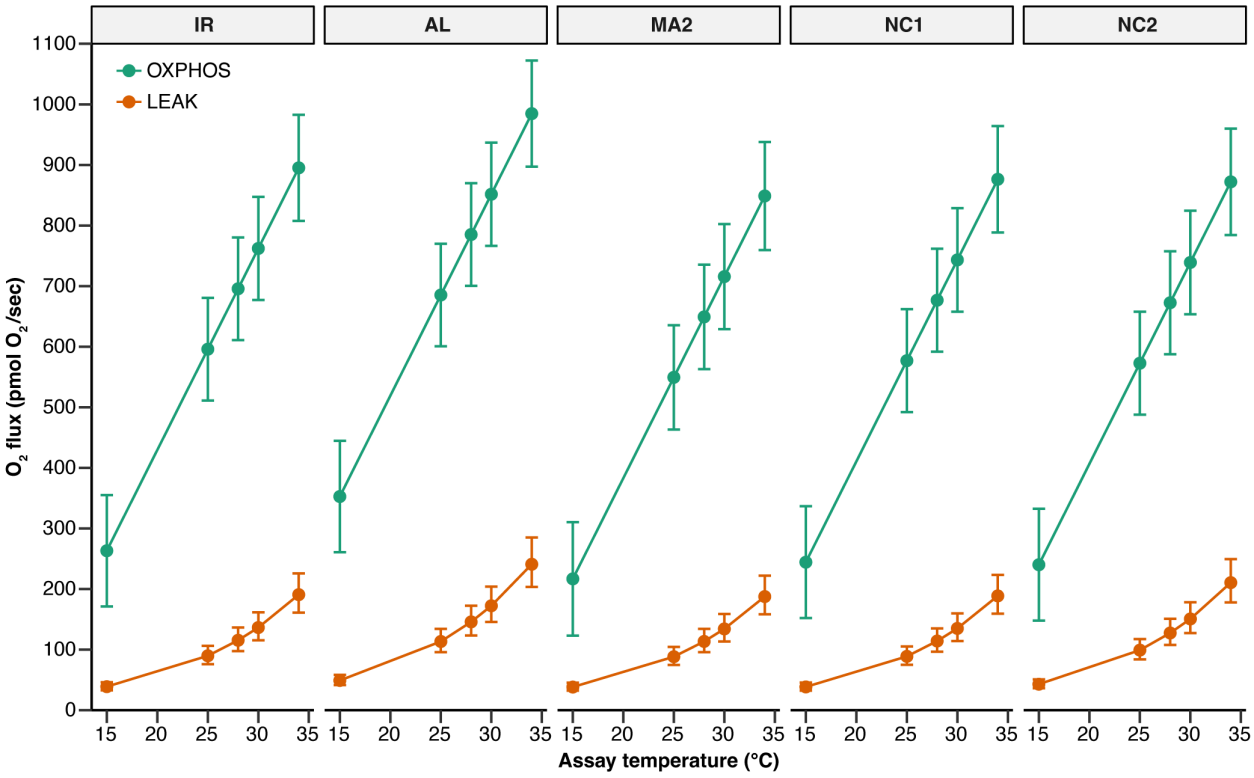

Figure 3.4. Estimated marginal means (EMM) for OXPHOS and LEAK respiration rates (± 95% confidence interval). Populations are arranged by latitude (north to south). EMMs and their confidence intervals for LEAK were untransformed so they could be plotted with OXPHOS EMMs.

