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# From Molecules to Whole Organisms: Insect Responses to Climate Change

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# FROM MOLECULES TO WHOLE ORGANISMS: INSECT RESPONSES TO CLIMATE CHANGE

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## ABSTRACT

Global atmospheric temperatures are rising at accelerated rates, exposing organisms to novel and potentially unsuitable changes in their environment. In order to survive changes in their thermal environments, organisms can employ physiological plasticity on short time scales or species can adapt over evolutionary time. Species may utilize one or both of these strategies to ensure survival; however, if they are incapable of responding to change, they may face extinction. Insect species may be some of the most vulnerable organisms experiencing climate change-induced alterations in their thermal environments because, as ectotherms, temperature influences nearly all of their physiological processes. By characterizing physiological responses to global change, we can begin to understand how organisms are currently responding to climate change and predict how organisms may respond in the future. My research aims to elucidate how insects respond to changes in their thermal environment by examining multiple physiological responses to winter warming in diapausing *Pieris rapae* butterflies, and the evolution of transcriptomic responses to heat shock in early *Drosophila melanogaster* embryos. Winter warming caused *P. rapae* butterflies to have compromised supercooling points, lowered cryoprotectant abundances, shifted metabolomes, more variable metabolic rates, and switches in energy fuel usage. While heat shock caused thermally sensitive early *D. melanogaster* embryos to exhibit changes in the abundance of thousands of gene transcripts, regardless of the region of origin. But, *D. melanogaster* embryos with higher thermal tolerance from tropical populations had higher abundance of key transcripts that encode proteins involved in the oxidative stress response. My results suggest that insects endure a broad suite of physiological consequences when the temperature increases. Will these species survive climate change? My work suggests that diapausing *P. rapae* may be threatened by winter warming, but also shows that some individuals can recover from winter warming. My work also demonstrates that the evolutionary genetic basis of heat tolerance in early *D. melanogaster* embryos is mediated at the level of the transcriptome, and suggests that *D. melanogaster* has the potential for adaptation to heat shock temperatures. However, to what extent these species will be challenged by future warming patterns, and if they will continue to evolve and adapt at a rate suitable for survival, needs to be further studied.

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## TABLE OF CONTENTS

# CHAPTER 1: INTRODUCTION

Global climate change has led to increased mean atmospheric temperatures, increased variation among temperature extremes, and increased frequency of extreme weather events (Allen et al., 2018). Over the next century, these patterns will further increase and expand; for example, it is predicted that global mean temperatures could increase by upwards of 4°C by the end of the 21$^{st}$ century (Meehl et al., 2007; Robinet and Roques, 2010). These patterns are concurrently affecting species, and will continue to do so in the future. However, the effects of climate change on global systems are not straightforward and universal but, rather, complex. Climate change can disproportionally affect species across seasons, temperature zones, latitudes, and altitudes (Bale et al., 2002; Bradshaw and Holzapfel, 2008; Buckley et al., 2017; Deutsch et al., 2008; Musolin, 2007). Numerous studies have examined the impacts of global warming-induced temperature increases on species' behavior, physiology, and range distribution (Bale et al., 2002; Hill et al., 2002; Hughes, 2000; Sgolastra et al., 2011). Results from these studies have shown both positive and negative effects of climate change on species from distribution expansions and increased population abundances, to higher susceptibility to disease and extinction. However, it is important to note that the magnitude of these responses to climate change is likely species, or even population-specific. Thus, it would be naïve to assume we have exhausted our knowledge to what extent global climate change has thus far affected species, and how it will continue to affect them in the future. Moving forward, we must continue to explore, and subsequently predict, how species respond to changes in their thermal environment looking through physiological, ecological, and genetic lenses.

Temperature is a strong force that drives the physiological, biochemical, and behavioral mechanisms of ectothermic organisms like insects (Kingsolver et al., 2004; Somero et al. 2017). This is due to the universal thermodynamic relationships that govern the stability of biological structures and reaction rates. Consequently, even acute increases in temperature, despite being within an organism's natural range, can lead to two to threefold increases in biochemical activity (Schulte, 2015; Tattersall et al., 2012). Due to their inherent sensitivity to temperature, changes in environmental temperature can have direct effects on insects' developmental rate, metabolic activity, thermal tolerance, fecundity, and survival (Colinet et al., 2015; García-Robledo et al., 2016; Robinet and Roques, 2010). However, seasonality is a key factor for multivoltine species of insects, and thus, plays a role in how insect species may respond to climate change.

Understanding an organism's physiology can be a powerful tool in predicting how species respond to changes in their thermal environments by looking at measurements such as thermal tolerance and gene expression. Insects present excellent systems for these types of measurements due to their quick generation times, wide global distributions, and the relative facility of rearing them under laboratory conditions that mimic ecological patterns. The research presented in these chapters uses two model systems among insects, *Pieris rapae* butterflies and *Drosophila melanogaster* flies.

*Pieris rapae*, or the cabbage white butterfly, is a globally abundant species with populations in temperate and sub-tropical environments. A recent study has uncovered the invasion and genetic history of this species, allowing for research that can describe the basis of differential phenotypes and physiological mechanisms among populations (Ryan et al., 2019). Such differences could be observed, for example, in how populations

of *P. rapae* maintain their overwintering strategy. In order to survive winter, late-season *P. rapae* pupae enter facultative diapause which is induced by drops in environmental temperature and decreased daylight hours (Danks, 2002; Kono, 1970; Nelson et al., 2010). However, *P. rapae* populations at higher and lower latitudes experience drastically different thermal environments during winter, and thus, one could predict, that climate change-induced winter warming could differentially affect them.

Diapause is not unique to *P. rapae* as a way to cope with cold stress and survival through sub-optimal conditions, as many temperate species of insects enter diapause, a dormancy stage characterized by depressed metabolic activity, developmental arrest, and elevated cold and stress tolerance (Denlinger, 2002; Salt, 1961; Saunders, 1971; Tauber and Tauber, 1978). As seen across different insect orders, all metamorphic life stages have evolved the ability for diapause and cold tolerance/hardiness (Danks, 1987; Nylin, 2013). However, the life stage in which a species diapauses is species-specific; individuals from the same species will always diapause in the same stage regardless of population, location or seasonal timing (Denlinger, 2002; Tauber et al., 1986). As described above, climate change has disproportionally affected seasons, with winters warming faster than any other season (NOAA, https://www.ncdc.noaa.gov/sotc/national/202001, accessed February 2020). Thus, there is the unique challenge of understanding how climate change may differentially affect these diapausing individuals, relative to individuals exposed to warming patterns during other seasons. To date, winter warming and its effects on overwintering physiology remain relatively understudied in diapausing insects, and thus leaves a large gap in our understanding of how climate change affects insects. Much of the ecological,

physiological, and behavioral challenges these overwintering organisms will face with climate change and their responses to said challenges remain to be described.

Similar to *P. rapae*, *Drosophila melanogaster* is a species of fly that lives across broad ranges of thermal environments worldwide. In the context of climate change and thermal tolerance, *D. melanogaster* is of particular interest given (1) its wide distribution globally, with both tropical and temperate populations, and (2) its four distinct life stages that might differentially respond to increased temperature.

In climate studies, the occurrence of acute warming, or warming on the order of minutes to hours, is often overlooked. Such warming patterns exist naturally in necrotic fruit, the environment of juvenile life stages of *D. melanogaster* (Roberts and Feder, 2000). On a given day, within a few hours, *Drosophila* embryos can be exposed to an increase in temperature upwards of 20°C (Roberts and Feder, 2000). *Drosophila* embryos are not as thermally tolerant as the other life stages (Lockwood et al., 2018), and thus, if these individuals are already living close to or at their thermal maxima, they may not be able to tolerate similar, cyclical increases in temperature if these patterns are exacerbated by global climate change.

Here, my aim was to describe the physiological mechanisms that underlie insects' responses to global climate change patterns. I used a combination of thermal tolerance and respirometry measurements, and metabolomic assays to test how diapausing *Pieris rapae* butterflies respond to acute and chronic warming events during their overwintering stage (Chapters 2 & 3). Furthermore, I used transcriptomic analyses to determine the underlying genetic basis of disparate thermal tolerance levels in temperate and tropical *Drosophila melanogaster* embryos (Chapter 4).

My results demonstrated that winter warming led to less negative supercooling points, lower abundance of cryoprotectant metabolites, and higher variation in metabolic activity in diapausing *P. rapae* pupae – patterns that could suggest the vulnerability of overwintering individuals to changes in their thermal environment as global climate change continues to increase winter temperatures. I also found considerable shared transcript abundances among tropical and temperate *D. melanogaster* embryos, suggesting that maternally loaded transcripts, regardless of region of origin or temperature at which embryos are shocked, play a demonstrative role in early zygotic development. However, I also found clear patterns in differential expression of transcripts across temperatures and regions suggesting, not only that these early embryos are actively expressing their own transcripts, but that there is active degradation of maternal transcripts accompanying these patterns.

My work provides strong evidence for the physiological consequences of increased temperature in the contexts of winter warming on overwintering butterflies and acute warming on early stage fly embryos. My novel research uncovers insects' physiological responses to climate change from transcripts to molecules to whole organisms. By understanding how these concurrent, ecologically relevant temperature patterns affect insect species, we can better understand and predict how species will respond to future warming patterns.

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# CHAPTER 2: WINTER WARMING CAUSES WIDESPREAD SHIFTS IN THE METABOLOME AND HINDERS SUPERCOOLING IN *PIERIS RAPAE* BUTTERFLIES


## ABSTRACT

Global climate change has the potential to negatively impact biological systems as organisms are exposed to novel temperature regimes. Increases in annual mean temperature have been accompanied by disproportionate rates of change in temperature across seasons, and winter is the season warming most rapidly. Yet, we still know little about how the direct effects of winter warming will alter the physiology of overwintering organisms. Here, we investigated the effects of winter warming stress on internal freezing temperatures (supercooling points) and metabolome profiles of diapausing *Pieris rapae* butterfly pupae. We show that after acute and chronic winter warming exposure, pupae had higher supercooling points and significant changes in metabolite abundances across the entire metabolome. Notably, there were warming-induced shifts in key biochemical pathways that likely support energy metabolism and cryoprotection. These physiological responses suggest that winter warming will threaten the survival of overwintering *P. rapae* pupae, and by extension winter warming may pose threats to other species that overwinter. Furthermore, we found population-specific supercooling profiles in *P. rapae*, as we observed significantly lower supercooling points in Vermont individuals relative to North Carolina individuals, with mean supercooling points matching local minimum temperatures of each location. Moving forward, future research should focus on species-wide responses to winter warming events, particularly in the context of local warming

patterns, to better predict how populations may differentially respond to changes in winter thermal environments.

## INTRODUCTION

Climate change will expose organisms to unpredictable thermal environments to which they may not be adapted through shifts in seasonality (i.e. later onset of winter and/or earlier onset of spring) and the increased frequency of temperature anomalies (García-Robledo et al., 2016; Kingsolver et al., 2011; Sinclair et al., 2016; Somero, 2010; Somero et al., 2017). Mean atmospheric winter temperatures are increasing at a faster rate than any other season (NOAA, https://www.ncdc.noaa.gov/sotc/national/202001, accessed February 2020; NOAA, https://www.ncdc.noaa.gov/cag/, accessed February 2020). According to the latest IPCC special report, winter temperatures have shown increased variability with both hotter mean temperatures and a higher frequency of extreme temperatures and weather events (Allen et al., 2018). Thus, it is imperative to characterize how overwintering organisms respond to winter warming conditions in order to predict how these species will respond to future climate conditions.

Diapause is an overwintering strategy for temperate insects, and relies on intrinsic physiological mechanisms that depress metabolic activity and confer cold tolerance (Denlinger, 2002; Salt, 1961; Saunders, 1971; Tauber and Tauber, 1978). A key trait that underlies cold tolerance during diapause is enhanced supercooling (Storey and Storey, 1988), which is the ability of organisms to lower the freezing point of their body solutions to below 0°C (Somero et al., 2017). Supercooling is achieved, at least in part, by manipulating the colligative properties of intra- and extra-cellular solutions through the synthesis and accumulation of cryoprotectant metabolites (Storey and Storey, 1988;

Storey and Storey, 1991). For example, larvae of the arctic beetle *Cucujus clavipes* have been observed to withstand temperatures of -100°C without freezing, which is largely made possible by the synthesis and accumulation of high concentrations of glycerol (4-6 mol $L^{-1}$) in their body solutions (Sformo et al., 2010).

Despite the intrinsic physiological mechanisms that enable diapausing insects to survive through months of extreme winter conditions, extrinsic factors such as temperature may influence diapause as well. Indeed, successful overwintering may depend on cold temperatures to maintain metabolic homeostasis (Hodek and Hodkova, 1988). The Arrhenius relationship predicts that increases in temperature will lead to exponential increases in the rates of biochemical reactions (Somero et al., 2017). Thus, if dormant animals rely on cold winter temperatures as a means to extrinsically regulate their physiological processes (Hodek and Hodkova, 1988; Storey et al., 2010), winter warming could lead to increases in biochemical activity that compromise their ability to survive. As a consequence, winter warming may cause diapausing insects to expend energy stores before spring emergence (Buckley et al., 2017) or to undergo shifts in metabolism that alter cryoprotective mechanisms like supercooling. If this is the case, winter warming patterns have the potential to adversely affect the overwintering physiology of animals that enter hibernation, torpor or diapause (Bale and Hayward, 2010; Bradshaw et al., 2010; Hahn and Denlinger, 2007).

Temperate species of insects that enter diapause in the winter may be particularly vulnerable to winter warming, as environmental temperature and seasonality can impact their physiology, as has been observed in previous studies (Arambourou and Robby, 2015; MacLean et al., 2017; Sinclair et al., 2012). Indeed, even subtle changes in

temperature can have major consequences on diapause development and subsequent spring eclosion success (Lehmann et al., 2018). Warmer pre-wintering temperatures led to greater expenditure of energetic reserves and an increase in yearly winter mortality in overwintering bees (*Osmia lignaria*) (Sgolastra et al., 2011). Similarly, variable temperature during autumn led to higher susceptibility of energy store loss in diapausing *Erynnis propertius* larvae, making this species, and other dormant ectothermic insects potentially vulnerable to metabolic activity shifts through diapause (Williams et al., 2012). Moreover, when adult blowflies (*Calliphora vicina*) were exposed to warmer autumn conditions (+5°C) during diapause induction, the subsequent larvae that entered diapause had lowered cold hardiness, compromised supercooling points (internal freezing point), and decreased survival (Coleman et al., 2014).

*Pieris rapae*, or the cabbage white butterfly, is a globally abundant species with populations found across five continents (Ryan et al., 2019). *Pieris rapae* diapause and overwinter in the pupal stage, and previous work has shown that North American (Ontario, Canada) *P. rapae* pupae can supercool to below -20°C and that supercooling point depends on thermal acclimation (Li et al., 2020). In addition, populations of *P. rapae* from Canada and Eastern Siberia differ in the responses of supercooling to thermal acclimation, suggesting genetic variation among populations in the plasticity of this trait (Li et al., 2020).

Although previous work has established the connection between the thermal environment and overwintering physiology across taxa, including *Pieris rapae*, no studies have characterized concurrent responses in supercooling and metabolomic profiles to winter warming. Thus, the extent to which winter warming will alter the intrinsic

mechanisms of cold tolerance, which may threaten the survival of overwintering organisms, cannot be predicted. To address this gap in knowledge, we tested whether increases in temperature, which approximate current and future winter warming scenarios, influence supercooling point and metabolomic profiles of *P. rapae* butterflies. Importantly, we characterized metabolomic profiles of pupae from which we also measured supercooling points, allowing us to directly correlate metabolomes to supercooling. We predicted that higher temperature experienced during diapause would adversely affect the ability for *P. rapae* pupae to supercool, and that these responses in supercooling would be underpinned by changes in metabolite abundances across the metabolome. Overall, our results suggest that diapausing *P. rapae* pupae exposed to either chronic or acute winter warming patterns may experience adverse effects on their overwintering physiology, particularly if winter temperatures continue to rise and have increased variability. The results from this research will provide ecologically relevant insight into the future of overwintering physiology, as we continue to understand the effects of winter warming at local scales, as well as global warming patterns, across seasons.

## MATERIALS AND METHODS

### Adult butterfly collections and maintenance

We collected approximately 40-50 male and female adult *Pieris rapae* butterflies in mid to late September in 2017 at two locations in northwestern Vermont at least 15 miles apart (44°29’48.52”N, 73°1220.19”W and 44°17’10.07”N, 73°14’07.11”W), while adult *P. rapae* butterflies were collected from two locations in North Carolina (35°36’19.47”N, 82°20-07.25”W and 35°36’31.57”N, 82°26’31.33”W). After collection, we kept adults in

mesh containers (Carolina Biological Supply, 11" diameter × 12" height) with 10 butterflies in each container under common garden conditions of 24°C, 12:12 Light:Dark photoperiod, 55% relative humidity, and with direct access to sunlight. We fed adults a diet of 10% honey solution on a sponge every 24 hours. After 48 hours post-collection, we isolated females in individual mesh containers, and gave them fresh organic kale leaves on which to oviposit. Fertilized eggs were collected every 24 hours, and placed into common garden juvenile rearing conditions.

**Juvenile stage rearing and diapause induction**

Upon oviposition, eggs were removed and placed into plastic containers (35.6cm length x 20.3cm wide x 12.4cm height) and into incubators (Percival model DR-36VL) set to 24°C and 55% relative humidity, with approximately 20 eggs in each container. *Pieris rapae* diapause in the pupal stage, with the larval stage as the sensitive stage or preparative stage (Richards 1940). To ensure all individuals entered diapause, we subjected all individuals to short-day photoperiods (8L:16D) starting at the embryonic stage. We replaced fresh organic kale leaves every day. Upon pupation, roughly 14 days post oviposition, we placed individuals into one of three winter warming treatments, ensuring that eggs from each female were represented in each treatment.

**Winter warming treatments in diapausing pupae**

We determined the winter warming treatments based from the historic data records of a local weather station in Burlington, VT (National Weather Service Forecast Office, https://w2.weather.gov/climate/local_data.php?wfo=btv, accessed February 2020). These patterns reflect historic, concurrent and predicted chronic and acute warming patterns

observed in Vermont winters (November-February) (Table 1). We isolated individual pupae into petri dishes (60 x 15mm) and kept them under one of the following conditions until Day 90 of pupal diapause or supercooling analysis (measured on Days 25, 50 and 75). The three temperature conditions consisted of a control treatment, chronic warming treatment, or acute warming treatment. We kept control individuals under a temperature regime with daily fluctuating temperature 4°C-8°C, representing autumn temperatures when individuals first enter diapause. We kept control individuals under this regime for the entire experiment. We kept the chronic warming individuals under a temperature regime of 7°C-11°C, representing a 3°C increase from the control. This pattern reflects both the long-term degree of warming pattern seen in Vermont over the last 50 years, as well as the predicted, continued pattern we expect to see over the next 50 years (National Weather Service Forecast Office, https://w2.weather.gov/climate/local_data.php?wfo=btv, accessed February 2020). We kept the acute warming individuals under the control conditions of daily fluctuating 4°C-8°C but with three, 24-hour warming events of fluctuating 18°C-23°C on days 25, 50 and 75. This temperature regime mimics the hottest recorded diurnal and nocturnal temperatures observed in Vermont winters (National Weather Service Forecast Office, https://w2.weather.gov/climate/local_data.php?wfo=btv, accessed February 2020). To measure standing population-level differences in diapause physiology, we only subjected individuals from North Carolina to the control treatment, thus, comparing them to the Vermont control treatment.

We weighed individual pupae every 2-4 days using a fine-scale balance to determine any differences in weight over the course of the 90-day experiment (Mettler Toledo XSE105), and report no statistical differences in weight over time for any of the three treatments (Type II ANOVA, $F_{1,41}$=1.62, $P$=0.21). Thus, we excluded weight as a factor in subsequent analyses.

**Supercooling point measurement**

We measured the internal freezing temperature or supercooling point (SCP) of the diapausing pupae based off the protocols described in Boychuk et al. (2015) and Sinclair et al. (2015) (Boychuk et al., 2015; Sinclair et al., 2015). To determine SCP, we placed pupae into individual 2-ml microcentrifuge tubes, attached to a type-K thermocouple wire (OMEGA Engineering), and then sealed it with parafilm. We equilibrated individuals in a circulating water bath (Polyscience PP15R-30) with Polycool HC -50 anti-freeze liquid, then kept individuals at 0°C for 10 minutes, and then cooled them from 0°C to -30°C at a rate of 0.5°C$min^{-1}$. We monitored body temperature using a thermometer and data logger program (OMEGA Engineering HH806AW). SCP was defined as the temperature at which intracellular ice formed, and was measured as the lowest temperature (°C) recorded before the detectable presence of an exothermic reaction (ice formation) in the temperature trace. We analyzed individual internal freezing temperatures on Days 25, 50 and 75 in the control (Vermont and North Carolina) and chronic warmed individuals, and 24-hours post-warming in the acute warmed (STA) individuals. We measured 4-7 individuals for each temperature by treatment combination. Immediately after SCP

analysis, we flash froze control and chronic warmed individuals in liquid nitrogen and preserved them at -80°C for metabolomics analysis.

We compared supercooling points of (1) control vs. chronic warmed, (2) control vs. acute warmed, and (3) Vermont vs. North Carolina populations (under control conditions) with a 2-way analysis of variance (ANOVA) reduced model. We modeled treatment, or population, and days in diapause as fixed effects. Pairwise differences between the control and warmed treatments were assessed with Dunnett's multiple comparison post hoc test. All supercooling point analyses were performed using GraphPad Prism 8.

**Global metabolomics sample preparation**

We used individual pupae preserved from the supercooling point analysis for global, untargeted metabolomics analysis. To determine the effect of chronic warming on metabolite abundance, we only used control and chronic warmed individuals (n=17 pupae per group) with at least 4 individuals represented at each timepoint (Days 25, 50 and 75). All samples were sent to the University of Florida Southeast Center for Integrated Metabolomics facility for analysis.

Samples were homogenized in 100 µL 5mM ammonium acetate and protein concentration of each sample homogenate was measured. All samples were normalized to 500 µg/mL protein concentration prior to extraction. Note that because samples were normalized to equal concentrations prior to metabolomics analysis, we did not normalize metabolite abundances to pupal weights. Extraction was performed using protein precipitation. Briefly, 50 µL normalized homogenate was spiked with a mixture of

internal standard. Proteins were precipitated by adding 400 µL of 8:1:1 acetonitrile:methanol:acetone. After mixing, proteins were allowed to precipitate for 15 min at 4°C. Supernatant from each sample was collected following centrifugation at 20,000xg for 10 min and dried under a gentle stream of nitrogen at 30°C. Samples were reconstituted with 50 µL of reconstitution solution consisting of injection standards and transferred to LC-vials for analysis.

**LC-MS analysis and data processing**

Untargeted metabolomics analysis was performed on a Thermo Q-Exactive Oribtrap mass spectrometer with Dionex UHPLC and autosampler. All samples were analyzed in positive and negative heated electrospray ionization with a mass resolution of 35,000 at m/z 200 as separate injections. Separation was achieved on an ACE 18-pfp 100 x 2.1 mm, 2 µm column with mobile phase A as 0.1% formic acid in water and mobile phase B as acetonitrile. The flow rate was 350 µL/min with a column temperature of 25°C. Injection volume was 2 µL.

MZmine 2.0 was used to identify features, deisotope, align features and perform gap filling to fill in any features that may have been missed in the first alignment algorithm. All adducts and complexes were identified and removed from the data set. This rendered a total of 14,379 features, which we analyzed for significant responses to winter warming (see below). We used MetaboAnalyst 4.0 (Chong et al., 2019) to normalize the mass spec peak intensities of metabolite features prior to statistical analyses. For each feature, peak intensity was log-transformed and normalized to the sample median. The data were auto-scaled to facilitate comparison among features.

**Statistical analysis of metabolomic data**

To test for differences in metabolite abundances between the control and chronic warmed pupae, we compared the normalized peak intensities, as a proxy for metabolite abundance, of all metabolite features identified by LC-MS. We conducted a principal components analysis to describe the major axes of variation in the dataset, and then tested whether the first principal component (PC1) significantly explained variation in supercooling point among the samples via least-squares linear regression. We then measured the number of metabolites with significantly different peak intensities via Type II 2-way ANOVA, with warming treatment and days in diapause modeled as fixed effects. Features in the positive and negative ion modes were analyzed separately. All p-values were corrected for false discovery via the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). All metabolite features with an FDR < 0.05 were considered to have significantly different abundances. Unless otherwise indicated, we performed all statistical analyses using GraphPad Prism 8 or R version 3.6.1.