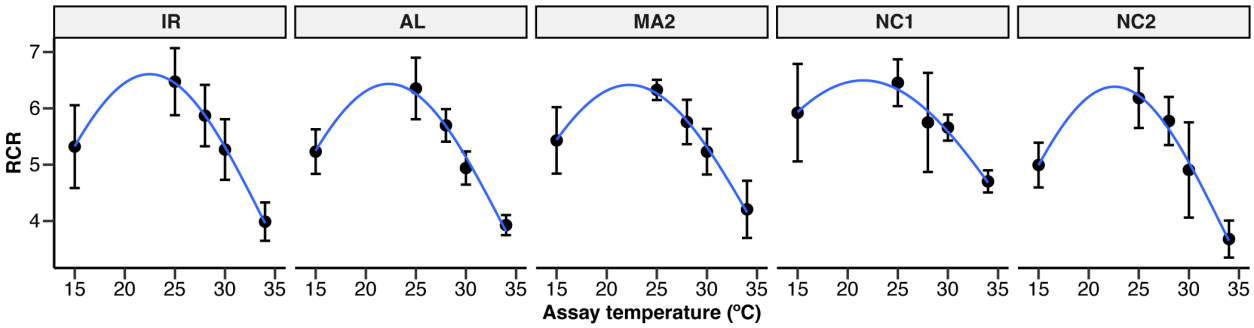

Figure 3.5. Mean RCR (± 95% confidence interval) at each assay temperature for each population. Populations are arranged by latitude (north to south). The blue line represents a Gaussian TPC fit to the RCR data for each population.

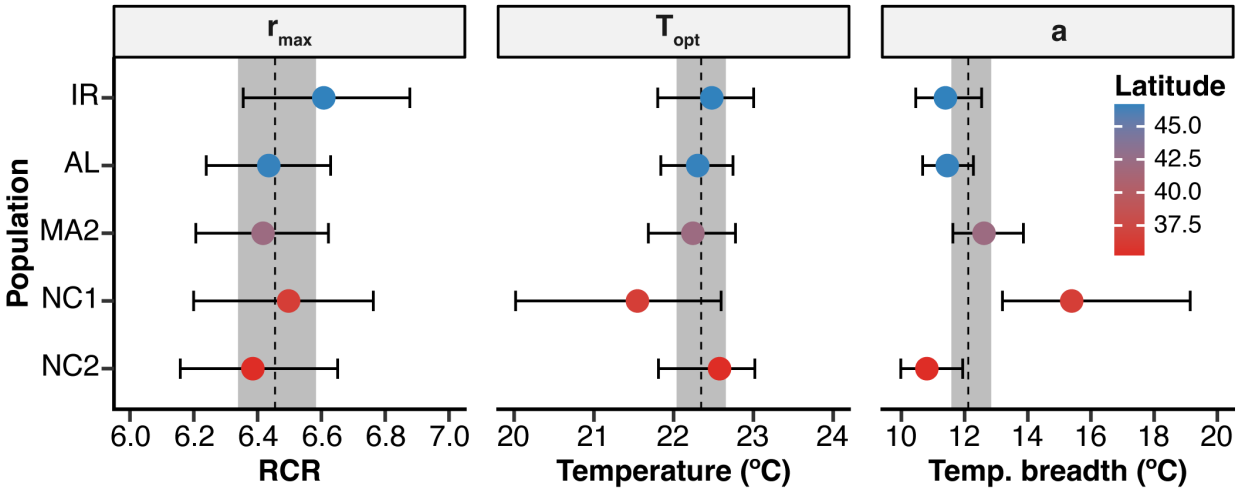

Figure 3.6. Parameter estimates from Gaussian TPCs for mitochondrial coupling efficiency (RCR). The dotted lines represent TPC parameter estimates from a single TPC where population data were pooled together and the vertical gray bars represent the 95% confidence interval. Points are colored by the latitude for each population.