**Metabolite annotation and pathway analysis**

We used the MS Peaks to Pathways module in MetaboAnalyst 4.0 (Chong et al., 2019) to annotate metabolome features and to conduct pathway analysis. Accurate annotation of untargeted metabolomics data is dependent upon a library of verified standards, which are often incomplete and not representative of the focal species (Li et al., 2013). The MS Peaks to Pathways approach subverts these shortcomings by identifying metabolite sets in the context of KEGG pathways. Metabolite annotation of features is based upon the mass-to-charge ratios in the context of pathways, whose compounds are found to respond in a coordinated manner to experimental manipulation (i.e., winter warming). Because

the goal of this study was to assess the physiological consequences of winter warming, we focused our pathway analysis and metabolite annotation on the features that were identified to change in abundance in response to chronic warming. We conducted the GSEA algorithm in the MS Peaks to Pathways module of MetaboAnalyst 4.0, which is a rank-based pathway enrichment test. Metabolite features were ranked based on the F-value from the treatment main effect from the 2-way ANOVA (see above). We used the *Drosophila melanogaster* KEGG pathway database, which is the only insect species for which KEGG pathway information is available, to identify significantly enriched pathways and metabolites in our dataset. Pathways with an FDR-corrected P-value less than 0.1 were considered significant, following the recommendations of the authors of the analysis software.

## RESULTS

### Supercooling point: effects of winter warming and population of origin

Chronic and acute winter warming caused significantly higher supercooling points in pupae on day 50 (Fig. 1A; 2-way ANOVA, $F_{2,43}$=6.566, $P$=0.0032; Dunnett's multiple comparison test, Day 25 – control vs. acute warming, $P$=0.2945, control vs. chronic warming, $P$=0.1610; Day 50 – control vs. acute warming, $P$=0.0070, control vs. chronic warming, $P$=0.0079; Day 75 – control vs acute warming, $P$=0.6917, control vs. chronic warming, $P$=0.8026). Supercooling point continuously decreased in Vermont control individuals over 75 days in diapause with the lowest observed, average supercooling points ($\bar{x}$ = -26.3 ± 0.3°C) on Day 75 (Fig. 1A; 2-way ANOVA, day factor, $F_{2,43}$=19.43, $P$<0.0001). In both the acute warming and chronic warming groups, supercooling point increased from day 25 to day 50 (Day 25 acute warming $\bar{x}$ = -21.0 ± 2.2°C and chronic

warming $\bar{x}$ = -20.7 ± 3.4°C to Day 50 acute warming $\bar{x}$ = -19.6 ± 3.9°C and chronic warming $\bar{x}$ = -19.2 ± 3.1°C). However, supercooling points decreased again in the warmed groups by Day 75 with decreased variance among individuals, mirroring the control group (acute warming $\bar{x}$ = -25.3 ± 2.9°C and chronic warming $\bar{x}$ = -25.6 ± 1.0°C) (Fig. 1A).

Supercooling points matched local environmental conditions in Vermont and North Carolina. Supercooling point was significantly lower (more negative) in Vermont individuals than North Carolina individuals across all three timepoints (Fig. 1B; 2-way ANOVA; population factor, $F_{1,26}$=67.35, $P$<0.0001; day factor, $F_{2,26}$=5.459, $P$=0.0105; population x day interaction, $F_{2,26}$=0.2307, $P$=0.7956). Supercooling point reached an average of -26.3 ± 0.3°C in Vermont pupae and -19.8 ± 4.0°C among North Carolina pupae at day 75, which corresponds to the disparate extreme low temperatures in these two locations—average extreme minimum temperatures in VT and NC are -29 to -26°C and -18 to -15°C, respectively (USDA, https://planthardiness.ars.usda.gov/, accessed February 2020). Although both populations depressed their internal freezing temperature over time, the Vermont pupae were able to depress their internal freezing to significantly lower temperatures than the North Carolina pupae.

**Responses of the metabolome to winter warming**

Untargeted metabolomics identified a total of 14,379 metabolite features in all pupae from the control and chronically warmed experimental groups. Of these, 1,370 showed significant changes in abundance (normalized peak intensity) through diapause, irrespective of winter warming treatment (2-way ANOVA, day factor, FDR < 0.01). 443

features showed significant changes in abundance in response to chronic winter warming (2-way ANOVA, temperature factor, FDR < 0.01), and 16 features showed significant changes in abundance through diapause and in response to warming. No features had abundances that depended on the interaction between day and treatment (2-way ANOVA, day x temperature interaction, all features had an FDR > 0.24).

Metabolite feature abundances representing individual metabolomes revealed that individuals cluster primarily by supercooling point which accounted for nearly 27% of the total variation in abundances of all 14,379 features among pupae (Fig. 2A). In addition, days in diapause accounted for 10% of the total variation in metabolomic profiles (Fig. 2A). The variation among metabolomes, as described by PC1, was strongly correlated to supercooling point (Fig. 2B; Least-squares linear regression of PC1 on SCP, $y = -15.48x - 23.3$, $R^2=0.73$, $P<0.00001$).

The coordinated changes in the metabolome that accompanied the chronic winter warming treatment constituted significant changes within 10 biochemical pathways (Fig. 3; Table S1). Chronic winter warming caused the abundances of metabolites in most (7 out of 10) of these pathways to decrease. Meanwhile, one pathway (arachidonic acid metabolism) showed increases in the abundance of its metabolites. Two pathways (valine, leucine and isoleucine biosynthesis and valine, leucine and isoleucine degradation) showed both increases and decreases in metabolite abundances, and thus these two pathways did not exhibit directionality in winter-warming-induced changes overall (Fig. 3). Three of the pathways—β-alanine metabolism, fructose and mannose metabolism, and glycine, serine and thronine metabolism—implicate the involvement of

previously described cryoprotectants, including β-alanine, sorbitol, and glycine (Michaud and Denlinger 2007; Lee 2010; Hahn and Denlinger 2011).

**Responses of putative cryoprotectants to winter warming**

Pupae with the lowest supercooling points had the highest abundances of three putative cryoprotectants, β-alanine, sorbitol, and glycine, and SCP was negatively correlated with the abundances of all of these metabolites (Fig. 4; Least-squares linear regression; β-alanine, $R^2 = 0.72$, $P < 0.00001$; sorbitol, $R^2 = 0.70$, $P < 0.00001$; and glycine, $R^2 = 0.55$, $P < 0.00001$). Moreover, all three of these metabolites showed significant decreases in abundance after chronic winter warming (Fig. 4; 2-way ANOVA, temperature main effect; β-alanine, $F_{1,30} = 17.16$, $P = 0.0003$; sorbitol, $F_{1,30} = 11.11$, $P = 0.002$; and glycine, $F_{1,30} = 11.36$, $P = 0.002$).

## DISCUSSION

Here, we measured key overwintering physiological traits in *Pieris rapae* pupae exposed to acute and chronic winter warming. We support the hypothesis that winter warming modifies the intrinsic physiological mechanisms that underlie supercooling, as warming led to less negative supercooling points that were accompanied by shifts in the metabolomic signatures of cryoprotection and other metabolic pathways. Because supercooling allows for the survival of temperate insects through sub-zero winter temperatures, winter warming threatens overwintering insects by raising their supercooling points. However, we also report variation among populations in supercooling that matches the historical thermal environment, which may reflect local adaptation between disparate populations of *P. rapae* from Vermont and North Carolina.

Thus, there is likely to be standing genetic variation in supercooling, upon which natural selection can act, which we predict will lead to evolutionary responses in supercooling to future winter warming conditions.

**Warming-induced changes in supercooling point**

We show that winter warming impaired supercooling—a key mechanism of cold tolerance. Strikingly, on Day 50, individuals from both warmed treatments had significantly higher supercooling points than control individuals. Elevated supercooling points for warmed individuals at Day 50, relative to those on Day 25, may represent the reprioritization of stored reserves, as warmed individuals allocated resources not to further depressing supercooling points at Day 50, but to another mechanism of diapause physiology, such as energy metabolism and/or stress tolerance (Hahn and Denlinger, 2011; Kukal et al., 1991). Similarly, increased thermal variability led to decreased cold hardiness on day 50 in diapausing cabbage root fly pupae, *Delia radicum* (Koštál and Šimek, 1995). This result, along with the data presented herein, suggests that individuals that have been in diapause for approximately two months may be particularly sensitive to thermal variability. However, our data further suggest that diapausing individuals may recover from extended warming (i.e. chronic) or repeated warming events (i.e. acute) by Day 75, as all individuals, regardless of treatment, had suppressed supercooling points at this last timepoint.

Importantly, our results suggest that warming induced changes in supercooling could threaten the survival of *P. rapae* pupae in nature. Although the observed supercooling points on day 50 remain relatively low (Day 50 acute warming $\bar{x}$ = -19.6 ±

3.9°C and chronic warming $\bar{x}$ = -19.2 ± 3.1°C), these supercooling points are within the range of winter temperatures in Vermont. Thus, the warmed pupae from this study could have easily frozen to death in the wild if atmospheric temperatures fell below the observed supercooling points. In addition, un-warmed *P. rapae* pupae from both Vermont and North Carolina exhibited a trend of decreasing supercooling points through diapause (Fig. 1). Supercooling points in many insect species have been shown to follow this same trajectory through diapause, which mirrors environmental temperatures in nature, as winter temperatures decrease from December through February (Bale, 2002; Hodkova and Hodek, 1988; Marshall and Sinclair, 2015; Pullin et al., 1991). Since warmed pupae broke with this trend on day 50 in diapause, it could signify a warming-induced mismatch between physiology and the environment. Particularly in the case of acute warming events such that winter warming may be punctuated by sudden drops in temperature that fall below an individual's supercooling point. If winters continue to increase in variation (Allen et al., 2018), with extreme temperatures at both cold and warm ends, this species, as well as other temperate diapausing insect species, may be unable to survive without rapid adaptation and/or phenotypic plasticity.

We note that extrapolating our results to what pupae experience in nature assumes the direct exposure of diapausing individuals to changes in atmospheric temperatures, which may or may not be a realistic assumption, depending on snow cover that could insulate insects against thermal fluctuations (Boychuk et al., 2015; Sinclair, 2001). However, winter warming is predicted to lead to loss or reduction in snow cover, which would subsequently expose diapausing individuals to fluctuating temperatures and to a higher number of freeze-thaw events (Bale and Hayward, 2010).

We also acknowledge that supercooling is a mechanism of cold tolerance that is most critical for species that are freeze avoidant—i.e., species that cannot survive if they experience internal freezing. Supercooling may or may not be important for overwintering survival in species that are freeze tolerant—i.e., species that can survive internal freezing. It remains to be determined whether freeze tolerant species will be challenged by winter warming; however, thermal acclimation influences cold tolerance in at least some freeze-tolerant insects (Li et al., 2020; Toxopeus et al., 2019), suggesting that winter warming may also impact species that are freeze tolerant and not only species that are freeze avoidant, as we have shown here.

Furthermore, it is important to note that the present study does not fully account for the diversity of potential mechanisms that could underlie supercooling. A critical factor that influences the supercooling point is osmolality, which we did not measure in this study. Dissolved solutes in an insect's hemolymph colligatively lower their freezing point by 1.86°C per osmole of solute (Denlinger and Lee, 2010), but other non-colligative mechanisms, such as ice-binding proteins, can contribute to the lowering of supercooling point as well (Meister et al., 2013). If non-colligative mechanisms contribute to supercooling in diapausing *P. rapae* pupae, then the metabolomic data we present herein would not fully describe the mechanisms that underlie the shifts in supercooling point that occurred in response to winter warming. Nonetheless, the strong correlation between the metabolome and supercooling is noteworthy and deserves further investigation. Future studies should measure osmolality (indicative of colligative mechanisms) and thermal hysteresis (indicative of non-colligative mechanisms) in

response to winter warming to provide further insights into the connection between putative cryoprotectant metabolites and supercooling in overwintering species.

Based on previous work (Li et al., 2020) and the results presented herein, it appears that North American *P. rapae* rely on supercooling as a primary mechanism of coping with extreme low temperatures during the winter. We report that the supercooling points of pupae from Vermont and North Carolina exhibited a pattern indicative of local adaptation—supercooling points matched the average extreme minimum winter temperatures in these locations, respectively. This correspondence between historic weather data and supercooling points is striking and suggests that natural selection has shaped the evolution of supercooling. Thus, supercooling is likely to be important for survival in these populations. Previous studies have also reported divergence of supercooling among populations of *Pieris rapae* (Li et al., 2020); however, it is important to note that supercooling point does not necessarily match the local environment. *Pieris rapae* pupae from London, Ontario, Canada had supercooling points that matched the local environment from which they were collected—i.e., average SCP was -24°C and the average extreme minimum winter temperatures of London, Ontario are -23°C to -20°C (Natural Resources Canada, http://www.planthardiness.gc.ca/, accessed February 2020; Li et al., 2020). However, this study also showed that a population of *P. rapae* from eastern Siberia had a relatively high SCP (-9.3°C), despite the harsh winter temperatures characteristic of that region (Li et al., 2020). Not surprisingly, given this high SCP, the Siberian pupae were freeze tolerant (i.e., they did not die when exposed to temperatures below their SCP), whereas pupae from Ontario were not freeze tolerant. Thus, *P. rapae* from eastern Siberia possess cold tolerance mechanisms that are distinct from

supercooling. Nonetheless, cold tolerance of Siberian *P. rapae* was influenced by thermal acclimation, suggesting that mechanisms other than supercooling could be affected by winter warming scenarios.

**Metabolomes underlie supercooling**

To our knowledge, our study represents the broadest survey of the metabolomic basis of supercooling to date; untargeted metabolomics allowed us to identify more than 14,000 metabolite features and directly correlate metabolite abundances to supercooling point. Our results reveal three major findings. First, supercooling in *P. rapae* diapausing pupae may be influenced by the abundances of thousands of metabolites, as a large proportion of the variance (27%) in abundances of metabolites across the whole metabolome and among pupae significantly correlated to supercooling point. Previous work has established a solid paradigm for interpreting the relationship between cold tolerance traits and metabolite abundance. Overwintering insects accumulate higher concentrations of key metabolites, or cryoprotectants, to lower the freezing point of intra- and extra-cellular solutions (Bale, 2002; Storey and Storey, 1988; Storey and Storey, 1991). Thus, it is perhaps not surprising to see correlations between metabolite abundance and supercooling, though most previous work has focused on characterizing a handful of candidate molecules. More recently, targeted metabolomics studies that measure tens-to-hundreds of metabolites simultaneously corroborate the relationship between metabolite abundance and various cold tolerance traits, including supercooling (Koštál et al., 2007; Koštál et al., 2011; Lehmann et al., 2018; Michaud and Denlinger, 2007). Our data provide a broadscale perspective on the key role of the metabolome in setting lower

thermal limits, which extends previous work to implicate the involvement of a wide array of molecular players.

Second, our data confirm the role of cryoprotectants in lowering the supercooling point, and that winter warming caused cryoprotectants to decrease in abundance. Pupae that had the lowest supercooling points also had the highest abundances of metabolites in pathways involved in the synthesis of putative cryoprotectants, including β-alanine metabolism, fructose and mannose metabolism, and glycine, serine and threonine metabolism (Fig. 3). Furthermore, warming caused the abundances of metabolites in these pathways to decrease. In accordance with these results, putative cryoprotectants that are produced by these pathways—i.e., β-alanine, sorbitol, and glycine—were of highest abundance in pupae with the lowest supercooling points (Fig. 4), and warmed pupae had some of the lowest levels of these compounds, particularly at day 50 in diapause (Fig. 4). These patterns suggest that the lack of these compounds may have driven the significantly higher internal freezing temperatures of warmed individuals on day 50 (Figure 1A). This fits with previous reports because β-alanine, sorbitol, and glycine have been shown to lower internal freezing point and increase in abundance in response to cold exposure in many insect species (Michaud et al., 2008; Michaud and Denlinger, 2007; Storey and Storey, 1990). In addition, sorbitol and glycine have been shown to stabilize macromolecular structures like proteins (Street et al., 2006; Yancey et al., 1982), suggesting other potential benefits of these compounds in addition to freezing point depression. An alternative hypothesis on the role of β-alanine is that it aids in survival at low temperatures by acting as an alternative end-product to lactic acid in anaerobic metabolism (Denlinger and Lee, 2010). However, this is an unlikely explanation for our

data, as the pattern of abundance of lactate (within the pyruvate metabolism pathway) across pupae is nearly identical to that of β-alanine—i.e., pupae with the lowest supercooling points had both higher β-alanine and higher lactate abundance levels relative to pupae with higher supercooling points.

Third, chronic winter warming (+3°C) caused shifts in core metabolic pathways, suggesting that even subtle changes in temperature cause changes in metabolism during diapause. Overwhelmingly, warming caused metabolite abundances to decrease; for example, metabolites within the fructose and mannose metabolism pathway, glycerolipid metabolism pathway, and pyruvate metabolism pathway were all significantly higher in control individuals (Fig. 3). Previous work has shown that metabolomes are dynamic, shift throughout diapause, and respond to temperature (Koštál et al., 2007; Koštál et al., 2011; Lehmann et al., 2018; Michaud and Denlinger, 2007). We noted shifts in the metabolome through time, as the second main axis (PC 2), which accounted for approx. 10% of the variation in metabolomic profiles among pupae, separated day 25 pupae from day 50 and day 75 pupae. But regardless of these metabolomic shifts that occurred through diapause, many of the changes in metabolomic profiles were induced by warming, particularly at day 50 in diapause. Moreover, many of the pathways that shifted in response to warming are involved in energy metabolism, such as pyruvate metabolism. Warming-induced decreases in the metabolites involved in pyruvate metabolism could indicate alterations in glycolysis or glycogenolysis, suggesting that ATP generating pathways could respond to winter warming exposure (Denlinger and Lee, 2010). This is of particular concern because diapausing pupae should be metabolically quiescent (dormant) and able to maintain stable metabolism throughout diapause. Yet, if warming

caused increases in energy metabolism that led to the decreased abundance of metabolites in ATP generating pathways, then this may mean that diapausing pupae deplete their energy reserves in response to winter warming. In addition, the maintenance of cold tolerance during diapause is dependent on the availability of energy reserves, as fuel sources (lipids, carbohydrates, and amino acids) are also used as anti-freezing cryoprotectants (Denlinger, 2002; Hahn and Denlinger, 2010; Storey and Storey, 2012).We did not assay total lipid or sugar content, nor did we measure metabolic rates; thus, the significance of these findings remains unresolved. Nevertheless, the characterization of energetics in the context of winter warming is likely to be a worthwhile avenue of future research.

Lastly, untargeted metabolomics elucidated patterns of metabolite abundances that we would not have otherwise seen if we had taken a targeted metabolomics approach. For example, the arachidonic acid metabolism pathway was the only pathway in which metabolites exhibited increased abundances in warmed pupae (Fig. 3). The specific function of arachidonic acid in diapausing *P. rapae* pupae remains to be determined, but based on what is known about the role of arachidonic acid in hibernating mammals, this result suggests that winter warming may impact the utilization of energy stores. Arachidonic acid is a long-chain fatty acid that has been shown to regulate the activity of peroxisome proliferator-activated receptor $\alpha$, a protein involved in mobilizing lipid stores (Wu et al., 2001) and a key regulator of lipid metabolism upon entrance into hibernation in ground squirrels, *Spermophilus tridecemlineatus* (Buck et al., 2002). Functional data on arachidonic acid in insects is lacking; thus, the potential role of arachidonic acid in regulating energetic processes during diapause remains obscure. It has

been shown that arachidonic acid is a key polyunsaturated fatty acid in the cellular membrane phospholipids of *Manduca sexta* (Ogg et al., 1991) and arachidonic acid is down-regulated in diapausing pupae of the flesh fly (*Sarcophaga crassipalpis*) following acute cold stress (Michaud and Denlinger, 2006). But, future study is needed to unravel the potential role of arachidonic acid in the context of diapause and environmental change in insects.

**Winter warming: good or bad?**

Whether winter warming will benefit or hinder overwintering organisms is currently under debate. Some research argues that warmer winter temperatures will result in beneficial effects on temperate species, as these warmer patterns could lead to increased survival, decreased cold-induced stress, and the ability to expand geographic ranges (Crozier, 2003). Cold stress lowers survival in ectothermic organisms; thus, the predicted 1°C-5°C increase in winter temperatures could increase survival through winter (Bale and Hayward, 2010). Although this prediction may be true for some species, based upon the data we present herein, not all overwintering organisms will benefit from winter warming. In *Pieris napi*, previous research has shown that chilling and cold temperatures are needed for endogenous diapause to maintain its developmental trajectory and to progress to post-diapause quiescence for spring emergence (Lehmann et al., 2017; Posledovich et al., 2015). One consequence of warming could be the lack of this transition into post-diapause development, leading to a longer diapause state, with cascading consequences. Anecdotally, in our study, warmed individuals had decreased

eclosion success relative to control individuals (unpublished data); thus, supporting these predictions.

One advantage of winter warming may be the earlier spring emergence of insects, including many butterfly and bee species (Bartomeus et al., 2011; Bosch, 2003); however, this phenomenon has potentially negative effects if it causes asynchrony with insects' host plants or if individuals experience severe environmental conditions post-emergence. It is more likely that warming will lead to entire shifts in the diapause program, including delays to diapause entry, decoupling of environmental cues (temperature and photoperiod) that maintain diapause, and/or the elimination of diapause completely (Bale and Hayward, 2010; Hodek and Hodkova, 1988). Studying diapause and other overwintering programs presents an opportunity to not only understand developmental biology across seasons, but to better predict the future of overwintering organisms as seasonal variation increases (Denlinger, 2008; Sinclair et al., 2003).

## CONCLUSION

Our study provides a molecular physiological perspective on the effects of temperature on the physiology of overwintering insects, thus providing insights into the challenges that species may endure as global winter temperatures increase and fluctuate with climate change. Future research exploring the effects of winter warming on overwintering organisms should address not only the direct effects of warming on physiological mechanisms and maintenance, as measured here, but also pre and post-winter development and subsequent reproductive success in the spring. Furthermore, research should focus on exposing populations to both local warming patterns and reciprocal transplant experiments to better understand population-level responses. This will allow us

to better predict the adaptive potential of populations of conspecifics. Such work will further our understanding of how winter warming will alter overwintering strategies.

## FIGURES AND TABLES

**Table 1: Burlington, Vermont climate data for winter months in 2019 and 2020.** Data represent observed monthly average winter temperatures (°C), as well as their deviation from the historic 40-year average. Also represented are the historic, highest recorded temperatures observed in winter months. All data were obtained from the National Weather Service Forecast Office. https://w2.weather.gov/climate/local_data.php?wfo=btv).

| Month | Monthly Average Temperature | Deviation from Historic Average Temperature (1981-2010) | Record High Observed Temperature |
|---|---|---|---|
| November 2019 | 0.5°C | -3.0°C | 23.9°C |
| December 2019 | -2.5°C | +0.9°C | 20.0°C |
| January 2020 | -3.3°C | +4.1°C | 18.9°C |
| February 2020 | -3.9°C | +1.9°C | 22.2°C |

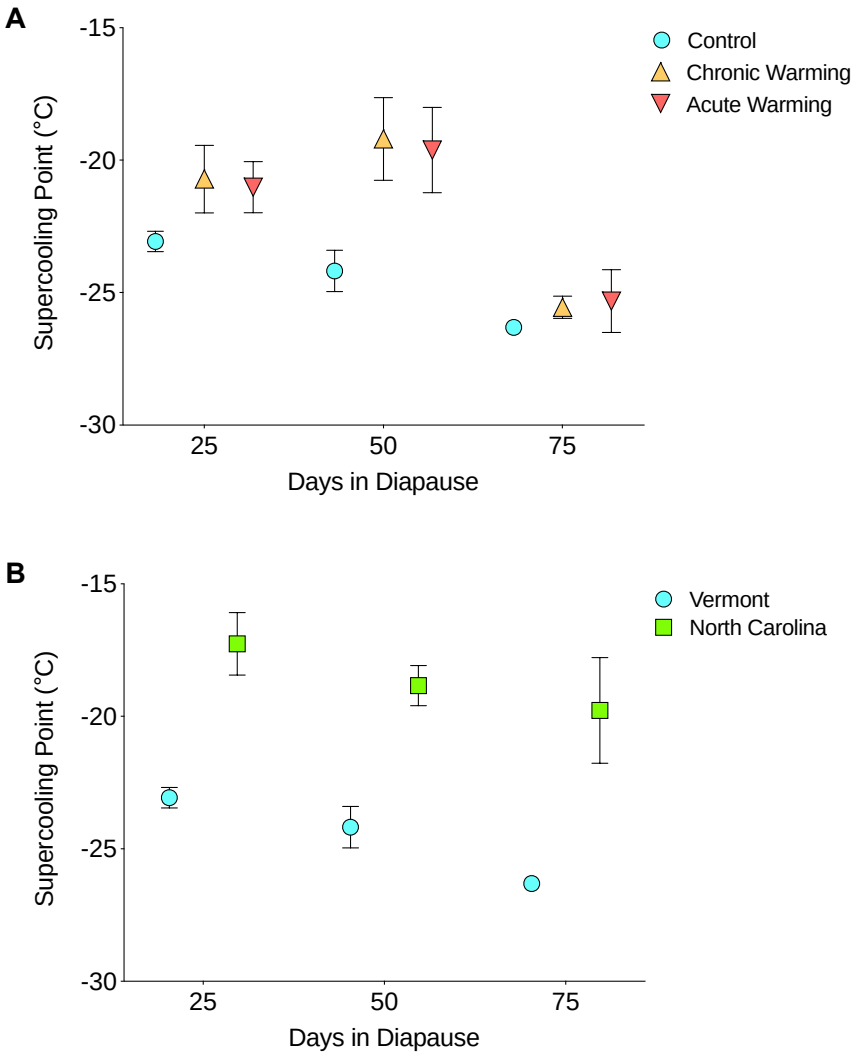


**Figure 1. Supercooling points (SCP) of diapausing *Pieris rapae* pupae exposed to winter warming conditions and from Vermont and North Carolina populations.** (A) SCPs (internal freezing temperature) were higher in pupae exposed to winter warming (2-way ANOVA; temperature factor, $F_{2,43}$=6.566, $P$=0.0032; day factor, $F_{2,43}$=19.43, $P$<0.0001; temperature x day interaction, $F_{4,43}$=1.217, $P$=0.3176; post-hoc Dunnett's multiple comparison test (Day 25 – control vs. acute warming, $P$=0.2945, control vs. chronic warming, $P$=0.1610; Day 50 – control vs. acute warming, $P$=0.0070, control vs. chronic warming, $P$=0.0079; Day 75 – control vs acute warming, $P$=0.6917, control vs.

chronic warming, $P$=0.8026. Diapausing pupae were exposed to one of three temperature treatments: control (4-7°C, n=18), acute warming (18-23°C, n=17), or chronic warming (7-11°C, n=17). SCP was measured on Days 25, 50, and 75 after diapause induction for the control and chronic warmed pupae, and 24-hr post warming (Days 26, 51, and 76) for the acute warmed individuals. SCP is presented as mean freezing temperature (°C) ± standard error of the mean, error bars for control individuals at Day 75 too small to be visible. (B) SCPs (internal freezing temperature) were higher in pupae from North Carolina (2-way ANOVA; population factor, $F_{1,26}$=67.35, $P$<0.0001; day factor, $F_{2,26}$=5.459, $P$=0.0105; population x day interaction, $F_{2,26}$=0.2307, $P$=0.7956. Diapausing pupae were exposed to control temperature regime (4-7°C) Vermont (n= 18) and North Carolina (n=14). SCP was measured on Days 25, 50, and 75 after diapause induction. SCP is presented as mean freezing temperature (°C) ± standard error of the mean, error bars for control individuals at Day 75 too small to be visible.

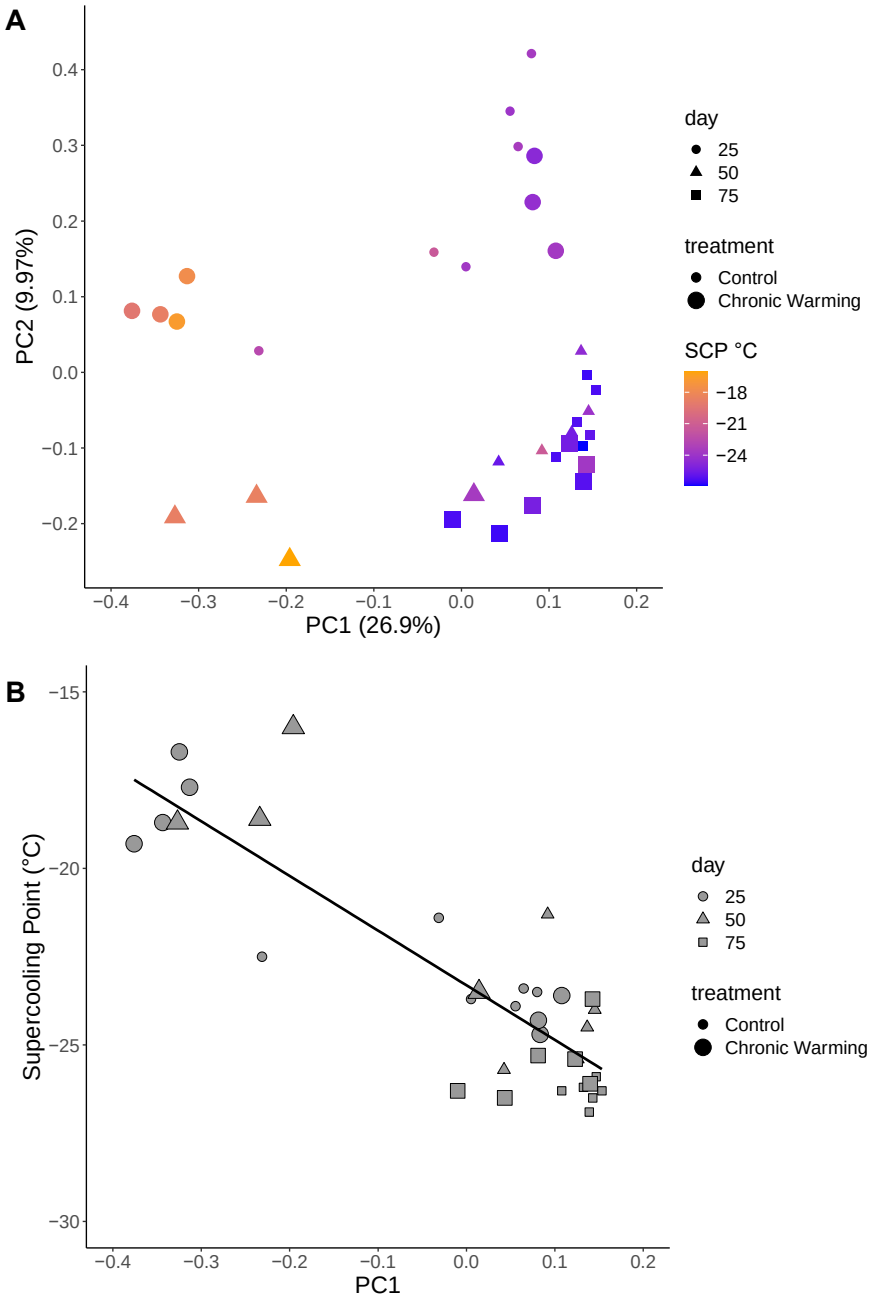


**Figure 2. Whole metabolomes cluster by supercooling point (SCP) and days in diapause.** (A) Principal components analysis of normalized intensities of 14,379 metabolite features among 34 pupae. Each point represents the metabolome of an individual pupa, collapsed in principal component space for the first two principal

components that describe 37% of the variation among metabolomes. 26.9% of the variation in metabolomes (PC1) separates pupae by SCP, and 9.97% of the variation (PC2) separates pupae by days in diapause. Day in diapause is indicated by shape, warming treatment is indicated by size, and SCP is indicated by color. (B) Variation among metabolomes is strongly correlated to supercooling point (SCP) (Least-squares linear regression of PC1 on SCP, $y = -15.48x - 23.3$, $R^2=0.73$, $P<0.00001$). Day in diapause is indicated by shape and warming treatment is indicated by size.

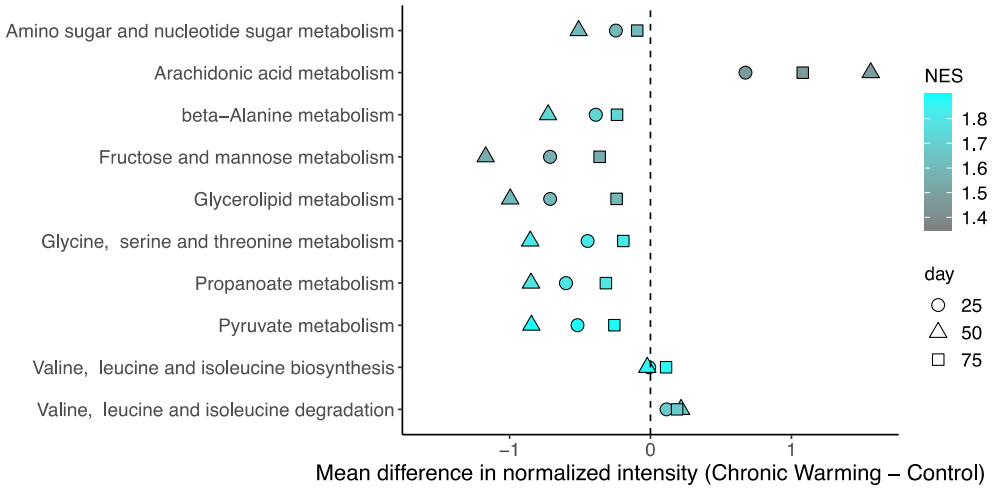


**Figure 3: Pathways significantly changed in response to winter warming.** Mean differences in normalized intensity (Chronic warmed – Control), averaged among all features in a given pathway and among pupae in a given day in diapause, for 10 pathways whose member KEGG compounds showed significant differences in normalized intensity in winter-warmed pupae. Represented in the 10 pathways are 153 features that mapped to 81 annotated KEGG compounds. Positive values (x-axis) indicate higher abundances of metabolites in warmed pupae, and negative values indicate lower abundances in warmed pupae, relative to controls. Days in diapause are indicated by the shapes, and normalized enrichment score (NES) is indicated by the color scale. Pathways with higher NES reflect greater proportions of metabolites that were found to be overrepresented in the pathway enrichment analysis. Pathways are listed in alphabetical order.

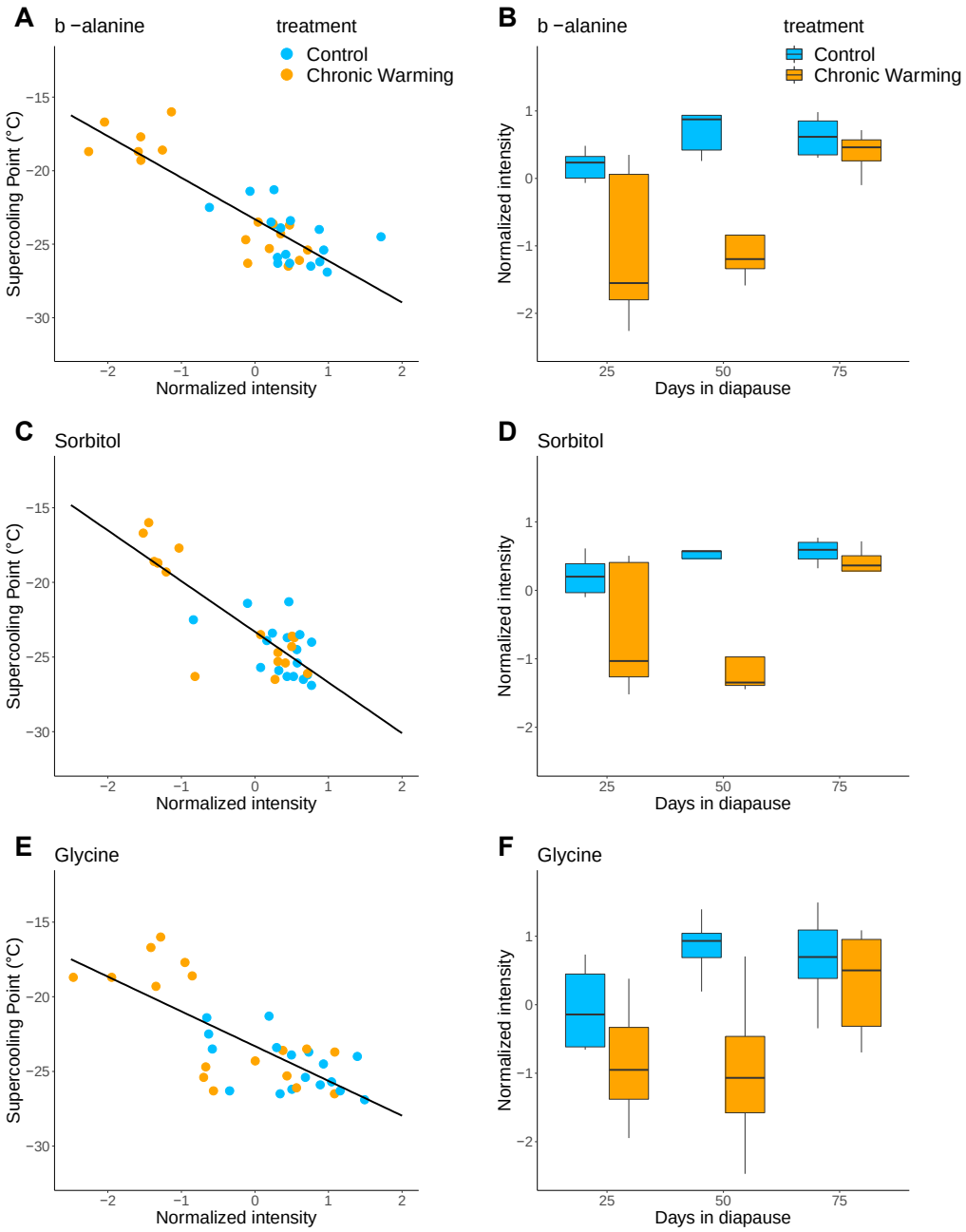


**Figure 4: Abundances of putative cryoprotectant metabolites were negatively correlated with supercooling point and reduced after winter warming.**

(A) β-alanine normalized intensity was negatively correlated with SCP (Least-squares linear regression, $y$ = -2.83$x$ – 23.31, $R^2$ = 0.72, $P$ < 0.00001). (B) β-alanine normalized

intensity was reduced after warming (2 way-ANOVA; temperature factor, $F_{1,30} = 17.16$, $P = 0.0003$; day factor, $F_{1,30} = 12.00$, $P = 0.002$; temperature x day interaction, $F_{1,30} = 2.34$, $P = 0.14$). (C) Sorbitol normalized intensity was negatively correlated with SCP (Least-squares linear regression, $y = -3.4x - 23.31$, $R^2 = 0.70$, $P < 0.00001$). (D) Sorbitol normalized intensity was reduced after warming (2 way-ANOVA; temperature factor, $F_{1,30} = 11.11$, $P = 0.002$; day factor, $F_{1,30} = 5.84$, $P = 0.02$; temperature x day interaction, $F_{1,30} = 0.10$, $P = 0.62$). (E) Glycine normalized intensity was negatively correlated with SCP (Least-squares linear regression, $y = -2.33x - 23.31$, $R^2 = 0.55$, $P < 0.00001$). (F) Glycine normalized intensity was reduced after warming (2 way-ANOVA; temperature factor, $F_{1,30} = 11.36$, $P = 0.002$; day factor, $F_{1,30} = 4.52$, $P = 0.02$; temperature x day interaction, $F_{1,30} = 2.30$, $P = 0.12$). Data represent mean normalized intensity of all features that matched a given metabolite (β-alanine: 3 features, sorbitol: 11 features, glycine: 1 feature).

**Table S1. KEGG pathways significantly enriched in the metabolomic response to winter warming (MS Peaks to Pathways, MetaboAnalyst)(Chong et al. 2019).**

| Pathway | Total Compounds | Compounds in Dataset | *P-val* | Adjusted *P-val* | NES |
|---|---|---|---|---|---|
| Glycine, serine and threonine metabolism | 25 | 17 | 0.010 | 0.079 | 1.81 |
| Amino sugar and nucleotide sugar metabolism | 34 | 20 | 0.010 | 0.079 | 1.607 |
| Pyruvate metabolism | 24 | 15 | 0.010 | 0.079 | 1.885 |
| Fructose and mannose metabolism | 18 | 13 | 0.010 | 0.079 | 1.562 |
| beta-Alanine metabolism | 13 | 8 | 0.011 | 0.079 | 1.724 |
| Valine, leucine and isoleucine degradation | 35 | 10 | 0.011 | 0.079 | 1.676 |
| Valine, leucine and isoleucine biosynthesis | 13 | 9 | 0.011 | 0.079 | 1.869 |
| Propanoate metabolism | 18 | 9 | 0.011 | 0.079 | 1.786 |
| Glycerolipid metabolism | 16 | 6 | 0.011 | 0.079 | 1.604 |
| Arachidonic acid metabolism | 10 | 2 | 0.014 | 0.087 | 1.473 |

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# CHAPTER 3: WINTER WARMING INCREASES VARIANCE IN METABOLIC RATE IN DIAPAUSING *PIERIS RAPAE* BUTTERFLIES

## ABSTRACT

Due to harsh environmental conditions and limited food availability, overwintering organisms are dependent on a store of energy reserves to sustain them until spring. To compensate for this limitation, metabolic activity of overwintering ectotherms is characteristically low. Winter warming associated with global climate change is expected to increase metabolism, and thus risk exhaustion of energetic reserves, due to increases in biochemical reaction rates. We used stop-flow respirometry to measure metabolic rate in response to temperatures representative of both acute and chronic winter warming events in diapausing *Pieris rapae* pupae. Metabolic rate increased with increasing temperature in diapausing individuals during warming exposure, indicating that metabolic rates in diapausing pupae are thermally sensitive to winter warming early in diapause, despite pupae exhibiting metabolic quiescence later in diapause. Yet, despite the increase in metabolic rates during warming, pupae did not exhibit cumulative changes in metabolic rate after weeks under warming conditions, and pupae recovered metabolic rates within 24-hours after short-term acute warming exposure. However, both chronic and acute warmed pupae had higher variance in metabolic rates relative to control individuals. Respiratory quotients indicated that warmed individuals also shifted their energy store usage from carbohydrates to proteins and/or mixed fuel sources during winter warming exposure, which could have downstream costs for diapause maintenance and adversely affect spring eclosion. These results suggest that diapausing individuals exposed to

variable winter temperatures could be vulnerable to metabolic challenges, thus, providing insight into the physiological consequences of winter warming on overwintering insects.

## INTRODUCTION

The latest climate report from the UN's Intergovernmental Panel on Climate Change estimates a global 1.0°C increase in average atmospheric temperatures since the industrial revolution, and predicts an additional increase of 1.5°C to 2.0°C by the year 2052 if global warming continues at its current rate (Allen et al., 2018). These increases in environmental temperatures may be particularly stressful for ectothermic organisms due to the temperature-dependency of their physiology (Deutsch et al., 2008; Huey et al., 2012; Somero, 2010). Acute increases in temperature within an organism's natural range can lead to two to threefold increases in rates of biochemical reactions (Schulte, 2015; Tattersall et al., 2012). To cope with thermal challenges induced by acute increases in temperature, changes in biological processes such as development, metabolism, and thermal tolerance have been observed in ectotherms (Colinet et al., 2015; García-Robledo et al., 2016; Robinet and Roques, 2010). These changes are perhaps not surprising, given that with each 1°C increase in environmental temperature metabolism increases by approximately 8% in ectotherms (Somero et al., 2017).

Although thermal challenges will likely be faced by organisms across space and time, both rates of environmental change and species vulnerability to warming are expected to vary by season (Bale and Hayward, 2010; Bradshaw and Holzapfel, 2010; Hahn and Denlinger, 2007). In fact, winters are warming faster than other seasons (NOAA, https://www.ncdc.noaa.gov/sotc/national/202001, accessed February 2020;

NOAA, https://www.ncdc.noaa.gov/cag/, accessed February 2020), with hotter mean temperatures, increased variability and a higher frequency of extreme, temperature anomalies (Allen et al., 2018). Winter warming has the potential to negatively impact temperate ectothermic organisms, but this has only been explored in a few studies (Arambourou and Robby, 2015; MacLean et al., 2017; Williams et al., 2012). Thus, there remains a large gap in our knowledge about the potential responses of insects to warmer winters.

Many temperate insects survive the winter by entering diapause (Denlinger and Lee 2010; Storey and Storey, 2012), a state of metabolic dormancy to conserve accumulated energetic reserves including lipids, carbohydrates, and amino acids (Hahn and Denlinger, 2011; Tomčala et al., 2006; Zhou and Miesfeld, 2009). Of these energetic reserves, most are predetermined and/or allocated for specific aspects of diapause maintenance or termination (Hahn and Denlinger, 2007; Hahn and Denlinger, 2011). Thus, most individuals face limited food availability in winter because they enter diapause with a fixed amount of energy reserves. Therefore, the energetic reserves that are present at the onset of dormancy must sustain insects not only through dormancy, but also post-dormancy, which for diapausing insects can include energetically costly processes such as metamorphosis, flight, and reproduction (Hahn and Denlinger, 2007; Ragland et al., 2009). Insects who either enter diapause with fewer reserves, run through reserves at a faster rate, or even switch to suboptimal reserves (i.e. reserves intended for a different process), could be more vulnerable to shifts in biochemical processes to compensate for insufficient reserves. Consequently, this constraint could result in ending

diapause early or dying during diapause or shortly thereafter, due to insufficient energetic reserves (Hahn and Denlinger, 2011).

Hypometabolism during winter dormancy is sustained by low winter temperatures that lower metabolic rate via the $Q_{10}$ effect (Hahn and Denlinger, 2007; Hahn and Denlinger, 2011). For temperate species of insects, metabolic rates drop significantly while in diapause (Hoback and Stanley, 2001), but hotter temperatures threaten this pattern (Angiletta, 2009; Dillon et al., 2010). Increased temperatures for both field and laboratory populations of diapausing *Eurosta solidaginis* larvae led to higher metabolic rates while in diapause, with decreased survival or decreased fecundity post-diapause (Hahn and Denlinger, 2007; Irwin and Lee, 2000; Irwin and Lee, 2003). Longer, warmer prewinter temperatures paired with longer periods of winter led to decreased metabolic reserves and subsequent decreased survival in diapausing *Rhagoletis pomonella* pupae (Feder et al., 1997). Furthermore, increased variable temperatures in autumn led to higher metabolic rates and higher energy store loss in diapausing *Erynnis propertius* larvae (Williams et al., 2014). These studies provide evidence for the potential consequences of pre-winter warming on overwintering metabolic activity and energy metabolism; however, there remains a gap in our understanding of how warming during winter dormancy affects metabolism and the usage of energetic reserves in overwintering insects.

The cabbage white butterfly *Pieris rapae* is a cosmopolitan species of butterfly found across five continents in temperate and sub-tropical zones (Ryan et al., 2019). The overwintering physiology of *P. rapae*, including metabolic rate, cold tolerance, and metabolite levels, has been well described across various populations of the species (Li et

al., 2020; Mikucki and Lockwood, 2020 (in review). Thus, *P. rapae* presents an ideal model system for studying the effects of winter warming on overwintering biochemical functions such as metabolic activity and energy reserve usage. *Pieris rapae* enters facultative diapause during the pupal stage, spending months in dormancy until spring eclosion (Richards, 1940). This dormancy period is facilitated by elevated cold and stress tolerance, and depressed metabolic activity (Denlinger and Lee, 2010). Previously, we have shown that diapausing *P. rapae* pupae exposed to acute and chronic winter warming had less negative supercooling points, lowered abundances of cryoprotectant molecules, and shifted metabolomic profiles (Mikucki and Lockwood, in review). These winter-warming-induced metabolomic profiles suggest that metabolic rate, and perhaps energy reserve usage, may also be sensitive to winter warming.

Because metabolic activity is intrinsically dependent on temperature, we predicted that metabolic rate of diapausing pupae would increase under winter warming conditions, and that warmed individuals would alter their energy reserve usage to compensate for increased metabolic demand. To test this prediction, we measured the effects of acute and chronic increases in temperature, representing winter warming scenarios, on metabolic rate during and after exposure (i.e. recovery) to winter warming in diapausing *Pieris rapae* pupae. We used stop-flow respirometry, and thus, we measured both carbon dioxide production and oxygen consumption, enabling the calculation of respiratory quotient to infer the macromolecular sources (i.e., carbohydrate, lipid, and/or protein) of energy fuel usage. Thus, our study provides an ecologically relevant insight into the metabolic challenges to coping with current and future winter warming conditions that will expose diapausing insects to novel thermal extremes.