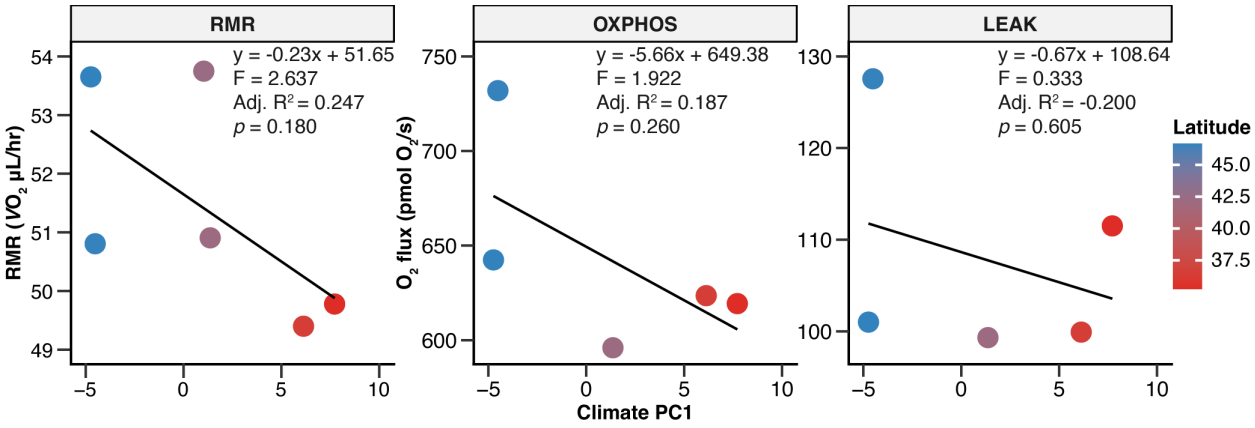

Figure 3.7. Linear regressions showing the relationship of metabolic rate at the whole-organism and mitochondrial level with climate PC1. Points are colored according to the latitude of each population.

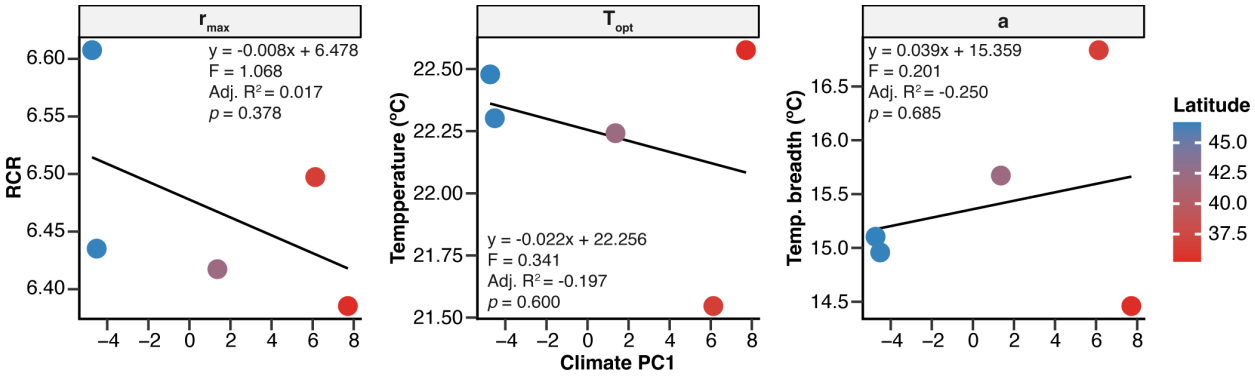

Figure 3.8. Linear regressions showing the relationship between TPC parameters for RMR with Climate PC1. Dots are colored according to the latitude of each population.