## MATERIALS AND METHODS

### Adult butterfly collections and juvenile stage rearing

We measured metabolic rates under three distinct experimental scenarios, each of which was conducted on separate sets of individuals. This was to avoid using pupae that had been exposed to another experimental condition that could impact their metabolic rate. We measured metabolic rates of diapausing *Pieris rapae* pupae under the following experimental scenarios: (1) in real-time during exposure to winter warming in early diapause (< 30 days), (2) after long-term chronic exposure to winter warming (over 90 days), and (3) 24 hours before and 24 hours after exposure to short-term acute winter warming at 3 timepoints during diapause (days 25, 50, and 75). To generate diapausing *Pieris rapae* pupae to test for the effects of real-time winter warming temperatures on thermal responses of metabolic rate (scenario 1), we collected approximately 40 adult *P. rapae* butterflies from two locations around Burlington, Vermont (44°28’51.94”N, 73°11’34.57”W and 44°20’46.59”N, 73°06’15.15”W) in July-August 2019. To generate diapausing *P. rapae* pupae to test the effects of winter warming patterns on metabolic rate after chronic warming exposure (scenario 2) and recovery from acute warming (scenario 3), we collected approximately 40 adult *Pieris rapae* butterflies from two locations in northwestern Vermont (44°29’48.52”N, 73°1220.19”W and 44°17’10.07”N, 73°14’07.11”W) in late September 2018. We kept all adults in mesh containers (Carolina Biological Supply, 11" diameter × 12" height, n=10 adults per container) under common garden conditions of 24°C, 12L:12D photoperiod, 55% relative humidity, and with direct access to sunlight in order to improve oviposition. We fed adults every 24 hours with a

diet of 10% honey solution on a sponge. After 48 hours, we isolated each female into an individual container with fresh, organic kale leaves for oviposition.

We collected fertilized eggs every 24 hours, and placed them into plastic containers (35.6cm length x 20.3cm wide x 12.4cm height; n≈20 eggs per container) in incubators (Percival model DR-36VL) with standard conditions of 24°C, 8L:16D photoperiod to induce pupal diapause, and 55% relative humidity. We fed larvae fresh organic kale leaves every day. Individuals were reared under these conditions until pupation, approximately 14 days post-oviposition. Upon pupation we placed all pupae into individual petri dishes (60 x 15mm) then we haphazardly moved them into temperature treatments, with offspring from each mother represented in each treatment.

**Temperature treatments for diapausing *P. rapae* pupae**

After pupation, all pupae were shipped to The University of Nebraska – Lincoln where all metabolic rate measurements were conducted. We acquired all necessary permits for transportation of live *P. rapae* from Burlington, Vermont to Lincoln, Nebraska (USDA permit P526P-16-02649). Over one day of travel, pupae were transported on ice, in coolers at 4°C with a photoperiod of 8L:16D kept using LED lights on timers. We subjected diapausing pupae to temperature treatments reflective of historic, current and predicted winter warming scenarios in Vermont USA during the winter months (November-February) (Table 1, see Chapter 2). We determined all winter warming treatments from historic data records from a local weather station in Burlington Vermont (National Weather Service Forecast Office, https://w2.weather.gov/climate/local_data.php?wfo=btv, accessed 2020) and from global

climate predictions (Allen et al., 2018) (Table 1). To determine metabolic rates during real-time exposure to winter warming temperatures (scenario 1), we kept all pupae at 4°C (in both Vermont and Nebraska) until metabolic rate measurement (Figure 1A). We note here, that early diapausing pupae (< 30 days old) were used in experimental scenario 1. The winter warming experiments (scenarios 2 and 3) consisted of control, chronic warming, and acute warming treatments. In Vermont, we kept the control group pupae under a temperature regime with daily fluctuating temperature 4°C-8°C, representing autumn temperatures when individuals first enter diapause, once in Nebraska, these individuals were kept in a temperature regime with a mean of 4°C (Figure 1B). In Vermont, we kept the chronic warming group pupae under a temperature regime of 7°C-11°C, representing a 3°C increase from the control; once in Nebraska the chronic warmed group were kept in a temperature regime with a mean of 8°C (Figure 1B). This pattern reflects both the long-term warming pattern seen in Vermont over the last 50 years, as well as the predicted, continued pattern expected for the next 50 years (Allen et al., 2018; National Weather Service Forecast Office, retrieved 2020). In Vermont, we kept the acute warmed pupae under the control conditions of daily fluctuating 4°C-8°C; once in Nebraska, they were kept in a temperature regime with a mean of 4°C with three one-day warming events with a mean of 23°C on Days 25, 50 and 75 (Figure 1C). This pattern reflects short-term warming events observed in Vermont during the winter months. For scenarios 2 and 3, we used diapausing pupae between the ages of 25 and 90 for metabolic rate measurements. We kept pupae used in all experiments under an 8L:16D photoperiod to maintain diapause.

**Metabolic rate measurements**

We weighed all pupae prior to metabolic rate analysis. To test for differences in metabolic rates across treatments, we estimated the scaling relationship between pupal mass and metabolic rate ($CO_2$ and $O_2$) using a Type II model regression in the "smatR" package in R (Wat et al., 2020). We found no effect of weight on metabolic rate for all temperature treatments ($P$=0.97 ($CO_2$); $P$=0.60 ($O_2$)), and thus, exclude weight as a factor in all further analyses. To measure metabolic rate, we used stop-flow respirometry (Sable Systems International, Las Vegas, NV) to measure carbon dioxide produced ($\mu lCO_2$/hr) and oxygen consumed ($\mu l$ $O_2$/hr) for all individuals. We pushed air through plastic respirometry chambers at a rate of 75mL/min. Any water vapor in the air was removed using magnesium perchlorate, and the levels of $CO_2$ and $O_2$ were measured using a Licor 7000 infrared detector (Licor, Lincoln, NE). We used the RM8 Intelligent Multiplexer to sequentially measure the airstream of eight respiratory chambers (Sable Systems International, Las Vegas, NV), seven of which had individual pupae, and one being an empty baseline chamber. For each experimental run, we measured the $CO_2$ and $O_2$ of individual pupae, each sampled three times for 120 minutes over a 360-minute period. For each individual, we used the final reading (3rd reading) for all analyses because it showed the most consistent measurement across replicate measures of a particular individual.

To measure metabolic rate during real-time winter warming (scenario 1), we placed individual pupae into 5-ml syringes in a circulating water bath set to one of three temperatures, 4°C, 11°C and 23°C, which represent the coldest temperature individuals were exposed to in the control group, the hottest temperature of the chronic warming

treatment (scenario 2), and the hottest temperature of the acute warming treatment, respectively (scenario 3). At 23°C, metabolic activity, $CO_2$ production and $O_2$ consumption, was measured for 15 diapausing pupae. At 4°C and 11°C, $CO_2$ production was measured for 15 individuals at each temperature. However, the $O_2$ consumption rate was too low to be detected for five individuals, so we only have $O_2$ consumption rates for 10 individuals at 4°C and 11°C. For this experiment, individuals were only measured once.

To measure metabolic rate in the chronically warmed pupae, after recovery from acute winter warming exposure, and in control animals (scenarios 2 and 3), we measured metabolic rate as described above, but we set the water bath temperature to 8°C, which was a temperature shared by all three winter warming regimes: control, chronic warmed, and acute warmed. Since control and chronic warmed individuals were kept under constant conditions (i.e. no warming events like the acute warmed treatment), we aimed to measure the change in metabolic rate over days in diapause, and not on set days like in the acute warming treatment. For the control and chronic warmed individuals, we measured metabolic rate at intervals over diapause, encompassing Day 30 to Day 90 (n=21 pupae per treatment). Again, in order to understand individual responses in metabolic rate to winter warming, we included individual as a random effect in our mixed effects model with repeated measures. However, due to the limitation of the respirometry equipment (we could only measure seven individual pupae per day) and the age of each pupae (many pupae had the same age), some individuals had repeated metabolic rate measurements over time while other individuals did not.

For the acute warmed treatment, we measured metabolic rate 24-hours pre and 24-hours post warming events on Days 25, 50 and 75 to characterize the change and/or recovery in metabolic activity due to the warming event (n=28 pupae). We wanted to understand individual responses in metabolic rate to winter warming, Thus, we included individual as a random effect in our mixed effects model with repeated measures. However, as stated above, we were limited in how many individuals we could measure at one time, and consequently, we have repeated metabolic measurements at each timepoint (Days 25, 50 and 75) for some individuals, while we do not have repeated measures for other individuals at each timepoint.

**Statistical analysis of metabolic activity**

We first analyzed all respirometry data using the Expedata software package (v. 1.1.15; Sable Systems International, Las Vegas, NV). We used the $CO_2$ and $O_2$ values from the baseline chamber to drift-correct metabolic activity of each individual, and then converted all raw metabolic activity values from parts per million to µL/hr (Hoekstra et al., 2018; Wat et al., 2020).

To determine whether metabolic rate changed during real-time winter warming exposure, we ran a Type II two-way Analysis of Variance (ANOVA), incorporating the fixed effects of temperature and age. We analyzed the relationship between age and metabolic rate for real-time winter warming exposure, and compared the slopes for all three temperatures using linear regressions. We also ran a Bartlett's test of homogeneity of variances to determine significant differences in variance (spread of data) between metabolic activity at the different temperatures. We further calculated $Q_{10}$ coefficients, or

the rate of change in activity based on an increase in temperature, using the formula: $Q10 = (\frac{R_2}{R_1})^{\frac{10}{(T_2-T_1)}}$, where $R_2$ was the average metabolic activity at the higher temperature ($T_2$), and $R_1$ was the average metabolic activity at the lower temperature ($T_1$).

By using stop-flow respirometry, we were also able to calculate the respiratory quotient (RQ), or the ratio of moles of carbon dioxide produced to moles of oxygen consumed ($CO_2/O_2$) for each individual. Here, we defined the RQ as the slope of the linear regression from the relationship between carbon dioxide production and oxygen consumption, as described in Berggen et al. 2012 and Münzer et al. 2019 (Berggren et al., 2012; Münzner and Berggren, 2019). Furthermore, we used respiratory quotients to infer which macronutrient energy stores pupae were using at the time of metabolic activity measurements which is determined from the relative number of carbons required to break down each macronutrient (Patel and Bhardwaj, 2019). A RQ value of 1 (equal ratio of $CO_2$ produced to $O_2$ consumed) indicates carbohydrates as the primary metabolic fuel, while a RQ value less than 1 indicates fat (≈ 0.7) or protein/mix of macronutrients (≈ 0.8) utilization (Hahn and Denlinger, 2011; Wat et al., 2020). All analyses were performed in R v. 3.6.2.

We used a log-likelihood ratio test to determine significant differences in metabolic rate due to treatment between the control and chronic warmed individuals. In R, using the lmer and nlme functions from the packages “lme4” and “nmle”, we ran a mixed effects models with repeated measures for metabolic activity recovery for the control and chronic warmed individuals after winter warming exposure, including the fixed effects, Day and Treatment, and the random effect, Individual. We compared models with and without the Treatment fixed effect, and due to the lack of statistical

significance between models and based on the given AIC values, we used the simpler model, excluding Treatment as an effect (Linear mixed model fit with REML; $CO_2$, $T$=4.35, $P$=0.0001; $O_2$, $T$=4.62, $P$<0.0001). We further ran a Bartlett's test of homogeneity of variances to determine significant differences in variance between the control and chronic warmed treatments.

Similarly, for the acute warmed individuals, we used a log-likelihood ratio test to determine significant differences in metabolic rate due to warming (pre and post). We ran a mixed effects model with repeated measures for metabolic activity recovery for the acute warmed individuals, pre and post warming events, which included the fixed effects, Day and Warming (pre or post), and the random effect, Individual. We compared models with and without the Warming fixed effect, and due to the lack of statistical significance between models and based on the given AIC values, we used the simpler model, excluding Treatment as an effect (Linear mixed model fit with REML; $CO_2$, $T$=5.34, $P$<0.0001; $O_2$, $T$=5.88, $P$<0.0001). We further ran a Bartlett's test of homogeneity of variances to determine significant differences in variance between the pre and post-acute-warmed treatments. Lastly, for the control and winter warming recovery treatments, we also determined RQ values for each treatment, as described above, using the slope of the linear regressions on metabolic activity.

## RESULTS

Metabolic rate significantly increased with increasing temperature during exposure to winter warming (Figure 2A & B; Type II ANOVA, $CO_2$ temperature factor $F_{2,39}$ = 262.40, $P$ < 0.0001; $O_2$ temperature factor $F_{2,29}$ = 254.93, $P$ < 0.0001). An increase of 7°C, from 4°C to 11°C, led to a 4-fold increase in carbon dioxide production and over a

3-fold increase in oxygen consumption (Table 2). An increase of 12°C, from 11°C to 23°C, led to a 3-fold increase in carbon dioxide production and a near, 3-fold increase in oxygen consumption (Table 2). Older pupae had lower metabolic rates for both carbon dioxide consumption and oxygen consumption (Figure 2C & D; Type II ANOVA, $CO_2$ age factor $F_{2,39} = 54.25$, $P < 0.0001$; $O_2$ age factor $F_{2,29} = 67.96$, $P < 0.0001$). This pattern was observed across all temperatures, (Figure 2C & D ; Least-squares linear regression; $CO_2$: 4°C, $y$= -0.125x +3.398, $R^2$= 0.54, $P$=0.001; 11°C, $y$= -0.250x + 8.121, $R^2$=0.79, $P$<0.0001; 23°C, $y$= -1.005x + 30.38, $R^2$=0.73, $P$<0.0001; $O_2$: 4°C, $y$= -0.165x + 4.211, $R^2$=0.21, $P$=0.187; 11°C, $y$ = -0.242x + 9.446, $R^2$= 0.24, $P$=0.15; 23°C, $y$= -1.199+ 36.49, $R^2$=0.76, $P$<0.0001), but was strongest at 23°C as the magnitude of the temperature effect was dependent on age (Type II ANOVA; $CO_2$, temperature x age factor, $F_{2,39} = 25.74$, $P$<0.0001; $O_2$, temperature x age factor; $F_{2,29} = 7.90$, $P$=0.002). There was significantly higher variance of metabolic rate at hotter temperatures for both carbon dioxide production and oxygen consumption (Figure 2; Bartlett's test of homogeneity of variances, $CO_2$, $P$<0.0001; $O_2$, $P$<0.0001). The highest degree of variance for metabolic rate between individuals was at 23°C ($CO_2$, $\bar{x}$ = 21.337 ± 6.767 μl/hr; $O_2$, $\bar{x}$ = 25.699 ± 7.913 μl/hr) relative to the metabolic activity at colder temperatures (4°C – $CO_2$, $\bar{x}$ = 1.860 ± 1.643 μl/hr; $O_2$, $\bar{x}$ = 2.138 ± 1.781 μ/hr; 11°C – $CO_2$, $\bar{x}$ = 4.928 ± 2.562 μl/hr; $O_2$, $\bar{x}$ = 5.166 ± 4.014 μl/hr).

Carbon dioxide production was strongly correlated with oxygen consumption for all *P. rapae* pupae measured during winter warming exposure (Figure 3, Least-squares linear regression; 4°C: $R^2$=0.70, $P$=0.003; 11°C: $R^2$=0.94, $P$<0.0001; 23°C: $R^2$=0.96, $P$<0.0001). The differences in slopes suggest differential energy reserves at 4°C

(i.e. control) and at 11°C and 23°C (i.e. warmed conditions) (Figure 3; Least-squares linear regression; 4°C: $y$= 1.175x – 1.142*;* 11°C: $y$= 0.845x – 0.070; 23°C: $y$= 0.836x – 0.158). At 4°C, individuals had a RQ ≈ 1.2, which indicates the use of carbohydrates to fuel metabolic activity, while at 11°C and 23°C, individuals had a RQ ≈ 0.84, which indicates the use of proteins and/or mixed fuel sources to fuel metabolic activity (Hahn and Denlinger, 2007).

There was no significant difference in metabolic rate between control and cumulatively chronic warmed *P. rapae* pupae (Figure 4; Log-likelihood ratio test; $CO_2$: *Chi-sq* = 2.62; Treatment effect: *P*=0.27; $O_2$: *Chi-sq* = 3.43; Treatment effect: *P*=0.18). Metabolic rate decreased over time in both control and chronic warmed individuals (Figure 4; Linear mixed model with repeated measures, Day factor ($CO_2$) production, *P*=0.05; Day factor ($O_2$), *P*=0.02). However, there was a significant difference in variance for metabolic rates between control and chronic warmed individuals (Figure 3; Bartlett's test of homogeneity of variances, $CO_2$ production: *P*=0.03; $O_2$ consumption: *P*=0.05). The chronic warmed treatment had higher variance among individuals relative to control individuals for both carbon dioxide production (Control, 0.0381μl/hr ± 0.0372 ; Chronic warmed, 0.0421μl/hr ± 0.0555) and oxygen consumption (Control, 0.0388 μl/hr ± 0.0367; Chronic warmed, 0.0375 μl/hr ± 0.0530).

Acute warmed individuals recovered from heat stress as there was no significant difference in metabolic rate between the pre and post-acute treatments (Figure 5; Log-likelihood ratio test; $CO_2$: *Chi-sq* = 2.81; Treatment effect, *P*=0.24; $O_2$: *Chi-sq* = 3.19; Treatment effect, *P* = 0.20). There was also no difference in metabolic rate over the three timepoints for pre and post-acute warmed pupae (Figure 5; Linear mixed model fit with

REML, $CO_2$: Day factor: $P$=0.86; $O_2$: Day factor: $P$=0.87). Mean metabolic rate was similar for pre and post-acute warmed pupae, but there was higher variance in metabolic rate observed post warming for both $CO_2$ production and $O_2$ consumption (Bartlett's test of homogeneity of variances, $CO_2$: $P$=0.01; $O_2$: $P$=0.01).

There was a highly-significant positive relationship between oxygen consumption and carbon dioxide production for *P. rapae* pupae from the control treatment and for all winter warming treatments after recovery from winter warming stress (Figure 6, Least-squares linear regression, Control: $R^2$=0.91; $P$<0.0001; Chronic warmed: $R^2$=0.84; $P$<0.0001; Pre-acute warming: $R^2$=0.96; $P$<0.0001; Post-acute warming: $R^2$=0.96; $P$<0.0001). These relationships did not differ among treatments as all treatments had slopes of approx. 1, which indicates the use of carbohydrates as a fuel source at the time of metabolic rate measurement for all individuals, regardless of temperature treatment (Figure 6; Least-squares linear regression, Control: $y = 0.965x + 0.0007$; Chronic-warmed: $y = 0.963x + 0.006$; Pre-acute warming: $y = 1.02x - 0.0045$; Post-acute warming: $y = 1.06x - 0.0045$) (Hahn and Denlinger, 2007).

## DISCUSSION

Here, we determined the impact of winter warming on the metabolic rate of diapausing *Pieris rapae* pupae during and after exposure to acute and chronic thermal stress. We present evidence for increased metabolic rate as a thermal response to winter warming exposure and increased variation in metabolic rate after chronic warming and after recovery from acute winter warming. We further report a change in respiratory quotients, indicative of a switch from carbohydrate catabolism to protein and/or mixed energy fuel utilization, as yet another thermal response to winter warming. However, our results also

suggest the potential for diapausing individuals to recover metabolic activity levels and respiratory quotients after warming exposure, which could be a key coping strategy in nature if warming continues to increase during the winter months.

Because metabolic rate was thermally responsive in diapausing *P. rapae* pupae, winter warming could pose physiological challenges to this species. During exposure to 11°C and 23°C, we saw two to four-fold increases in carbon dioxide production and oxygen consumption, and such an exponential increase in respiration would require more fuel to meet the increased energetic demand. A depressed metabolic rate is a key trait for survival in insect species that diapause through the winter (Denlinger and Lee, 2010; Storey and Storey, 1990). Lower metabolic rates conserve limited energy reserves (Hahn and Denlinger, 2007; Hahn and Denlinger, 2011), and low temperatures directly depress metabolic rates during diapause, via the $Q_{10}$ effect, to levels lower than 10% of the metabolic rate of non-diapausing individuals (Storey and Storey, 2012; Hoback and Stanley, 2001). Similar increases in metabolic rate with increases in temperature have been shown in a population of diapausing *P. rapae* pupae from London, Ontario (Li et al., 2020). Thus, the patterns that we report on herein are likely to be indicative of a broadscale physiological response in this species and not specific to the population of *P. rapae* that resides in Vermont. However, it is important to note that over the longer term, our data indicate that the thermal effects of warming on metabolic rate diminish as pupae age, such that we did not see a significant effect of chronic warming on metabolic rate. Nor did we see a lasting effect of acute warming on metabolic rate after 24 hours of recovery in older pupae. The discrepancy between these sets of results may be reconciled

by considering the possibility that diapausing pupae become less thermally sensitive as diapause progresses (see below).

Age plays a strong role in individual responses to thermal stress on metabolic rate, which could dictate their vulnerability to winter warming. Our results suggest that diapausing pupae are most thermally sensitive early in diapause. It has been hypothesized that insects are insensitive to temperatures early in diapause (i.e. autumn) to avoid development at heightened rates before temperatures drop in winter (Lehmann et al., 2016; Tauber and Tauber, 1976). Our results contradict this observation, and instead support the prediction that individuals early in diapause are vulnerable to warming-induced changes in their metabolic rate. Recent climate reports are noting that winters are arriving later, and that individuals will be exposed to pre-winter warming temperatures (Sgolastra et al., 2011). Diapausing insects that rely on photoperiodic cues for diapause induction, rather than low temperatures, are likely to be subject to hotter temperatures in the early stages of their diapause program, and might suffer downstream consequences later on (Bale and Hayward, 2010). Previous work examining the effect of autumnal warming on diapausing *Erynnis propertius* larvae showed that warming early in diapause led to higher metabolic rate and energy store loss (Williams et al., 2014). Furthermore, longer winter warming duration, starting at the onset of overwintering, was found to negatively impact survival and fat body depletion in *Osmia lignaria* bees (Bosch and Kemp, 2003). Our study suggests that warming early in diapause may make individuals more vulnerable to increased metabolic rate. We support this pattern in two ways: (1) we observed higher metabolic rate early in diapause for both the real-time winter warming (scenario 1) and cumulative effect of chronic winter warming stress (scenario 2), and (2)

we observed that individuals were less thermally sensitive in their metabolic rate later in diapause (scenarios 1, 2, and 3, see below). It is important to note that our characterization of "early diapause" was distinct from "late diapause" because we used three sets of pupae for our winter warming scenarios: (1) real-time exposure to winter warming in early diapause (30 days), (2) after long-term chronic exposure to winter warming (90+ days) and (3) 24 hours after exposure to short-term acute winter warming (25-75 days). We further note that because we did not measure metabolic rates in real-time in response to increased temperature in later-stage diapause (scenario 1), we cannot say if the pupae warmed in early diapause had any downstream, negative effects on metabolic rate or other components of the diapause program. Thus, a limitation of the present study is that we do not know if we have fully characterized the potential overlap between these winter warming scenarios, which needs to be further explored.

Higher inter-individual variation in metabolic rate among warmed individuals constitutes increased phenotypic variance. This pattern was most pronounced early in diapause for chronic warmed pupae, which as stated above, suggests that pupae may be more vulnerable to thermally induced increases in metabolic rate early in diapause. In a previous study, we found that there was greater variation among supercooling points early in diapause (Day 25 and Day 50) for chronic warmed individuals as compared to supercooling points later in diapause (Day 75) (Mikucki and Lockwood, in review). These observations suggest that the timing of winter warming may be critical in determining the physiological costs of warming on diapausing individuals, and further suggest vulnerability at the onset of diapause due to winter warming before transition into diapause maintenance (Lehmann et al., 2018). Contrary to the chronic warming group,

higher variance among the post-acute warming individuals occurred later in diapause (Days 50 and 75). This pattern may have been driven by a few individuals with high post-warming metabolic rate. This suggests that different warming patterns have the same, general effect on metabolic rate (i.e., greater variance), but that it occurs at different points in diapause. Global climate change is leading to two main conditions of winter warming: (1) increased mean temperatures (i.e. chronic warming) and (2) higher frequency of hotter temperature anomalies (i.e. acute warming) (Allen et al. 2018). The worst-case scenario for overwintering individuals would be if these patterns co-exist together under future warming scenarios. Thus, diapausing insects may be at risk of having higher variance in metabolic rate during the early, mid, and even later stages of diapause, depending on the confluence of chronic and acute warming conditions, which could have downstream, negative effects on development, diapause termination, and spring eclosion (Hahn and Denlinger, 2007; Hahn and Denlinger, 2011; Lehmann et al., 2018).

Because warming increased phenotypic variance in metabolic rate, winter warming in nature may lead to the opportunity for natural selection to act on this trait. Thus, exposure to winter warming events may lead to evolutionary responses in populations of *P. rapae*, especially if winters continue to vary in their winter warming patterns. Ultimately, this pattern of increased phenotypic variance in response to winter warming suggests that some individuals will perish while others survive. Here, we do note that some individuals show the potential for metabolic recovery from acute winter warming stress and that some individuals were unperturbed by continuous chronic warming as diapause progressed. However, it remains to be determined whether the

source of these disparate thermal responses has a genetic basis. If the thermal response of metabolic rate during diapause does have a genetic basis, we predict future evolutionary shifts as natural selection favors pupae that are able to maintain low metabolic rates in the face of winter warming.

It should be noted that these experiments were conducted over less than three months (≈75-80 days), whereas in northern latitudes, *P. rapae* can stay in diapause for upwards of eight months (≈240 days) (Richards, 1940). We predict that the observed pattern of recovered metabolic rate after warming exposure is representative of longer periods of diapause in this species. However, we do not yet know the extent to which winter warming can affect metabolic rate over longer periods of dormancy. Indeed, there are some species of diapausing insects that can remain in prolonged states of diapause for years (Danks, 1992). Thus, the effect of winter warming on metabolic rate over longer periods of diapause needs to be explored further.

Our data on real-time responses of metabolic rate to warming in early diapause suggest that pupae not only increased their metabolic rates but also changed their energy source. If this is a broadscale phenomenon, then it could lead to significant metabolic costs for insects exposed to winter warming. Many diapausing insects primarily rely on stored lipids to fuel oxidative metabolism, which is an efficient means of producing ATP over long periods of dormancy and is supported by observed respiratory quotients around 0.7 (Hahn and Denlinger 2011; Somero et al., 2017). There have been observed instances of diapausing insects, such as *Eurosta solidaginis*, using carbohydrates as their main energy fuel, especially under hypoxic conditions (Danks, 2000; Han and Bauce, 1993; Storey and Storey, 1986). This form of anaerobic metabolism often uses glycogen or the

disaccharide trehalose (Sinclair, 2015). However, stored protein reserves, as suggested for our pupae measured at 11°C and 23°C, are not typically used as a main energy source for metabolism during diapause (Sinclair, 2015). Proteins accumulated before diapause, and stored during diapause, are often associated with diapause cessation and post-diapause development (Hahn and Denlinger, 2007). Thus, by using proteins to fuel metabolic activity during winter warming exposure, our pupae may be (1) using sub-optimal energy stores and/or (2) using energy stores that should otherwise be reserved for late-stage diapause functions. However, since amino acids breakdown into different intermediates, assigning a single RQ to the oxidation of proteins is difficult, and consequently, 0.8 is only estimated to be the respiratory quotient for protein catabolism, but not explicitly. An RQ of 0.8 can also be categorized as mixed energy sources, often referring to the combined utilization of carbohydrate and fat catabolism (Patel and Bhardwaj, 2019). Shifts and/or combinations of energy stores have been documented in diapusing insects (Hahn and Denliner, 2007; Hahn and Denlinger, 2011); however, it is oftentimes associated with changes in diapause maintenance (i.e. transition from pre-diapause stage to diapause stage) (Marron et al., 2003; Schneiderman and Williams, 1953). No studies to date have demonstrated shifts in respiratory quotients (i.e. energy stores) as a result of winter warming, a pattern revealed in our study. This suggests that winter warming directly affects energy store use and conservation, and could potentially affect diapause progression, maintenance, and termination.

It is important to note that while we observed shifts in RQ in real time in response to warming in early diapause, we did not observe sustained shifts in RQ after chronic warming or acute warming later in diapause. This discrepancy may have been due to the

greater thermal sensitivity of early stage pupae as compared to later stage pupae, as discussed above for the effects of warming on metabolic rate. Alternatively, these trends in RQ may simply be a result of the temperature at which we measured metabolic rate. We assayed metabolic rate in the chronic and acute warming experiments at 8°C, for both the treatments and the controls, which is a temperature in between two of the temperatures at which we measured metabolic rate in the real-time exposure experiment, 4°C and 11°C. Accordingly, the observed RQ values at 8°C (approx. 1) were midway between the RQ values at 4°C (1.18) and 11°C (0.85). Thus, it is possible that the RQ reflects real-time responses to temperature in diapausing *P. rapae*; although, we cannot determine this definitively based on the present data because we did not assay metabolic rates at multiple temperatures across all of the experiments that we report on herein. Future work should expose diapausing pupae to winter warming events across a broader timeframe of the diapause program (0-90+ days) and measure metabolic rate, and RQ, at multiple temperatures in real time. Such experiments would fill gaps in our understanding about the transition in metabolic rate sensitivity through diapause, as well as the shifts in respiratory quotient and energy reserve usage in response to increases in temperature in diapausing insects under conditions of winter warming.

## CONCLUSION

Diapause is a complex dormancy program linking metabolic activity and metabolism to other key traits like cryoprotectant synthesis and cold tolerance (Lee and Denlinger, 2010; Sinclair, 2015). By measuring thermal responses in metabolic rate during exposure to winter warming stress, after long-term exposure, and after recovery from acute winter

warming stress, we determined short-term and long-term alterations in metabolism. Our results suggest potential alterations to key metabolic processes during the diapause state as a consequence of winter warming, but also that individuals may respond to these warming events and recover. Future studies should address metabolic challenges experienced by overwintering species that diapause in other life stages, including egg, larval and adult stages, to better understand if these stages experience differential, metabolic responses to winter warming. Furthermore, future studies should focus on understanding the effects of winter warming on the metabolic activity of diapausing species that enter dormancy for prolonged periods of time (> 1 year). Filling in these gaps in knowledge could help us better predict which species may be most vulnerable to physiological challenges in the face of global climate change.

## FIGURES AND TABLES

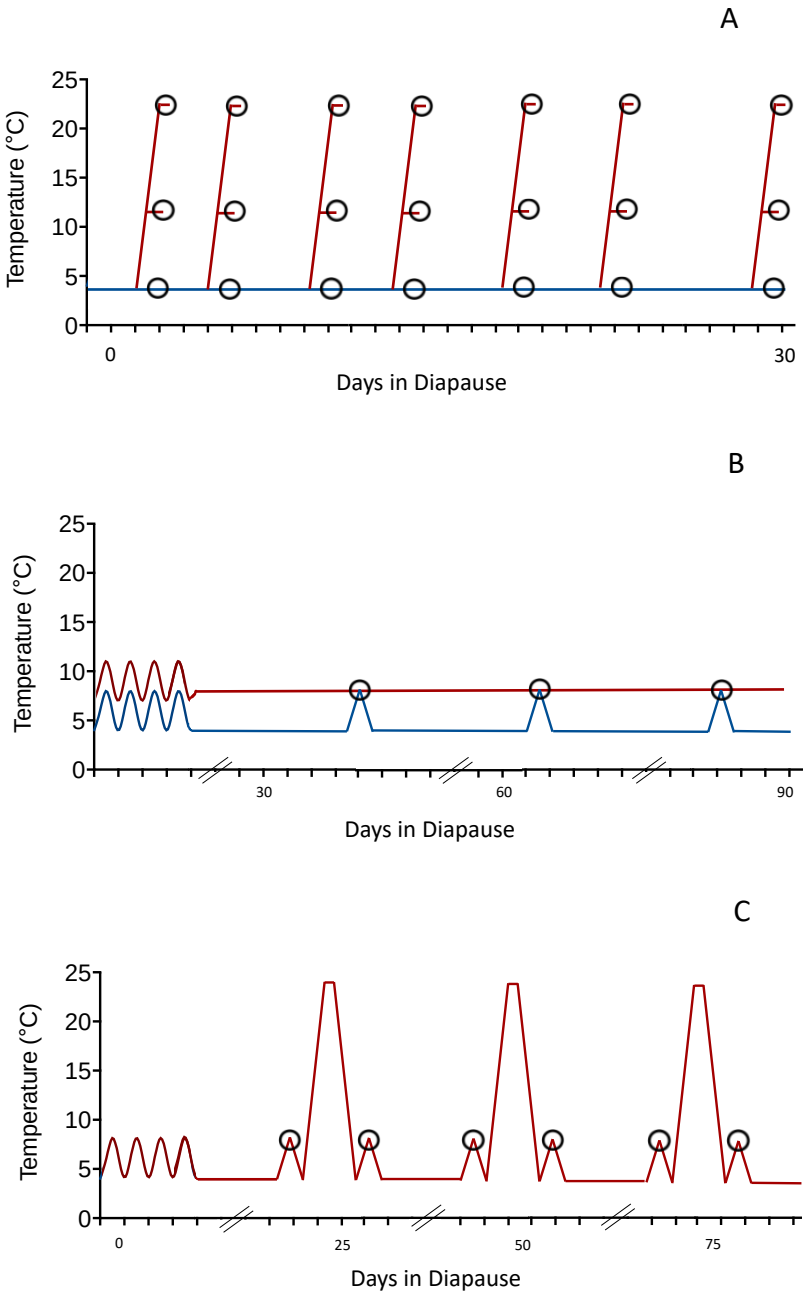


**Figure 1: Schematic designs for the winter warming metabolic rate experiments on diapausing *Pieris rapae* pupae.** (A) Metabolic rate during winter warming exposure. Individuals were kept at a constant 4°C until metabolic rate measurement at 4°C, 11°C or

23°C. Individuals were measured over the first 30 days of diapause. This experiment had no repeated measurements. Circles represent time of metabolic rate measurement. (B) Cumulative effect of chronic winter warming stress in control (blue) and chronic warmed (red) individuals. Prior to transport to Lincoln, NE, control individuals were kept under a daily fluctuating temperature regime of 4°C-8°C. Upon arrival in Lincoln, control individuals were kept at a constant 4°C. Prior to transport to Lincoln, NE, chronic warmed individuals were kept under a daily fluctuating temperature regime of 7°C-11°C. Upon arrival in Lincoln, warmed individuals were kept at a constant 8°C. Metabolic rate was measured at 8°C over 90 days in diapause. Circles represent time of metabolic rate measurement. This experiment had some repeated measurements, and individual was modeled as a random effect. (C) Recovery from acute winter warming stress. Prior to transport to Lincoln, NE, acute warmed individuals were kept under a daily fluctuating temperature regime of 4°C-8°C. Upon arrive in Lincoln, acute warmed individuals were kept at a constant 4° until exposed to a 24-hour warming of 23°C on Days 25, 50 and 75. Metabolic rate was measured at 8°C 24-hours pre and 24-hours post-warming. The experiment had repeated measurements. Circles represent time of metabolic rate measurement.

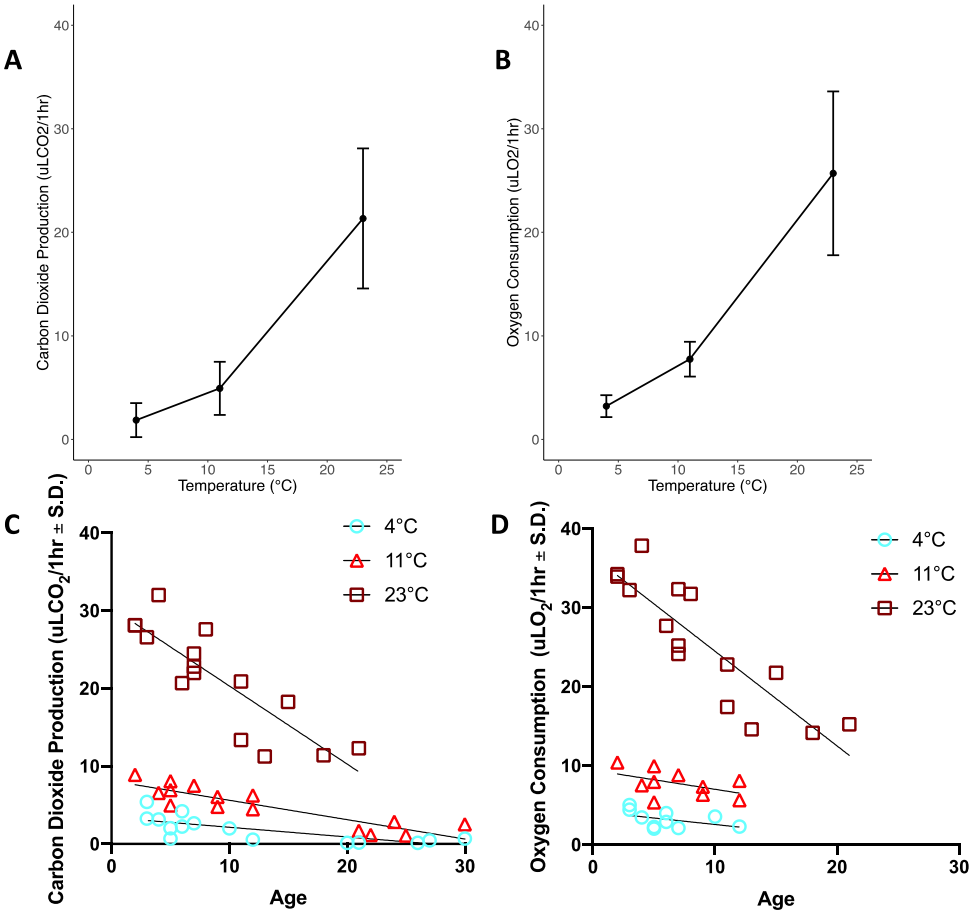


**Figure 2: Metabolic rate exponentially increased in diapausing *Pieris rapae* pupae during exposure to winter warming but was overall, lower in older pupae.** (A) Carbon dioxide production increased with increasing temperature (Type II ANOVA; temperature factor $F_{2,39}$ = 262.40, $P$<0.0001) with increased variation in metabolic rate at hotter temperatures (Bartlett's test of homogeneity of variances, $P$<0.0001). Carbon dioxide consumption (μlCO2/hr) measured using stop-flow respirometry at three temperatures: 4°C, 11°C, and 23°C (n = 15 pupae at all temperatures). Error bars represent standard deviations. (B) Oxygen consumption increased with increasing temperature (Type II ANOVA; temperature factor $F_{2,29}$ = 254.93, $P$<0.0001) with increased variation in metabolic rate at hotter temperatures (Bartlett's test of

homogeneity of variances, $P<0.0001$). Oxygen consumption ($\mu lO_2$/hr) measured using stop-flow respirometry at three temperatures: 4°C, 11°C, and 23°C (n = 10 pupae at 4°C and 11°C, n = 15 pupae at 23°C). Error bars represent standard deviations. (C) Metabolic rate ($CO_2$) decreased in older pupae but was dependent on temperature (Least-squares linear regression; 4°C, $y= -0.125x +3.398$, $R^2= 0.54$, $P=0.001$; 11°C, $y= -0.250x + 8.121$, $R^2=0.79$, $P<0.0001$; 23°C, $y= -1.005x + 30.38$, $R^2=0.73$, $P<0.0001$; Type II ANOVA; age factor $F_{1,39} = 54.25$, $P<0.0001$, temperature x age factor, $F_{2,39} = 25.74$, $P<0.0001$). (D) Metabolic rate ($O_2$) decreased in older pupae but was dependent on temperature (Least-squares linear regression; 4°C, $y= -0.165x + 4.211$, $R^2=0.21$, $P=0.187$; 11°C, $y = -0.242x + 9.446$, $R^2= 0.24$, $P=0.15$; 23°C, $y= -1.199+ 36.49$, $R^2=0.76$, $P<0.0001$). 10 pupae excluded due to low oxygen measurement; Type II ANOVA; age factor $F_{1,29} = 67.96$, $P<0.0001$, temperature x age factor; $F_{2,29} = 7.90$, $P=0.002$).

**Table 2: $Q_{10}$ coefficients for metabolic rate during winter warming.** Fold-change increases in rate in carbon dioxide production and oxygen consumption based on increases in temperature: 4°C to 11°C and 11°C to 23°C (n = 15 pupae for $CO_2$ measurements at all temperatures, n = 10 pupae for $O_2$ measurements at 4°C and 11°C, and n = 15 pupae for $O_2$ measurements at 23°C).

| | $CO_2$ | $O_2$ |
|---|---|---|
| **4°C-11°C** | **4.088** | **3.525** |
| **11°C-23°C** | **3.360** | **2.716** |

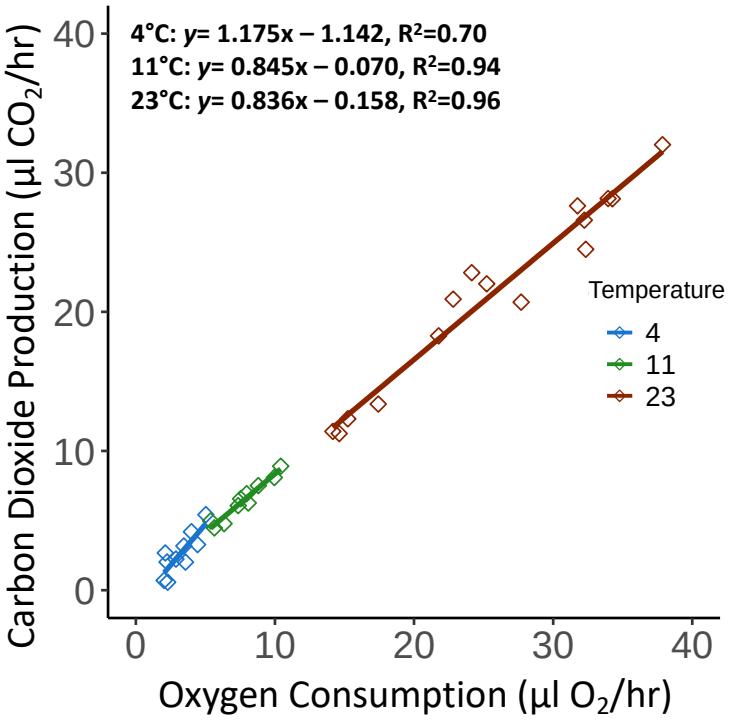


**Figure 3: Respiratory quotients (RQ = $CO_2/O_2$) shifted during winter warming thermal stress for diapausing *Pieris rapae* pupae.** There was a strong, direct relationship between carbon dioxide production and oxygen consumption (RQ = $CO_2/O_2$) for pupae exposed to winter warming temperatures at 4°C, 11°C, and 23°C (Least-squares linear regression; 4°C: $y$= 1.175x – 1.142, $R^2$=0.70, $P$=0.003; 11°C: $y$= 0.845x – 0.070, $R^2$=0.94, $P$<0.0001; 23°C: $y$= 0.836x – 0.158, $R^2$=0.96, $P$<0.0001). Metabolic activity was measured using stop-flow respirometry as oxygen consumption and carbon dioxide production (µl/hr) (n = 35 pupae, 10 pupae excluded from analysis due to low oxygen consumption measurement). Error bars represent 95% confidence intervals. All respiratory quotients calculated as the slope of the linear regression for the relationship between carbon dioxide production and oxygen consumption (i.e. the ratio of $CO_2$ produced to $O_2$ consumed).

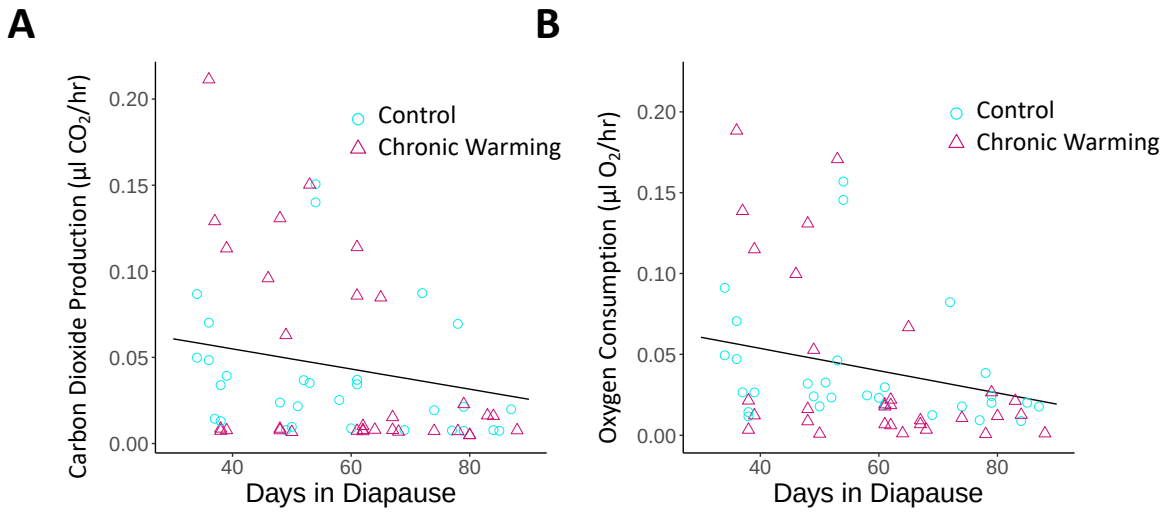


**Figure 4: Chronic warming caused no change in metabolic rate overall, but led to greater variance in metabolic rate.** (A) There was no difference in metabolic rate ($CO_2$) between control and chronic warmed individuals (Log-likelihood ratio test; *Chi-sq* = 2.62; Treatment effect: *P*=0.27). Carbon dioxide production decreased over time for both control and chronic warmed treatments (Linear mixed model fit with REML; line represents predicted mixed model fit, *T*=4.35, *P*=0.0001; Day factor: *P*=0.05) with higher variance for chronic warmed pupae (Bartlett's test of homogeneity of variances, *P*=0.03). Carbon dioxide production (µl/hr) measured using stop-flow respirometry at 8°C. Control individuals maintained at fluctuating 4-8°C temperature regime over time and chronic warmed individuals maintained at fluctuating 7-11°C temperature regime over time (n=21 pupae per treatment). Error bars represent 95% confidence intervals. (B) There was no difference in metabolic rate ($O_2$) between control and chronic warmed individuals

(Log-likelihood ratio test; *Chi-sq* = 3.43; Treatment effect: *P*=0.18). Oxygen consumption decreased over time for both control and chronic warmed treatments (Linear mixed model with repeated measures; line represents predicted mixed model fit, *T*=4.62, *P*<0.0001; Day factor: *P*=0.02) with higher variance for chronic warmed pupae (Bartlett's test of homogeneity of variances, *P*=0.05). Oxygen consumption (μl/hr) measured using stop-flow respirometry at 8°C. Control individuals maintained at fluctuating 4-8°C temperature regime over time and chronic warmed individuals maintained at fluctuating 7-11°C temperature regime over time (n=21 pupae per treatment). Error bars represent 95% confidence intervals.

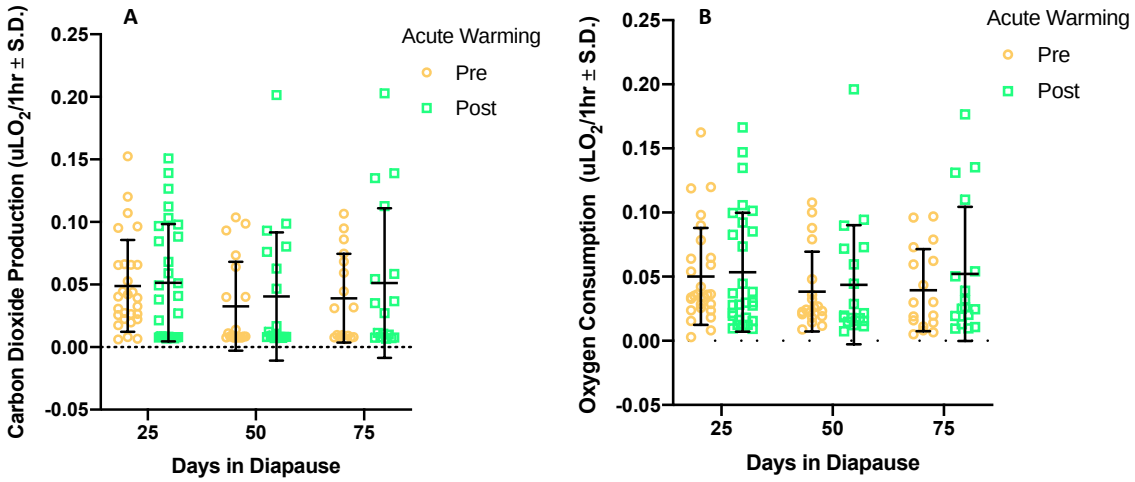


**Figure 5: Metabolic rate recovered from acute warming overall, but acute warming caused greater variance in metabolic rate.** (A) There was no difference in metabolic rate ($CO_2$) pre and post-acute warming (Log-likelihood ratio test (*Chi-sq* = 2.81; Warming effect, *P*=0.24). There was no difference in carbon dioxide production over the three timepoints for acute warmed individuals (Linear mixed model fit with REML, Day factor, *P*=0.86) but there was higher variance in carbon dioxide production post-warming (Bartlett's test of homogeneity of variances, *P*=0.01). Carbon dioxide production (µl/hr) measured using stop-flow respirometry at 8°C. Pre-warming metabolic activity was measured 24-hours before one-day warming event at fluctuating 18-23°C, and post-warming metabolic activity was measured 24-hours after one-day warming event (n=28 pupae). Error bars represent standard deviations. (B) There was no difference in metabolic rate ($O_2$) pre and post-acute warming (Log-likelihood ratio test (*Chi-sq* = 3.19; Warming effect, *P*=0.20). There was no difference in oxygen consumption over the three timepoints for acute warmed individuals (Linear mixed model fit with REML, Day factor, *P*=0.87) but there was higher variance in carbon dioxide production post-warming

(Bartlett's test of homogeneity of variances, *P*=0.01). Lines connect an individual pre and post warming on Days 25, 50 and 75. Oxygen consumption (μl/hr) measured using stop-flow respirometry at 8°C. Control individuals maintained at fluctuating 4-8°C temperature regime over time and chronic warmed individuals maintained at fluctuating 7-11°C temperature regime over time (n=28 pupae per treatment). Error bars represent standard deviations.