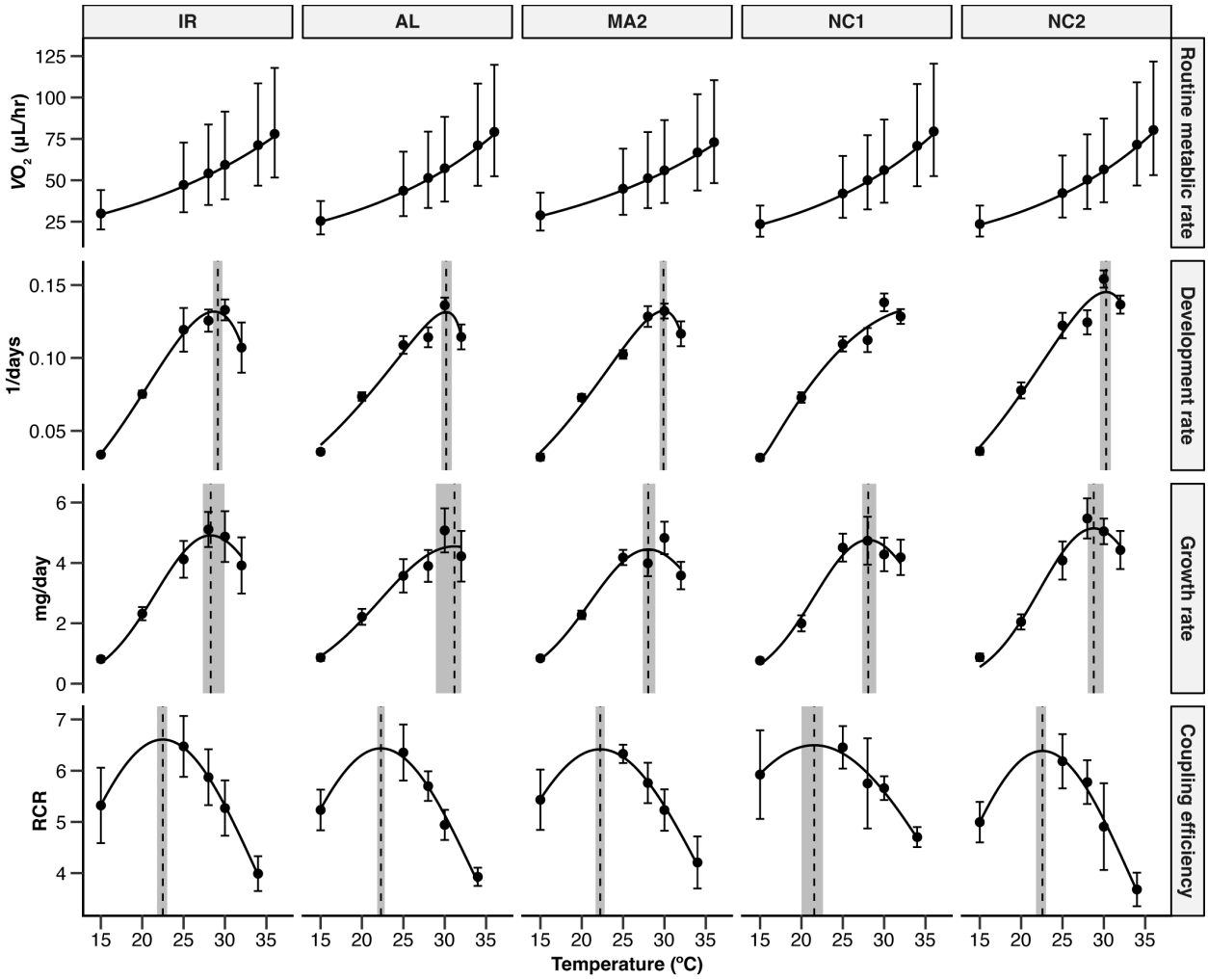

Figure 3.9. Synthesis of whole-organism metabolic rate, larval development rate, growth rate, and mitochondrial coupling efficiency for five *L. dispar* populations. Populations are arranged by latitude (north to south). Points represent performance trait means (± 95% confidence interval). Dashed lines and shaded areas represent T_{opt} and its 95% confidence interval.