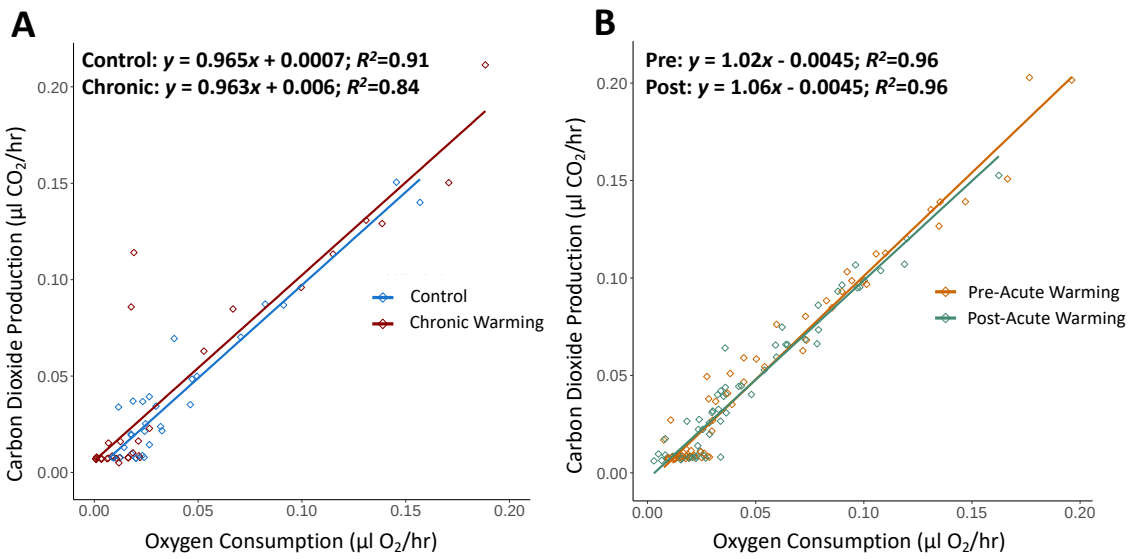


**Figure 6: Respiratory quotient ($CO_2/O_2$) was not affected by chronic winter warming and recovered from acute warming. (A)** There was a significant positive relationship between carbon dioxide production and oxygen consumption (RQ = $CO_2/O_2$) for diapausing *Pieris rapae* pupae exposed to control and chronic warming treatments (Least-squares linear regression; Control: $y = 0.965x + 0.0007$; $R^2=0.91$; $P<0.0001$; Chronic warmed: $y = 0.963x + 0.006$; $R^2=0.84$; $P<0.0001$). Metabolic activity of diapausing *P. rapae* pupae measured using stop-flow respirometry as oxygen consumption and carbon dioxide production (µl/hr) at 8°C. Control individuals maintained at fluctuating 4-8°C temperature regime over time and chronic warmed individuals maintained at fluctuating 7-11°C temperature regime over time (n=21 pupae per treatment). (B) There was a strong, direct relationship between carbon dioxide production and oxygen consumption (RQ = $CO_2/O_2$) for diapausing *Pieris rapae* pupae pre and post-exposure to acute winter warming treatment (Least-squares linear regression; Pre-acute warming: $y = 1.02x - 0.0045$; $R^2=0.96$; $P<0.0001$; Post-acute warming: $y = 1.06x - 0.0045$; $R^2=0.96$; $P<0.0001$ ). Metabolic activity of diapausing *P.*

*rapae* pupae measured using stop-flow respirometry as oxygen consumption and carbon dioxide production (μl/hr) at 8°C. Pre-warming metabolic activity was measured 24-hours before one-day warming event at fluctuating 18-23°C, and post-warming metabolic activity was measured 24-hours after one-day warming event (n=28 pupae). All respiratory quotients were calculated as the slope of the linear regression for the relationship between carbon dioxide production and oxygen consumption (i.e. the ratio of $CO_2$ produced to $O_2$ consumed). All error bars represent 95% confidence intervals.

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# CHAPTER 4: MATERNAL MRNAS UNDERLIE HIGHER THERMAL TOLERANCE IN TROPICAL VS. TEMPERATE *DROSOPHILA MELANOGASTER* EMBRYOS

## ABSTRACT

Acute thermal stress events pose cellular challenges for ectotherms that can threaten their survival. In nature, early-stage *Drosophila melanogaster* embryos, which are more thermally sensitive than later stages in development, are frequently exposed to variation in their thermal environment. Regional differences in heat tolerance of early *D. melanogaster* embryos (0-1hr old) have shown that tropical embryos have significantly higher heat tolerance that temperate embryos; however, the transcriptomic basis of their enhanced tolerance has not been identified. In order to fill this gap in knowledge, we sequenced mRNA from early *D. melanogaster* embryos from tropical and temperate regions that were acutely exposed to a range of temperatures (25°C-36°C). Only 828 transcripts differentiated the transcriptomes of tropical vs. temperate embryos. Functional enrichment analysis of these differentially abundant transcripts indicated that tropical embryos had higher abundances of maternally loaded transcripts that encode proteins involved in the oxidative stress response. Higher abundance of these transcripts could provide tropical embryos with a better coping mechanism to heat-induced oxidative stress, relative to temperate embryos, increasing their survival under hotter temperatures. Our results also demonstrate that, in response to acute heat stress, embryos exhibited changes in the abundance of 4,534 gene transcripts, most of which were shared responses among temperate and tropical embryos. Further, despite the fact that previous research has shown that early zygotes are transcriptionally inactive, we observed a strong heat

shock response in embryos, indicating that they actively expressed zygotic transcripts by 2-hours post-fertilization. As global temperatures continue to rise, organisms may meet or exceed their upper thermal limits, causing increases in mortality. By characterizing the genetic basis of thermal tolerance, we can better predict how populations of *D. melanogaster* will differentially respond and adapt to future warming.

## INTRODUCTION

All life is inherently sensitive to temperature due to the basic thermodynamic relationships that govern the stability of biological structures (Somero et al., 2017). Even short-term, acute, thermal stress events can damage cellular structures by disrupting macromolecules, thereby compromising cellular functions (Richter et al., 2010). Thermal effects on macromolecules include protein unfolding and the loss of protein function, protein aggregation, disruption of the cytoskeleton, alterations of intracellular transport processes, and the disorganization and localization of organelles, all of which can be toxic to the cell (Lockwood et al., 2017; Richter et al., 2010). Not surprisingly, all organisms possess a coping strategy to thermal stress known as the heat shock response (HSR) or cellular stress response (Feder and Hofmann, 1999; Tomanek, 2010). This molecular response to stress is characterized by the activation of a signaling pathway that leads to the up-regulation of gene loci including heat shock proteins, molecular chaperones that help to refold proteins and prevent protein aggregation, as well as the down-regulation of gene loci involved in house-keeping functions such as development and reproduction (Buckley et al., 2006; Leemans et al., 2000; Lockwood et al., 2010). Although these cellular mechanisms of thermal stress tolerance aid in whole-organism

thermal tolerance, individuals still remain vulnerable to thermal stress events, especially under the threat of global climate change.

The physiological basis of thermal adaptation of heat tolerance may be mediated by the evolution of transcriptional regulation (i.e., the HSR) (Richter et al., 2010; Sørensen et al., 2003). Changes in the regulation of the transcriptome can lead to increases or decreases in whole-organism heat tolerance (Kassahn et al., 2009). Variation in transcriptional regulation has been the target of thermal selection in many species, such that heat tolerant species and populations have often evolved their higher heat tolerance by adjusting their regulation of the transcriptome (González-Schain et al., 2016; Lockwood et al., 2010; Qin et al., 2008). Oftentimes, higher heat tolerance is correlated with higher expression of heat shock proteins (HSPs) (Bettencourt et al., 2002; Mckechnie et al., 1998; McMillan et al., 1998; Wang et al., 2016); however, this is not ubiquitous (Rako et al., 2007). Thus, the evolution of transcriptional regulation is a common theme of thermal adaptation of heat tolerance, though the exact details may vary among taxa.

Due to the aforementioned relationship between temperature and cell function, thermal tolerance is a key physiological trait for the survival of organisms to variable thermal environments, especially thermally-sensitive ectotherms (Buckley and Huey, 2016; Kellermann et al., 2012; Terblanche et al., 2011). Early ectothermic embryos are particularly susceptible to changes in temperature (Kuntz and Eisen, 2014). Indeed, early stage *Drosophila melanogaster* embryos (0-1hr old) are more thermally sensitive than other life stages (Gilchrist et al., 2008; Walter et al., 1990; Welte et al., 1993). As an immobile, juvenile stage, *D. melanogaster* embryos are at the mercy of the environment

where their mothers lay them, and oftentimes these environments dramatically fluctuate in temperature throughout the day. Within hours, the temperature of a piece of necrotic fruit, the preferred oviposition site for *D. melanogaster*, can increase by 20°C (Roberts and Feder, 2000). Unlike adult flies, *D. melanogaster* embryos cannot avoid thermal extremes through behavioral thermoregulation (Dillon et al., 2009) or through a robust heat shock response, as the HSR is vastly reduced in early embryos due to their reduced levels of transcriptional activity (Graziosi et al., 1980; Lockwood et al., 2017; Welte et al., 1993). In fact, early embryos of nearly all multi-cellular species are dependent upon maternal mRNAs and proteins, which are deposited into the egg during oogenesis and are responsible for the early stages of post-fertilization embryonic development, including fundamental processes like biosynthesis, mitotic division, cell fate and developmental patterning (De Renzis et al., 2007; Lockwood et al., 2017; Tadros and Lipshitz, 2009). In *D. melanogaster*, it is not until two hours post-fertilization that the zygote starts transcribing its own genome and actively degrades maternal transcripts in a process called the naternal to zygotic transition (Bashirullah et al., 2003; Bushati et al., 2008; Edgar and Datar, 1996; Tadros and Lipshitz, 2009). Thus, any acute increases in temperature during the first few hours of an embryo's life could push them past their upper thermal limits if they are not supplied with the proper stress mechanisms from their mother (Lockwood et al., 2017).

The early embryonic stage of *D. melanogaster* presents a unique phenomenon in thermal tolerance as it is the only life stage for which systematic differences are seen between tropical and temperate regions (Lockwood et al., 2018). This provides evidence for thermal adaptation across populations of *D. melanogaster*, which are only recently

diverged (15,000 years ago) (Li and Stephan, 2006); yet, the genetic and physiological mechanisms that underlie this pattern of life-stage-specific thermal adaptation remain uncharacterized. Because variation in transcriptional regulation has been shown to be an underlying physiological mechanism of variation in whole-organism heat tolerance in many species, we sought to characterize the transcriptomes of tropical and temperate *D. melanogaster* early embryos, and hereby uncover potential molecular physiological mechanisms of adaptive thermal evolution. Thermal adaptation of embryonic heat tolerance among populations of *D. melanogaster* could manifest in a number of ways at the level of the transcriptome. Tropical embryos may have higher heat tolerance because they possess transcripts that encode proteins that are involved in heat stress coping mechanisms, such as molecular chaperones. The differential regulation of the expression of such genes could have evolved in heat tolerant populations via alterations in the loading of mRNAs into embryos by females, or by modifications to zygotic gene expression, such that early embryos are transcriptionally responsive to heat stress—a mechanism that has heretofore never been described. More broadly, a fundamental question in evolutionary genetics is whether phenotypic variance is caused by few genes of large effect or many genes of small effect, and it remains to be determined which of these possibilities characterizes the evolutionary process by which thermal adaptation proceeds. In the present study, are tropical fly embryos more heat tolerant because they have evolved to differentially regulate the expression of thousands of gene loci? Or rather, have they achieved higher heat tolerance by modifications to the expression of relatively few genes? To address these questions, we used high-throughput mRNA sequencing to determine transcript abundances of early embryos from tropical and

temperate regions that were acutely exposed to a range of heat shock temperatures. Our results provide evidence that across the entire transcriptome, embryos largely overlap in the abundance of thousands of gene transcripts, but that differences in transcript abundances across regions could confer the disparate levels of thermal tolerance observed across *Drosophila* from temperate and tropical regions. With these results, we can begin to understand the molecular underpinnings of thermal tolerance among diverged populations, aiding us in our pursuit of understanding how organisms may evolve to respond to climate warming.

## MATERIALS AND METHODS

### Fly strains

To compare transcript abundances in tropical and temperate *Drosophila melanogaster* embryos, we propagated five tropical and five temperate iso-female lines of *D. melanogaster* as described in Lockwood et al. (2018) (Lockwood et al., 2018). All tropical lines were originally established from stock populations acquired from the UCSD Stock Center at the University of California, San Diego with origins from Bombay, India (BO) (Stock number: 14021-0231.45); Accra, Ghana (GH) (Stock number: 14021-0231.182); Monkey Hill, St. Kitts (SK) (Stock number: 14021-0231.34) ; Chiapas de Corzo, Chiapas, Mexico (CP) (Stock number: 14021-0231.22); and Guam, USA (GM) (Stock number: 14021-0231.198). All Vermont (VT) populations were originally collected, established, and gifted from K.L. Montooth from East Calais, VT (Cooper et al., 2014). Iso-female lines were established by taking single females whose progeny were then inbred for several generations to isogenize the genetic variability within each

of the lines (genotypes). This is a well-established technique to minimize the potential for lab evolution because it removes genetic variability among individuals within a line (Cooper et al., 2014). We maintained all topical and temperate lines at controlled densities of 50-100 adults per vial (95mm x 25mm). We maintained all fly populations under common-garden conditions in incubators (DR-36VL, Percival Scientific Inc., Perry, Iowa, USA) set to 25°C and a 12L:12D light cycle on a cornmeal-yeast-molasses medium for at least 2 generations prior to collecting embryos for embryonic heat shock exposure. This represents an ecologically relevant and realistic temperature that both tropical and temperate *D. melanogaster* flies are exposed to in their natural environments.

**Embryonic heat shock**

Heat shocks were designed to mimic the natural, acute increases in temperature of the necrotic fruit that *D. melanogaster* oviposit their eggs on in the wild (Roberts and Feder, 2000). We allowed three- to five-day-old adult flies (n ≈ 100 pairs) from each of the 10 lines to lay eggs on grape juice agar plates (60 x 15 mm) dabbed with active baker's yeast for one hour at 25°C. We then wrapped the egg plates with 0-1h old embryos with parafilm, and submerged them in a circulating water bath (A24B, Thermo Scientific, Waltham, MA, USA) for 45-minutes at one of four temperatures: 25°C, 32°C, 34°C, and 36°C. Once put into the water bath, the temperature of the plates increased at a rate of $+0.4°C^{-1min}$ until reaching the target temperature (Lockwood et al., 2018). We selected these heat shock temperatures from known lethal temperature ($LT_{50}$) data for early embryos (0-1h) at these temperatures, and this temperature range covers the disparate thermal tolerance traits observed in tropical and temperate embryos (Lockwood et al.,

2018). We repeated the heat shocks for a minimum of three replicates per treatment (genotype x temperature).

After the temperature shock, we rinsed the eggs from each plate with 1x embryo wash solution (0.2%NaCl, 0.02% Triton X-100), for approximately 30 seconds in order to remove residual yeast. We then dechorionated the embryos by washing with a 50:50 solution of water and bleach for one minute, followed by a rinse in $dH_2O$ for 30 seconds. We removed residual water by blotting with a Kimwipe. We pooled eggs ($n \geq 30$) from each plate, placed them into 1.5ml microcentrifuge tubes, and immediately flash froze them in liquid nitrogen. All pools of embryos were preserved at -80°C until further transcriptomic analysis.

**RNA extraction and sequencing**

We extracted total RNA by first homogenizing the pooled embryos in 250ul of trizol (Tri-reagent, Sigma Life Science, St. Louis, MO, USA), and centrifuging all samples at 4°C at 6,000g for one minute (Sorvall ST89, Thermo Scientific). We then added an additional 150ul of trizol (Tri-reagent, Sigma Life Science) and 150ul of chloroform (Sigma Life Science) to each sample, followed by centrifugation at 4°C at 12,000g for ten minutes (Sorvall ST89, Thermo Scientific). Lastly, we precipitated each sample using isopropanol and ethanol. To determine the quantity of RNA in each extraction, we used a Nanodrop spectrophotometer (NanoDrop 2000, Thermo Scientific). To assess the quality of RNA, we used agarose (2%) gel-electrophoresis (EL-200, Walter, Plymouth, MI, USA) to visualize bands of RNA. We only sequenced samples that contained >1 ug of

RNA and that exhibited strong bands, corresponding to intact 28S and 18S ribosomal RNAs.

We sent all samples (n = 120) to Novogene (Sacramento, CA, USA) for 150 base-pair paired-end mRNA sequencing. Further quality control of each RNA sample was performed by Novogene using a Nanodrop spectrophotometer, agarose gel electrophoresis, and the Agilent 2100 BioAnalyzer (Santa Clara, CA, USA). Only 110 of the 120 samples passed this second round of quality control; thus, we only report results from 110 samples. The samples excluded were as follows: BO at 34°C, BO at 36°C, CP at 36°C, GH at 32°C, GM at 32°C, VT12 at 25°C, VT2 at 32°C, VT2 at 36°C (x2), and VT9 at 25°C.

After quality control, libraries were prepped by first enriching the mRNA using oligo(dT) beads, then cDNA was synthesized using mRNA templates and random hexamer primers. Double-stranded cDNA libraries were prepared using Novaseq 6000 (S4 Flowcell) (Illumina, San Diego, CA, USA). Library quality was then assessed using Qubit 2.0 Fluorometer (Thermo Scientific), Agilent 2100 BioAnalzyzer, and q-PCR.

We checked the quality of paired-end raw sequence reads using FastQC (v. 0.11.5) (Andrews, 2010). We trimmed the forward and reverse reads using Trimmomatic (v. 0.36) (Bolger et al., 2014) to remove adapter sequences and low quality leading and trailing bases by cropping the first 12 bases of each read, and the 20 leading and trailing bases. We also scanned the reads with a 6-base sliding window, cutting the read when the average quality per base was below a Phred score of 20. Lastly, we dropped reads below lengths of 35 bases.

**Quantification, normalization, and statistical comparisons of transcript abundances**

We used salmon (v. 1.1.0) to map transcripts to the latest *Drosophila melanogaster* transcriptome (FlyBase v. r6.34) and to quantify the abundance of transcripts for each of the 110 embryonic samples (Patro et al., 2017). We used R (v. 4.0.0) and the package "DESeq2" to normalize read counts and perform statistical analyses of transcript abundances among experimental groups (Love et al., 2014). To determine transcripts with significantly different abundances due to heat shock temperature or region of origin (tropical or temperate), we used the likelihood ratio test within the DESeq function. From these tests, we generated lists of differentially abundant transcripts based on a cutoff value of FDR<0.05 for both factors and the interaction between factors. We note that we did not consider statistical models that incorporate genotype as an explanatory factor because such models would not describe regional patterns of thermal tolerance. It is possible that idiosyncratic transcriptomic patterns confer thermal tolerance for a particular genotype, but identifying such patterns of transcriptional regulation was not the goal of the present study because such mechanisms are not likely to be the main targets of natural selection that have consistently maintained higher thermal tolerance across broad geographical regions in the tropics over the past 15,000 years.

Using the package "Pheatmap" in R, we generated heatmaps for each of the temperature and regions models incorporating all of the significantly differentially abundant transcripts for each unique list. We conducted a principal components analysis to describe the major axes of variation in transcript abundances among all samples.

**Gene Ontology Analysis**

We used WebGestalt (Web-based gene set analysis tool kit) for functional enrichment analysis for significantly differentially abundant transcripts based on temperature and region (Wang et al., 2017). We converted differentially abundant transcripts to known genes using the *D. melanogaster* genome (FlyBase v. r6.34), only using unique, non-overlapping hits for enrichment analysis. We conducted an over-representation analysis (ORA) with a minimum of five genes and a maximum of 200 genes per category, focusing solely on biological process. We deemed categories with FDR < 0.1 to be significantly enriched, following recommendations of the WebGestalt authors (Wang et al., 2017). For temperature, we determined enriched functional categories for the following groups: "Up All", where transcripts had significantly higher abundance at the heat shock temperatures relative to 25°C, and "Down All", where transcripts had significantly lower abundance at the heat shock temperatures relative to 25°C. We also determined functional categories for each individual heat shock temperature relative to 25°C, including: "Up 32°C, Down Rest" where transcripts were significantly higher at 32°C, but significantly lower at 34°C and 36°C, and "Down 32°C, Up Rest" where transcripts were significantly lower at 32°C, but significantly higher at 34°C and 36°C. This was also done for 34°C and 36°C. For region, we found enriched functional categories for the transcripts with higher abundance in the tropical and temperate embryos, respectively.

## RESULTS

### Conserved transcript abundances across all embryos and temperatures

Among the 29,686 unique transcripts identified, transcript abundances among all embryos largely overlapped, regardless of heat shock temperature or region of origin, as no clear clusters were identified by heat shock temperature or region of origin along PC1 (22% total variance) or PC2 (14% total variance) (Figure 1).

### Similar transcriptional responses to heat shock among tropical and temperate embryos

Across heat shock temperatures, 4,534 transcripts changed in abundance with increasing temperature in early *D. melanogaster* embryos, regardless of region of origin (Figure 2; Likelihood Ratio test (LRT), temperature factor, FDR < 0.05). Of these transcripts, most decreased in abundance with increasing temperature (n=2,793); however, many transcripts increased in abundance with increasing temperature (n=671) (Figure 3; LRT, temperature factor, FDR < 0.05). The top transcripts that were significantly different in abundance by heat shock temperature were almost exclusively transcripts that encode heat shock proteins (LRT, temperature factor, FDR < 2.0E-31), which increased in abundance with increasing temperature. These included *Hsp68* (Figure 4A; LRT, temperature factor, FDR = 8.08E-58), *Hsp70Bb* (Figure 4B; LRT, temperature factor, FDR = 2.87E-51), *Hsp70Bbb* (Figure 4C; LRT, temperature factor, FDR = 2.81E-41), *Hsp70Bc* (Figure 4D; LRT, temperature factor, FDR = 3.37E-37), *Hsp22* (Figure 4E; LRT, temperature factor, FDR = 6.49E-36), and *Hsp23* (Figure 4F; LRT, temperature factor, FDR = 5.96E-36). Additionally, a transcript that encodes fs(1)N, also increased in

abundance with increasing heat shock temperature in both tropical and temperate regions (Figure 5; LRT, temperature factor, FDR = 1.19E-31). All other top temperature-sensitive transcripts encoded uncharacterized protein coding genes involved in gene expression (regulation of transcription) or otherwise unknown functions which were inferred from the gene ontology annotations on FlyBase (Gaudet et al., 2011). These transcripts had the highest average abundance at 36°C (LRT, temperature factor, FDR = 3.04E-42, FDR = 1.65E-39, and FDR = 3.71E-37, respectively).

Fifty-eight gene ontology functional categories were enriched among the 671 transcripts that had significantly higher abundance at heat shock temperatures relative to 25°C ("Up all") (Table 1; Over-representation analysis (ORA) for biological process; FDR < 0.1). Among these functional categories, many indicate that all embryos, both temperate and tropical, exhibited a strong heat shock response. These include the gene ontology (GO) functional categories of protein folding (FDR = 6.82E-09), response to topologically incorrect protein (FDR = 8.04E-05), and regulation of protein stability (FDR = 0.095). Furthermore, functional categories involved in early development were significantly enriched, including axis specification (FDR = 2.71E-07), cell cycle phase transition (FDR = 1,91E-04), and cortical cytoskeleton organization (FDR = 7.73E-04.

Eighty-four functional categories were enriched among the 2,793 transcripts that had significantly lower abundance at heat shock temperatures relative to 25°C ("Down all") (Table 2; ORA; FDR < 0.1). Transcripts within these categories were largely involved in developmental processes, including embryo development (FDR = 1.09E-05), animal organ formation (FDR = 2.63E-05), and head development (FDR = 8.01E-04). Transcripts were also found within the adult behavior pathway (FDR = 8.56E-05).

Fifty-five GO categories were enriched among the 308 significant transcripts for "Up 32°C, Down Rest", in which transcripts were found to be more abundant at 32°C relative to all other temperatures (Table 3; ORA; FDR < 0.1). Meanwhile 13 categories were enriched among the 316 significant transcripts for "Down 32°C, Up Rest", in which transcripts were found to be less abundant at 32°C relative to all other temperatures (Table 4; ORA; FDR < 0.1). Genes within these sets indicate both up and down regulation of developmental processes even with a slight heat shock. No functional categories were enriched among the 16 significant transcripts for "Up 34°C, Down Rest", in which transcripts were found to be more abundant at 34°C relative to all other temperatures, or for the 1 significant transcript for "Down 34°C, Up Rest" that was least abundant at 34°C (ORA; FDR < 0.1). Only two functional categories were enriched among the 204 significant transcripts for "Up 36°C, Down Rest", in which transcripts were found to be more abundant at 36°C relative to all other temperatures. These included the GO categories stat cascade (FDR = 0.077) and epigenetic regulation of gene expression, (FDR = 0.057). Furthermore, only one functional category, DNA confirmation change (FDR = 0.029), was enriched among the 225 significant transcripts for "Down 36°C, Up Rest" where transcripts were found to be less abundant at 36°C relative to all other temperatures.

No transcripts were found to be significantly different in abundance for the interaction between temperature and region (Likelihood Ratio test, temperature x region interaction, FDR < 0.05). In other words, tropical embryos exhibited no significant differences from temperate embryos in the transcriptomic changes that were induced by increasing temperature.

**Relatively few transcriptional differences between tropical and temperate embryos**

Based on region of origin, 828 transcripts were found to be significantly different in abundance (Figure 6; LRT, region factor, FDR <0.05). Of these transcripts, 473 were more abundant in embryos from the tropics, and 355 were more abundant in temperate embryos (Figure 6; LRT, region factor, FDR <0.05). The top transcripts that were significantly different in abundance by region (FDR < 3.0E-14) included 3 transcripts that encode NADH dehydrogenase (ND-15). One of these *ND-15* transcripts was more abundant than the other two transcripts in all embryos, by more than an order of magnitude, and it was more abundant in tropical embryos than temperate embryos (Figure 7A; LRT, region factor, FDR = 9.66E-17). However, temperate lines had higher abundance of ND-15 for two transcripts with overall lower counts for all lines (Figure 7B & 7C; LRT, region factor, FDR = 9.66E-17 & FDR = 1.25E-26, respectively). Transcripts of *Nek2* (Figure 7D; LRT, region factor, FDR = 5.74E-16) and *Jabba*, (Figure 7E; LRT, region factor, FDR = 1.02E-14) were significantly more abundant in temperate lines. All other top significantly different transcripts by region encoded uncharacterized protein coding genes in small molecule metabolism, cell organization gene expression or otherwise unknown functions which were inferred from the gene ontology annotations on FlyBase (LRT, region factor, FDR = 6.64E-18, FDR = 2.06E-17, FDR = 2.39E-17, FDR = 1.46E-11, and FDR = 4.45E-11, respectively) .

Nine functional categories were enriched for the significantly more abundant transcripts found in the tropical region (Table 5; ORA; FDR < 0.1). Across the tropical region, individuals had higher abundance of transcripts involved in response to stressors,

notably, oxidative stress (FDR = 0.002), response to wounding (FDR = 0.076), and response to extracellular stimulus (FDR = 0.096) (Table 5). Tropical flies had higher abundance of key transcripts in the oxidative stress category, relative to temperate individuals. This included a transcript encoding Ucp4A, a maternally loaded uncoupling protein that protects mitochondria from oxidative stress (Echtay, 2007) (Figure 8A; LRT, region factor, FDR = 0.021), a transcript encoding isocitrate-dehydrogenase [NADP], a cytosolic enzyme that is involved in the oxidative stress response by providing reducing equivalents to the glutathione pathway (Bayliak et al., 2017) (Figure 8B; LRT, region factor, FDR = 0.049), and a transcript encoding *rl* or rolled, which encodes a critical protein for cell signaling in the MAP-K pathway (Kim et al., 2006) (Figure 8C; LRT, region factor, FDR = 1.47E-06). Furthermore, increased abundance in tropical embryos was found for a transcript of *sesB* (*sesB-RA*), or stress-sensitive B which encodes an adenine nucleotide translocase (Rikhy et al., 2003) (Figure 8D; LRT, region factor, FDR = 0.02). However, one transcript of *Sod3* (*Sod3-RB*), which encodes superoxide dismutase 3, an enzyme that converts oxygen free radicals to hydrogen peroxide (Jung et al., 2011), was more abundant in tropical embryos (Figure 8E; LRT, region factor, FDR = 0.002). Meanwhile, with considerably fewer counts than *Sod3-RB* overall, another transcript (*Sod3-RA*) was more abundant in temperate embryos (Figure 8F; LRT, region factor, FDR = 0.002).

Only three functional categories were enriched for the transcripts that were significantly more abundant in temperate embryos (Table 6). These categories included pathways in cellular processes: ribonucleoprotein complex subunit organization (FDR =

0.012), protein acylation (FDR = 0.033), and protein localization to membrane (FDR = 0.091).

## DISCUSSION

Here, we used transcriptomics to examine the genetic basis of disparate thermal tolerance between tropical and temperate early *D. melanogaster* embryos. Although embryos overlapped in overall abundance of transcripts across the transcriptome, we identified thousands of genes that were induced upon heat shock by all embryos, regardless of region, whereas the patterns that differentiated the regions constituted relatively few genes. Notably, both tropical and temperate embryos showed transcriptomic patterns of a heat shock response with the increased abundance of heat shock proteins at hotter temperatures, suggesting the expression of zygotic transcripts at this early stage, which is a novel finding. Tropical embryos had significantly higher abundance of maternally loaded transcripts that encode proteins involved in oxidative stress, which could be a key mechanism of the higher thermal tolerance observed in tropical embryos. This provides evidence for evolutionary adaptation of transcriptional regulation across thermal gradients at a global scale.

Thermal adaptation has not led to broadscale evolution of the transcriptome. At least at the level of transcriptional regulation, we do not see evidence that many genes of small effect underlie the evolution of heat tolerance in fly embryos. In fact, the transcriptomes of early embryos were largely similar, despite acute heat stress and diverged regions of origin. This is a surprising result in the context of the well-established role of transcriptional responses in the cellular response to heat stress (Richter et al., 2010). In fact, in other species (Gasch et al., 2000; Lockwood et al., 2015) or life

stages (Brown et al. 2014), heat stress can cause changes in the expression of as much as a third of the transcriptome. To our knowledge, no studies have characterized embryological responses to the relatively extreme acute heat stress conditions that we have used in the present study; however, *Drosophila* embryonic developmental processes have been shown to be unperturbed across a range of more benign temperatures, from 18°C to 29°C (Houchmandzadeh et al., 2002). Thus, it seems that a potential explanation for the pattern of overall transcriptional homogeneity across heat shock temperatures and among embryos from different geographic regions may be the highly conserved nature of early embryonic development (Davidson, 1986), such that developmental processes have evolved to be robust to environmental and mutational perturbations—i.e., embryonic transcriptomes are canalized (von Heckel et al., 2016; Waddington, 1942). Indeed, gene expression in early development is highly evolutionarily conserved within the *D. melanogaster* species, as well as across the *Drosophila* genus, particularly among maternally loaded transcripts (Omura and Lott, 2020).

But despite the overwhelmingly overlapping variance in transcriptomic patterns, many thousands of transcripts exhibited changes in abundance following heat stress in all embryos, indicating that early embryos from tropical and temperate regions share the potential for zygotic transcription as a response to thermal stress. This is a surprising result, given that previous studies have shown that for the first few hours of life, embryos are dependent on maternally-loaded levels of transcripts and proteins to protect them from thermal stress events (Omura and Lott, 2020). In fact, it is well-established that in all multi-cellular organisms early zygotes are thought to be transcriptionally inactive (Tadros and Lipshitz, 2009). In light of our results, it may be that early embryos are more

transcriptionally active than previously described, such that they can induce changes in gene expression if exposed to acute environmental stress.

Given our results of increased abundance of HSP-encoding transcripts at hotter temperatures, we demonstrate the ability of early zygotes to actively express zygotic genes that encode for the heat shock response. Thus, early embryos may not be dependent solely on maternal transcripts for protection from heat stress and can instead induce the expression of HSPs themselves. The heat shock response is a conserved response to cellular stress across taxa, and is characterized by shifts in gene expression across the transcriptome which includes a suite of HSPs (Buckley et al., 2006; Kültz, 2005; Leemans et al., 2000; Lockwood et al., 2010; Selander et al., 2015). Thermal stress causes protein unfolding which can have adverse cellular effects, including protein aggregation and loss of function (Richter et al., 2010). This response can be mitigated by the expression of HSPs which protect the cell by acting as molecular chaperones that can refold denatured proteins by binding to and sequestering them, helping to ensure survival (Feder et al., 1996; Somero et al., 2017). It should be noted that a previous study identified a somewhat muted, though significant, increase in the expression of HSPs in early *Drosophila* embryos (Graziosi et al., 1980). However, to our knowledge, ours is the first study to document a wide-spread transcriptional response to temperature in early embryos.

The ability for early embryos to undergo a transcriptomic response to heat stress could be due to developmental progression. Early onset of zygotic transcription after acute heat shock could suggest a shift in the maternal to zygotic transition (MZT). This could be a result of the positive relationship between temperature and reaction rates

(Somero et al. 2017), such that embryos develop more quickly in hotter temperatures (Chong et al., 2018). We saw increased abundance of transcripts involved in developmental processes at hotter temperatures, including pathways in cellular organization, and morphogenesis, which support this pattern. We also observed that 2,793 transcripts decreased in abundance with increasing temperature across embryos from both regions. The transcripts that decreased in abundance most likely represent maternal transcripts that were being degraded (Atallah and Lott, 2018; Omura and Lott, 2020) because they were present at highest abundances at 25°C. The degradation of these maternal transcripts may be an outcome of the onset of zygotic transcription, which involves the concomitant degradation of maternal mRNAs when the zygotic genome begins to be expressed (Tadros and Lipshitz, 2009).

Alternatively, these transcriptomic responses to increasing temperature could signify a more specific molecular physiological response to heat stress that is distinct from the transcriptomic patterns that would result from merely speeding up development rate. The most robust changes in gene expression that we observed were among transcripts that encode molecular chaperones, or heat shock proteins (HSPs), and the induction of heat shock genes does not take place during normal development (Brown et al., 2014; Casas-Vila et al., 2017; Lefebvre and Lécuyer, 2018). Meanwhile, the degradation of thousands of maternal transcripts could be a component of the heat shock response, which characteristically involves the down-regulation of thousands of genes (Richter et al., 2010). In the canonical model of the heat shock response, the expression of HSPs increases dramatically, but the expression of thousands of other genes is reduced (Somero et al. 2017). This reduction in gene expression serves to halt the production of

proteins that could unfold, denature, and cause further risk to the cell (Richter et al., 2010), as could be the case in our study. Thus, observed transcriptional responses to heat stress support a strong heat shock response in early *D. melanogaster* embryos that is distinct from transcriptional characteristics of the MZT; however, it may be difficult to discern if these possibilities are co-occurring or if one is occurring more than the other.

Our data indicate that, at least in terms of transcriptomic variation, relatively few genes are involved in thermal adaptation of acute heat tolerance between tropical and temperate *Drosophila* embryos. Previous studies have shown similar trends in the evolution of transcriptional regulation among species, such that overall transcriptomic responses to heat stress are conserved, but key differences in the expression of few genes underlie heat tolerance (Dilly et al., 2012; Lockwood et al., 2010). Based upon the set of genes that differentiate tropical and temperate *Drosophila* embryos, our results support the interpretation that embryos from tropical populations are better equipped to prevent cellular damage during heat stress by maintaining higher abundances of transcripts encoding proteins that allow them to cope with oxidative stress. Although temperate embryos had higher abundance of some transcripts involved in oxidative stress, such as *Sod3-RA*, these transcripts were not highly abundant in embryos from either region, and thus, may not signify a main source of protection from oxidative stress. Furthermore, GO analyses indicated that transcripts that were of higher abundance in temperate embryos were involved in cellular processes that don't appear to be directly related to thermal tolerance (i.e., ribonucleoprotein complex subunit organization, protein acylation, and protein localization to membrane), and thus, it is not clear if these transcripts are helpful

in mounting a cellular defense against heat stress, particularly since they don't correlate with higher heat tolerance at the whole-organism level.

Thus, mitigating the effects of oxidative stress may be a key physiological mechanism underlying thermal adaptation in early *Drosophila* embryos. Thermal stress can lead to oxidative stress with changes in cellular redox potential and increased production of reactive oxygen species (ROS) and free radicals (Kültz, 2005; Somero et al., 2017). Reactive oxygen species can cause cell damage by oxidizing DNA, RNA, lipids, and/or proteins. Oxidized DNA and RNA can cause mutagenesis and cell death (Keyer et al., 1995), oxidized fatty acids can cause changes in membrane permeability (i.e. leaky membranes) (Schönfeld and Wojtczak, 2008) and alterations to membrane-specific lipid-protein interactions (Tomanek, 2015), while oxidized lipids and polyunsaturated fatty acids can cause the production of reactive aldehydes and alkanes, radical amino acids, and other molecules that are cytotoxic (Lushchak, 2014). Key molecular players in mitigating cellular defenses against antagonistic ROS and free radicals include proteins like superoxide dismutase (*Sod3*), which converts oxygen free radicals to hydrogen peroxide and restores redox balance (Fridovich, 1998). Others include uncoupling proteins, like Ucp4A, that allow proton reentry into the mitochondria without the need for ATP synthesis (Echtay, 2007; Somero et al., 2017), and thus maintain the flow of electrons through the electron transport system (ETS) to prevent the univalent reduction of oxygen (i.e., the production of ROS) prior to oxidative phosphorylation. Furthermore, isocitrate dehydrogenase [NADP] (Idh) plays important roles in redox balance and the scavenging of ROS (Bayliak et al., 2017; Somero et al., 2017). The combination of higher abundance of transcripts that encode these oxidative

stress response proteins in tropical embryos could increase survival, and therefore, thermal tolerance. Thermal stress tolerance and oxidative stress tolerance are inherently linked across taxa (Abele et al., 2002; De Zoysa et al., 2009; Teixeira et al., 2013; Tomanek and Zuzow, 2010). This relationship includes the increased expression of HSPs during and after oxidative stress events to prevent cellular damage (Kalmar and Greensmith, 2009; Tomanek, 2015). Furthermore, HSPs can act as sensors of cellular redox changes, activating ROS scavengers like Sod, catalase, and peroxidases to prevent the depolarization of membranes, preserving cellular integrity (Madeira et al., 2013). Under direct temperature stress, the interplay between thermal stress tolerance and oxidative stress tolerance is key in the survival of organisms (Heise et al., 2006); however, to our knowledge, no studies have identified oxidative stress response loci as the main targets of thermal selection as we report here.

In the present study, we present evidence that maternal effects (i.e., maternally loaded protein-coding mRNAs) provide the means by which heat tolerance is higher in tropical embryos than in temperate embryos. This finding is somewhat inconsistent with a previously published study to come out of our laboratory, in which we tested the role of maternal effects in embryonic thermal tolerance in the F1 progeny from mating crosses between one tropical and one temperate genotype, Chiapas and VT12, respectively (Lockwood et al., 2018). These previous results showed that thermal tolerance of the F1 progeny of this cross matched that of the parental tropical genotype, regardless of whether the mother or the father was of the tropical genotype. This result suggested that embryonic thermal tolerance was not due to maternal effects, but rather that higher embryonic thermal tolerance was a dominant trait overall. We note that in the present

study transcript abundances of mRNAs encoding oxidative stress response proteins in the Chiapas vs. VT12 genotypes showed patterns that were consistent with the patterns present in the other tropical and temperate genotypes, such that Chiapas embryos consistently had higher abundances of these transcripts than VT12 embryos. There was one exception to this pattern, the transcript *Ucp4A*, for which VT12 was an outlier from the other temperate genotypes, having similar abundances of *Ucp4A* as Chiapas. The relatively high levels of *Ucp4A* provided by VT12 mothers would help to explain the high heat tolerance of VT12 x Chiapas F1 hybrids, if in fact VT12 mothers consistently load this transcript when crossed to males of a tropical genotype. However, these transcriptomic patterns do not explain the overall lower heat tolerance of homozygous VT12 embryos. It could be that maternally loaded transcripts work in concert with other cellular stress response mechanisms, and that by themselves they do not account for the full extent of the variance in whole-organism embryonic thermal tolerance. We note that although oxidative stress response transcripts may aid embryos in combatting cellular damage against heat stress, they are likely not the sole players in mitigating (or differentiating) whole-organism level thermal tolerance. Transcriptomics provides insight into one level of biological organization that may underlie thermal tolerance, but cannot reveal all potential avenues that could confer higher thermal tolerance. Other levels of biological regulation of stress responses, such as the proteome, are likely to be important as well. Future work should examine other potential mechanisms to more fully characterize the molecular and cellular bases of this instance of life-stage-specific thermal adaptation in *Drosophila melanogaster*.

# CONCLUSION

As atmospheric temperatures continue to rise due to climate change, *D. melanogaster* embryos could face temperatures outside their thermal maxima, and given our results, these embryos may or may not possess the genetic material needed for survival depending on their region of origin. Based on our results, which determined patterns of evolutionary thermal adaptation of heat tolerance in diverged embryos, we can predict how *D. melanogaster* may survive hotter temperatures induced by climate change. The populations described here are recently diverged over a short evolutionary time-scale yet show the potential for rapid evolution of thermal tolerance at the embryonic stage. This was regulated by relatively few genes underlying heat tolerance; thus, evolution may not need to modify large-scale physiological responses to heat stress to be able to achieve higher thermal tolerance. For *D. melanogaster*, higher heat tolerance may only require higher abundances of oxidative stress proteins. Future research could examine the role of these genes that were identified as significantly more abundant in tropical embryos for thermal tolerance, including *Idh*, *Ucp4A*, and *rl*, using RNA interference in tropical embryos and/or over-expression in temperate embryos to determine the extent to which any particular gene contributes to whole organism thermal tolerance. Furthermore, the potential for adaptive maternal effects should be examined by isolating maternal effects by conducting transcriptomics on offspring from reciprocal crosses of more thermally tolerant tropical genotypes with less thermally tolerant temperate genotypes. This will allow for further understanding of the genetic mechanisms that have diverged between temperate and tropical populations.

## FIGURES AND TABLES

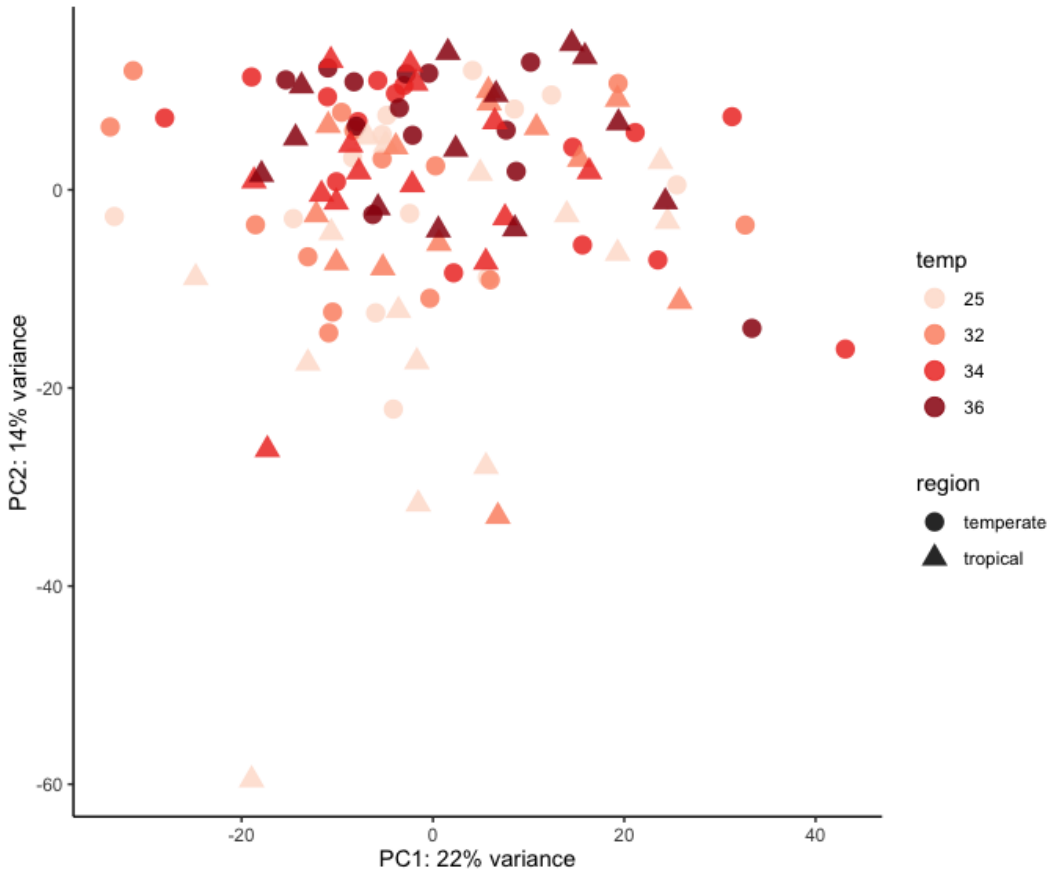


**Figure 1: Full transcriptomic abundances do not group 0-1h *Drosophila embryos* by region or heat shock temperature.** Principal components analysis of normalized transcript counts for all 29,686 transcripts found among the 110 embryonic samples. Each point represents complete transcriptomic abundance for each sample, collapsed in principal component space for the first two principal components that describe 36% of the total variation in transcript abundance. Increasing heat shock temperature is represented by the increasing color gradient, and region of origin is represented by the shape of the symbol.