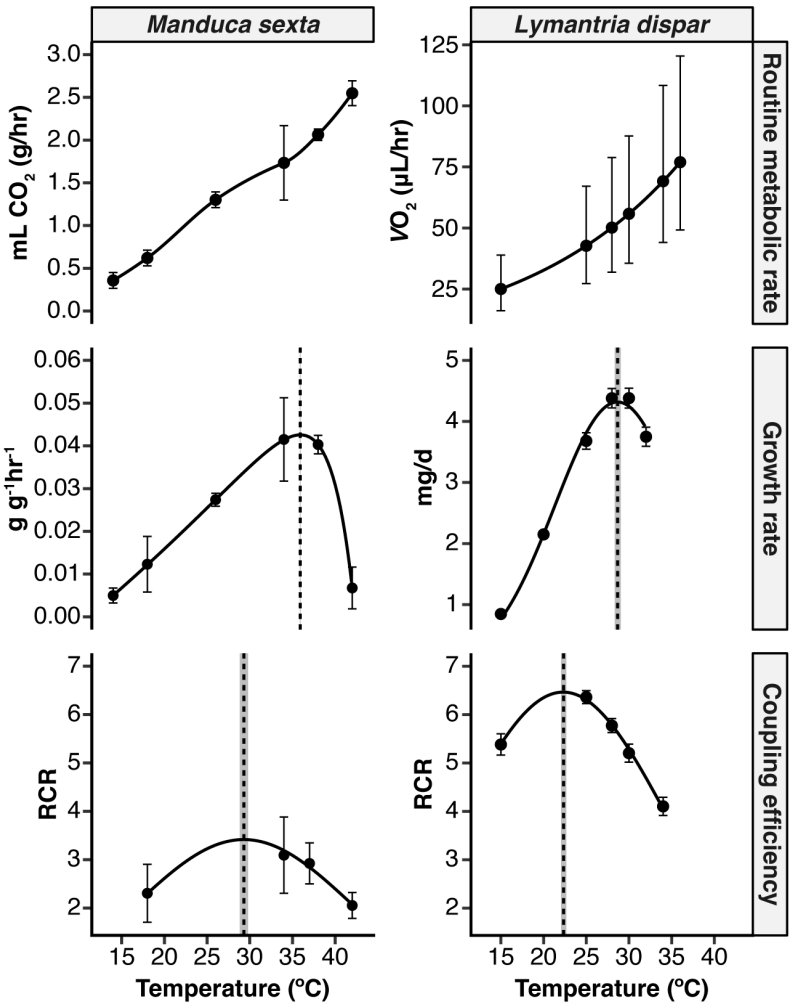

Figure 3.10. Synthesis of whole-organism metabolic rate, growth rate, and mitochondrial coupling efficiency for wild *L. dispar* populations and *M. sexta* laboratory strains. *L. dispar* thermal performance data for MR-T and RCR were pooled from the populations used in this study. *L. dispar* thermal performance data for growth were also pooled which included the six populations from this study as well as the other eight populations from Thompson et al. (2021). Data for *M. sexta* were replotted from Kingsolver and Woods (1997) and Martinez et al. (2016). Points represent trait means. Error bars for 95% confidence intervals. RMR and growth rates for *M. sexta* are mass-specific rates. Dashed lines and shaded areas for TPCs represent T_{opt} and the 95% confidence interval.

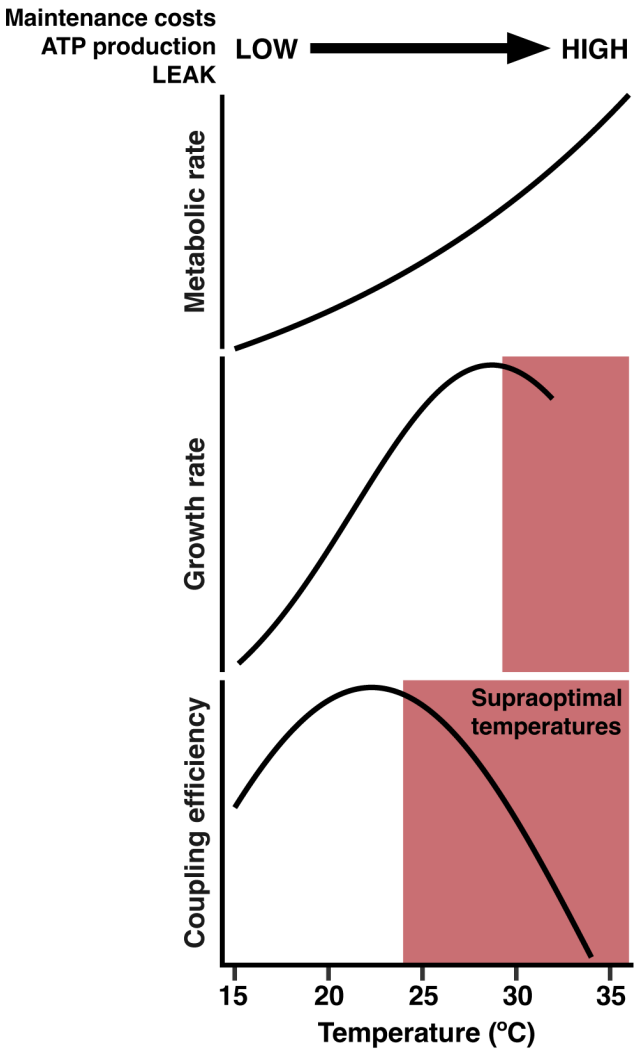

Figure 3.11. Conceptual diagram of the mitochondrial efficiency hypothesis based on observations from *L. dispar* populations. Maintenance costs to fuel essential biological processes increase with temperature and rely on ATP produced by oxidative phosphorylation (OXPHOS). As OXPHOS increases with temperature and ATP demand, leak respiration (LEAK) also increases. At supraoptimal temperatures (shaded region) LEAK increases at a faster rate than OXPHOS due to its higher temperature sensitivity causing a decrease in coupling efficiency which also increases maintenance costs. As maintenance costs continue to increase, less energy becomes available for growth/development causing a rapid decline in these rates.

3.9 Appendix

3.9.1 Supporting tables

Table S3.1. Summary of fixed and random effects from the linear mixed model used to compare population RMR. Bolded p values represent significant effects at $\alpha = 0.05$.

Parameter	Coefficient	95% CI	t	p
Fixed effects				
Intercept	1.858	1.725, 1.991	27.475	**<0.001**
Mass	0.656	0.605, 0.706	25.325	**<0.001**
Temperature	0.023	0.021, 0.026	19.508	**<0.001**
Population				
AL	Reference			
IR	0.123	0.027, 0.220	2.508	**0.012**
MA1	-0.022	-0.119, 0.074	-0.457	0.647
MA2	0.115	0.020, 0.211	2.366	**0.018**
NC1	-0.061	-0.156, 0.034	-1.255	0.210
NC2	-0.066	-0.161, 0.029	-1.365	0.173
Temp.× Population				
AL	Reference			
IR	-0.004	-0.007, 0.000	-2.072	**0.039**
MA1	0.002	-0.002, 0.005	0.981	0.327
MA2	-0.004	-0.007, -0.001	-2.409	**0.016**
NC1	0.002	-0.002, 0.005	1.033	0.302
NC2	0.002	-0.001, 0.005	1.218	0.224
Random effects				
Sample hour (n = 3)	0.083			
Residual	0.11			
Fit				
Marginal R^2	0.678			
Conditional R^2	0.794			

3.9.2 Thermal performance curve models

Gaussian model

$$\mathbf{rate} = \mathbf{r_{max}} \times \mathbf{exp}^{(-0.5(\frac{|\mathbf{temp}-\mathbf{T_{opt}}|}{\mathbf{a}})^{\mathbf{2}})}$$

rate = performance metric

r_{max} = maximum rate at thermal optimum

temp = temperature in ºC

T_{opt} = Thermal optimum in ºC

a = related to thermal performance breadth

Lobry-Rosso-Flandros model

$$\mathbf{rate} = \mathbf{r_{max}} \times \frac{(\mathbf{temp}-\mathbf{T_{max}}) \times (\mathbf{temp}-\mathbf{T_{min}})^{\mathbf{2}}}{(\mathbf{T_{opt}}-\mathbf{T_{min}}) \times ((\mathbf{T_{opt}}-\mathbf{T_{min}}) \times (\mathbf{temp}-\mathbf{T_{opt}}) - (\mathbf{T_{opt}}-\mathbf{T_{max}}) \times (\mathbf{T_{opt}}+\mathbf{T_{min}}-\mathbf{2} \times \mathbf{temp}))}$$

rate = performance metric

r_{max} = maximum rate at thermal optimum

temp = temperature in ºC

T_{opt} = Thermal optimum in ºC

T_{min} = low temperature at which rates become negative

T_{max} = high temperature at which rates become negative

Jöhnk model

$$\mathbf{rate} = \mathbf{r_{max}}(1 + \mathbf{a}((\mathbf{b}^{\mathbf{temp}-\mathbf{T_{opt}}} - 1) - \frac{\mathbf{ln(b)}}{\mathbf{ln(c)}}(\mathbf{c}^{\mathbf{temp}-\mathbf{T_{opt}}} - 1)))$$

rate = performance metric

r_{max} = maximum rate at thermal optimum

temp = temperature in ºC

T_{opt} = Thermal optimum in ºC

a = parameter with no biological meaning

b = parameter with no biological meaning

c = parameter with no biological mean