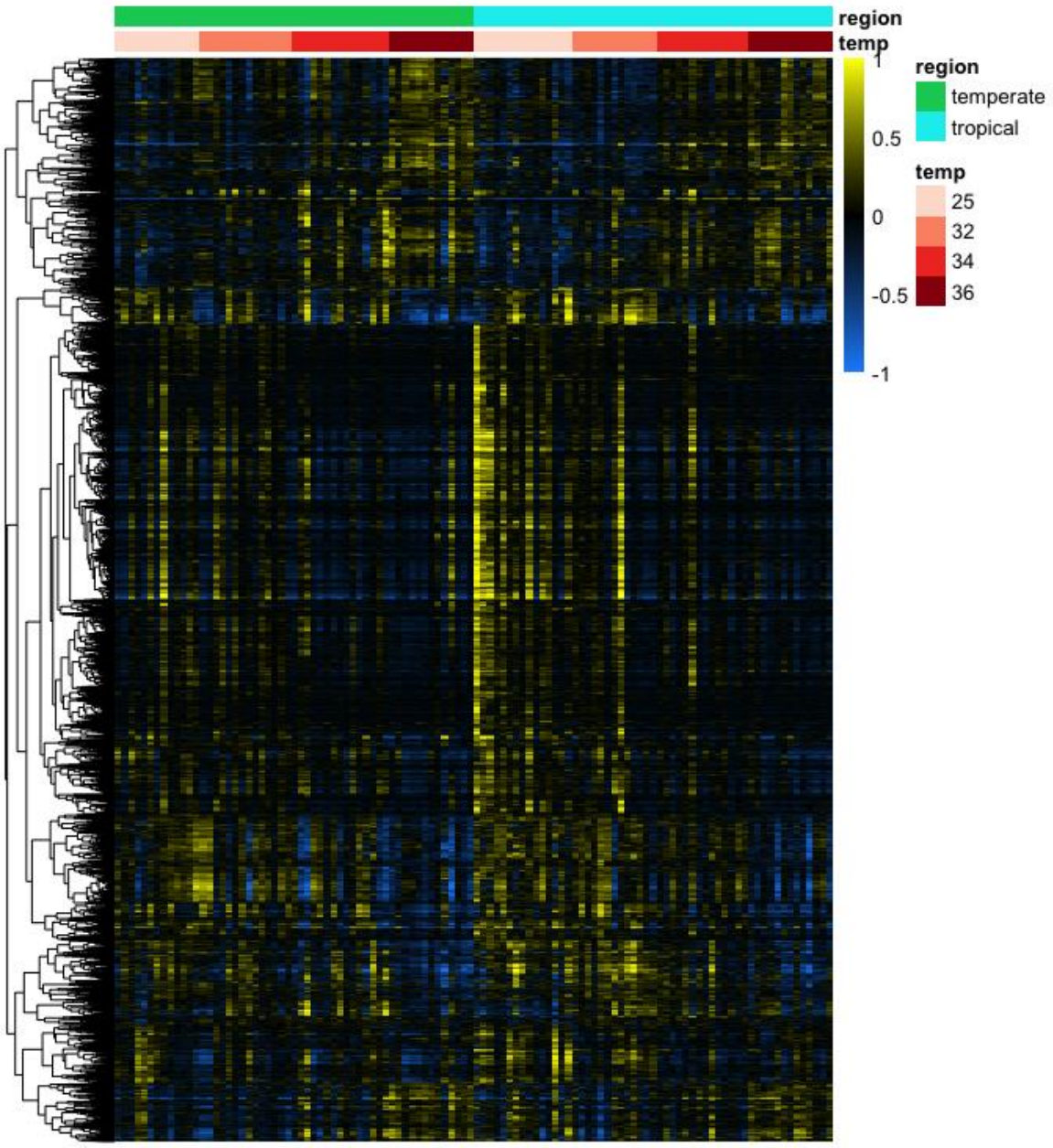


**Figure 2: Heat shock led to differential transcript abundances in *D. melanogaster* embryos, regardless of region of origin.** Heat map of the normalized transcript abundance counts ($log_2$-ratio) of 4,534 transcripts (rows) that were significantly differentially abundant with heat shock temperature across the 110 embryonic samples (columns) (LRT, temperature factor, FDR <0.05). Transcripts are clustered using a

hierarchical clustering algorithm. The gradient scale bar indicates the deviation from row means across all 110 samples, with blue indicating down-regulation in abundance, yellow indicating up-regulation in abundance, and black no change in abundance from the mean. Each sample is represented by region of origin and heat shock temperature

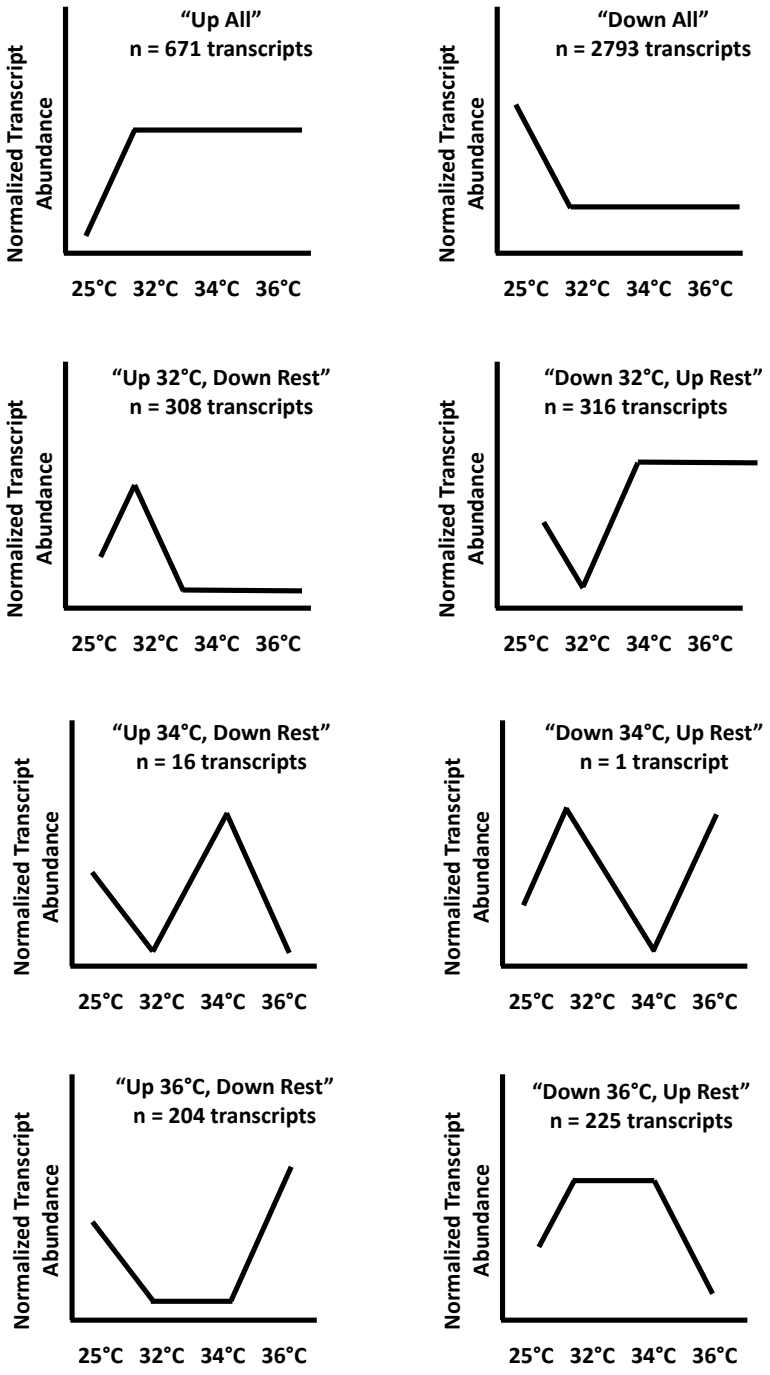


**Figure 3: Differential abundance of gene transcripts was found across all heat shock**

**temperatures relative to 25°C in *Drosophila melanogaster* embryos (0-1hr) regardless of region of origin (temperate vs. tropical)** (LRT, temperature factor, FDR < 0.05, n=4,534 transcripts).

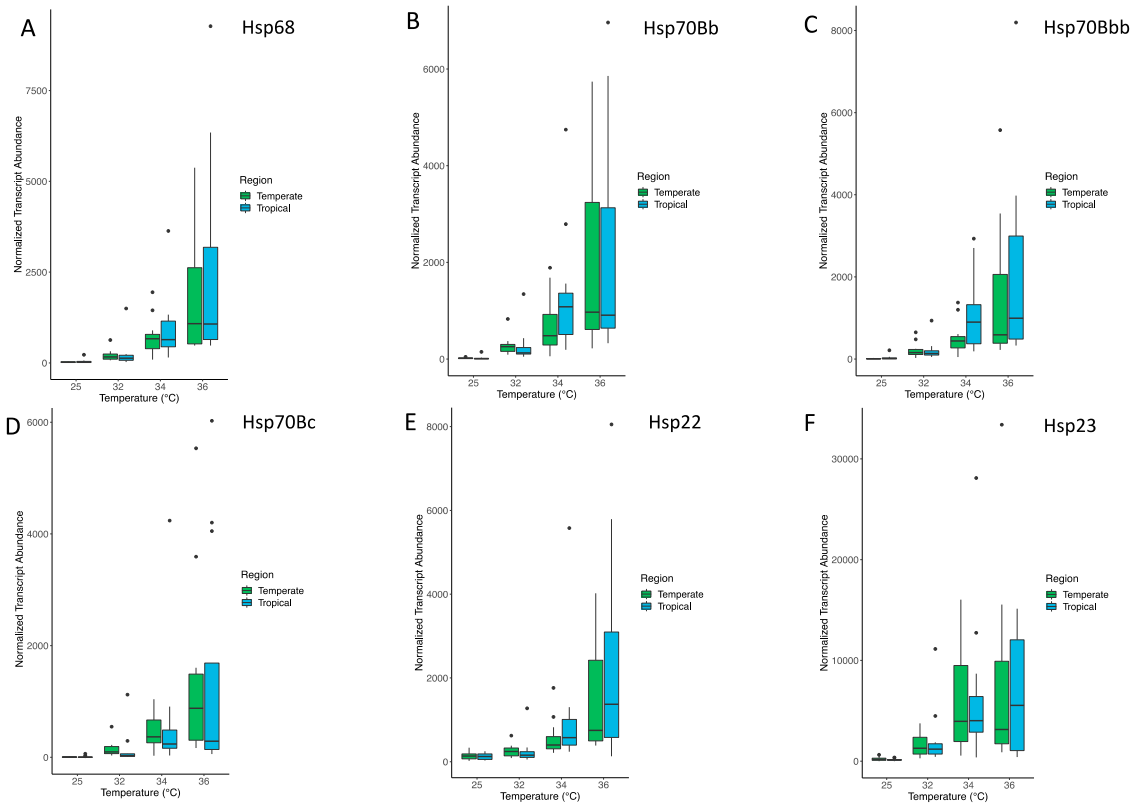


**Figure 4: Tropical and temperate embryos had significantly higher abundance of transcripts that encode heat shock proteins (HSPs) with increasing heat shock temperature.** (A) Normalized transcript abundances for *Hsp68* for early temperate and tropical *D. melanogaster* embryos (0-1hr) across heat shock temperatures (LRT; temperature factor, FDR = 8.08E-58). (B) Normalized transcript abundances for *Hsp70Bb* for early temperate and tropical embryos (0-1hr) across heat shock temperatures (LRT; temperature factor, FDR = 2.87E-51). (C) Normalized transcript abundances for *Hsp70Bbb* for early temperate and tropical embryos (0-1hr) across heat shock temperatures (LRT; temperature factor, FDR = 2.81E-41). (D) Normalized transcript abundances for *Hsp70Bc* for early temperate and tropical embryos (0-1hr) across heat shock temperatures (LRT; temperature factor, FDR = 3.37E-37). (E)

Normalized transcript abundances for *Hsp22* for early temperate and tropical embryos (0-1hr) across heat shock temperatures (LRT; temperature factor, FDR = 6.49E-36). (F) Normalized transcript abundances for *Hsp23* for early temperate and tropical embryos (0-1hr) across heat shock temperatures (LRT; temperature factor, FDR = 5.96E-36). Note that y-axis scales are different for each plot.

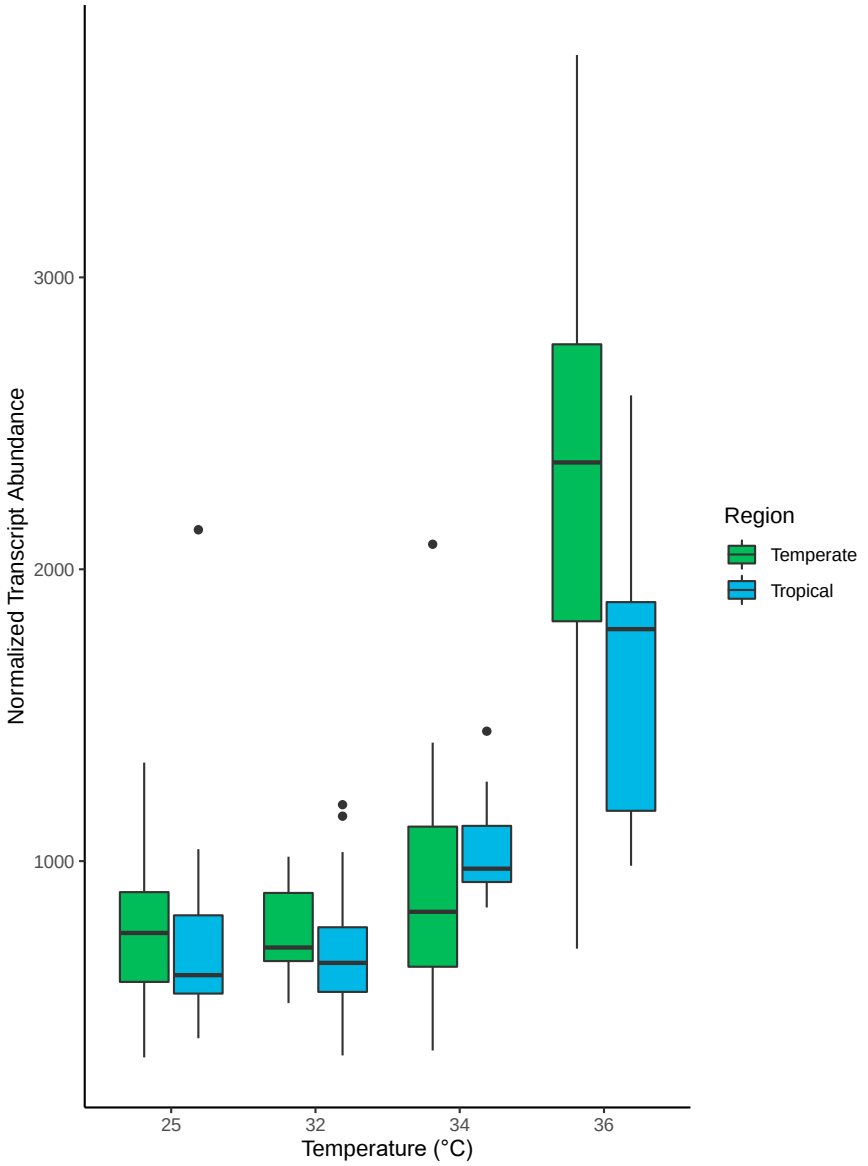


**Figure 5: Tropical and temperate embryos had significantly higher abundance of the transcript encoding fs(1)N, which protects the vitelline membrane, with increasing heat shock temperature**. Normalized transcript abundances for early temperate and tropical *D. melanogaster* embryos (0-1hr) across heat shock temperatures (LRT; temperature factor, FDR = 1.19E-31).

**Table 1:** Gene ontology (GO) categories identified for the 671 significant transcripts that had higher abundance at heat shock temperatures relative to 25°C ("Up All") (Webgestalt; Over-representation analysis (ORA) for biological processes; FDR < 0.1).

| GeneSet | Description | Number of Enriched Genes | FDR |
|---|---|---|---|
| GO:0006457 | protein folding | 25 | 6.82E-09 |
| GO:0009994 | oocyte differentiation | 26 | 1.03E-06 |
| GO:0009798 | axis specification | 26 | 2.04E-05 |
| GO:0006403 | RNA localization | 25 | 5.52E-05 |
| GO:0044770 | cell cycle phase transition | 19 | 1.91E-04 |
| GO:0016197 | endosomal transport | 15 | 0.00126612 |
| GO:0034976 | response to endoplasmic reticulum stress | 12 | 0.00201997 |
| GO:0051169 | nuclear transport | 17 | 0.00201997 |
| GO:0035966 | response to topologically incorrect protein | 9 | 0.00201997 |
| GO:0007300 | ovarian nurse cell to oocyte transport | 8 | 0.00235017 |
| GO:0061919 | process utilizing autophagic mechanism | 19 | 0.00396872 |
| GO:1990778 | protein localization to cell periphery | 10 | 0.00396872 |
| GO:0010927 | cellular component assembly involved in morphogenesis | 17 | 0.0042959 |
| GO:0007028 | cytoplasm organization | 11 | 0.0042959 |
| GO:0030703 | eggshell formation | 15 | 0.00526024 |
| GO:0030865 | cortical cytoskeleton organization | 10 | 0.01091541 |
| GO:0016482 | cytosolic transport | 9 | 0.01130686 |
| GO:0051129 | negative regulation of cellular component organization | 17 | 0.01169354 |
| GO:0017038 | protein import | 13 | 0.01511357 |
| GO:0006836 | neurotransmitter transport | 15 | 0.01627073 |
| GO:0035148 | tube formation | 7 | 0.01671067 |
| GO:0022411 | cellular component disassembly | 15 | 0.01822361 |
| GO:0014070 | response to organic cyclic compound | 12 | 0.02554591 |
| GO:0006997 | nucleus organization | 10 | 0.02982966 |

| | | | |
|---|---|---|---|
| GO:0007293 | germarium-derived egg chamber formation | 11 | 0.02982966 |
| GO:0007264 | small GTPase mediated signal transduction | 17 | 0.03014077 |
| GO:0051338 | regulation of transferase activity | 15 | 0.03014077 |
| GO:0051235 | maintenance of location | 12 | 0.03058317 |
| GO:0010256 | endomembrane system organization | 16 | 0.03598182 |
| GO:0042157 | lipoprotein metabolic process | 9 | 0.03598182 |
| GO:0007349 | cellularization | 11 | 0.04110699 |
| GO:0016333 | morphogenesis of follicular epithelium | 7 | 0.04377652 |
| GO:0007219 | Notch signaling pathway | 15 | 0.04377652 |
| GO:0048193 | Golgi vesicle transport | 13 | 0.04863789 |
| GO:0072593 | reactive oxygen species metabolic process | 6 | 0.05355786 |
| GO:1903509 | liposaccharide metabolic process | 7 | 0.05368813 |
| GO:0007277 | pole cell development | 5 | 0.05368813 |
| GO:0043631 | RNA polyadenylation | 5 | 0.05368813 |
| GO:0043543 | protein acylation | 12 | 0.05536729 |
| GO:0040029 | regulation of gene expression, epigenetic | 16 | 0.06780536 |
| GO:0033993 | response to lipid | 8 | 0.07543333 |
| GO:0043062 | extracellular structure organization | 8 | 0.07543333 |
| GO:0006354 | DNA-templated transcription, elongation | 6 | 0.08908386 |
| GO:0099504 | synaptic vesicle cycle | 11 | 0.08908386 |
| GO:0008213 | protein alkylation | 9 | 0.08908386 |
| GO:0032196 | transposition | 4 | 0.09111003 |
| GO:0007034 | vacuolar transport | 6 | 0.09111003 |
| GO:0015931 | nucleobase-containing compound transport | 12 | 0.09111003 |
| GO:0043473 | pigmentation | 11 | 0.09111003 |
| GO:0006352 | DNA-templated transcription, initiation | 9 | 0.091169 |
| GO:0001505 | regulation of neurotransmitter levels | 12 | 0.091169 |
| GO:0050803 | regulation of synapse structure or activity | 14 | 0.09212245 |
| GO:0072657 | protein localization to membrane | 11 | 0.0937133 |
| GO:0031647 | regulation of protein stability | 6 | 0.09557233 |

| | | | |
|---|---|---|---|
| GO:2000241 | regulation of reproductive process | 10 | 0.09557233 |
| GO:0071824 | protein-DNA complex subunit organization | 11 | 0.09807782 |
| GO:0031123 | RNA 3'-end processing | 8 | 0.09807782 |
| GO:0071166 | ribonucleoprotein complex localization | 6 | 0.09807782 |

**Table 2:** Gene ontology (GO) categories identified for the 2,793 significant transcripts that had lower abundance at heat shock temperatures relative to 25°C ("Down All") (Webgestalt; Over-representation analysis (ORA) for biological processes; FDR < 0.1).

| GeneSet | Description | Number of Enriched Genes | FDR |
|---|---|---|---|
| GO:0006040 | amino sugar metabolic process | 52 | 1.88E-10 |
| GO:0006022 | aminoglycan metabolic process | 52 | 1.60E-08 |
| GO:0003013 | circulatory system process | 25 | 3.71E-08 |
| GO:0010469 | regulation of signaling receptor activity | 37 | 2.39E-06 |
| GO:0048859 | formation of anatomical boundary | 23 | 7.41E-06 |
| GO:0015850 | organic hydroxy compound transport | 20 | 7.41E-06 |
| GO:0007498 | mesoderm development | 34 | 1.02E-05 |
| GO:0008643 | carbohydrate transport | 17 | 1.09E-05 |
| GO:0019932 | second-messenger-mediated signaling | 38 | 1.09E-05 |
| GO:0009792 | embryo development ending in birth or egg hatching | 53 | 1.09E-05 |
| GO:0006836 | neurotransmitter transport | 42 | 1.44E-05 |
| GO:0010817 | regulation of hormone levels | 32 | 2.21E-05 |
| GO:0001505 | regulation of neurotransmitter levels | 41 | 2.21E-05 |
| GO:0048863 | stem cell differentiation | 32 | 2.44E-05 |
| GO:0048645 | animal organ formation | 22 | 2.63E-05 |
| GO:0007626 | locomotory behavior | 49 | 4.89E-05 |
| GO:0072359 | circulatory system development | 33 | 5.59E-05 |
| GO:0030534 | adult behavior | 43 | 8.56E-05 |
| GO:0042063 | gliogenesis | 28 | 8.82E-05 |
| GO:0015849 | organic acid transport | 33 | 1.12E-04 |
| GO:0099504 | synaptic vesicle cycle | 35 | 1.12E-04 |
| GO:0010876 | lipid localization | 38 | 1.30E-04 |
| GO:0007219 | Notch signaling pathway | 43 | 2.05E-04 |
| GO:0009636 | response to toxic substance | 49 | 2.05E-04 |
| GO:0048732 | gland development | 47 | 2.20E-04 |
| GO:0050890 | cognition | 41 | 3.28E-04 |
| GO:0019748 | secondary metabolic process | 29 | 5.66E-04 |

| | | | |
|---|---|---|---|
| GO:0070848 | response to growth factor | 29 | 5.66E-04 |
| GO:1901615 | organic hydroxy compound metabolic process | 44 | 6.09E-04 |
| GO:0060322 | head development | 35 | 8.01E-04 |
| GO:0001655 | urogenital system development | 23 | 8.05E-04 |
| GO:0055123 | digestive system development | 29 | 0.00104867 |
| GO:0035272 | exocrine system development | 33 | 0.00127283 |
| GO:0042303 | molting cycle | 28 | 0.00143507 |
| GO:0035218 | leg disc development | 28 | 0.0016632 |
| GO:0090130 | tissue migration | 42 | 0.00209536 |
| GO:0034330 | cell junction organization | 26 | 0.00277801 |
| GO:0003014 | renal system process | 10 | 0.00281884 |
| GO:0002164 | larval development | 38 | 0.00331143 |
| GO:0007631 | feeding behavior | 17 | 0.00341653 |
| GO:0051604 | protein maturation | 26 | 0.00352132 |
| GO:0048568 | embryonic organ development | 17 | 0.00411805 |
| GO:0099003 | vesicle-mediated transport in synapse | 29 | 0.00541069 |
| GO:0048511 | rhythmic process | 37 | 0.00583645 |
| GO:0035214 | eye-antennal disc development | 19 | 0.00623361 |
| GO:0061458 | reproductive system development | 22 | 0.00699072 |
| GO:0043171 | peptide catabolic process | 10 | 0.00708173 |
| GO:0044057 | regulation of system process | 18 | 0.00708173 |
| GO:0051705 | multi-organism behavior | 9 | 0.00711473 |
| GO:0010171 | body morphogenesis | 10 | 0.00940781 |
| GO:0043473 | pigmentation | 29 | 0.01032074 |
| GO:0098727 | maintenance of cell number | 24 | 0.01418521 |
| GO:0042391 | regulation of membrane potential | 19 | 0.01557825 |
| GO:0007398 | ectoderm development | 10 | 0.01598067 |
| GO:0050795 | regulation of behavior | 23 | 0.01643989 |
| GO:0007164 | establishment of tissue polarity | 25 | 0.01843627 |
| GO:0042044 | fluid transport | 11 | 0.02186777 |
| GO:0051098 | regulation of binding | 9 | 0.0226522 |
| GO:0007568 | aging | 36 | 0.02350708 |
| GO:0031098 | stress-activated protein kinase signaling cascade | 21 | 0.02981082 |
| GO:1901568 | fatty acid derivative metabolic process | 10 | 0.03099928 |
| GO:0008037 | cell recognition | 26 | 0.03171766 |

| | | | |
|---|---|---|---|
| GO:0042440 | pigment metabolic process | 28 | 0.03275448 |
| GO:0007422 | peripheral nervous system development | 15 | 0.03475421 |
| GO:0007548 | sex differentiation | 24 | 0.04004098 |
| GO:0032101 | regulation of response to external stimulus | 31 | 0.0434739 |
| GO:0035329 | hippo signaling | 12 | 0.04391845 |
| GO:0009410 | response to xenobiotic stimulus | 11 | 0.04395844 |
| GO:0140053 | mitochondrial gene expression | 26 | 0.04841041 |
| GO:0035215 | genital disc development | 13 | 0.04841041 |
| GO:0104004 | cellular response to environmental stimulus | 19 | 0.04912204 |
| GO:0001738 | morphogenesis of a polarized epithelium | 25 | 0.05080639 |
| GO:0043900 | regulation of multi-organism process | 34 | 0.05080639 |
| GO:0030537 | larval behavior | 13 | 0.0539732 |
| GO:0060856 | establishment of blood-brain barrier | 7 | 0.05462204 |
| GO:0050803 | regulation of synapse structure or activity | 34 | 0.06228112 |
| GO:0009611 | response to wounding | 27 | 0.0643694 |
| GO:0010035 | response to inorganic substance | 17 | 0.06742066 |
| GO:0003012 | muscle system process | 10 | 0.06834654 |
| GO:0046677 | response to antibiotic | 19 | 0.07007366 |
| GO:0097164 | ammonium ion metabolic process | 13 | 0.07779506 |
| GO:0044092 | negative regulation of molecular function | 37 | 0.08576424 |
| GO:0008544 | epidermis development | 14 | 0.09213445 |
| GO:0009100 | glycoprotein metabolic process | 29 | 0.09621601 |

**Table 3:** Gene ontology (GO) categories identified for the 308 significant transcripts that had higher abundance at 32°C relative to all other temperatures ("Up 32°C, down rest") (Webgestalt; Over-representation analysis (ORA) for biological processes; FDR < 0.1).

| GeneSet | Description | Number of Enriched Genes | FDR |
|---|---|---|---|
| GO:0009798 | axis specification | 20 | 2.36E-07 |
| GO:0060322 | head development | 14 | 3.70E-05 |
| GO:0048863 | stem cell differentiation | 12 | 3.70E-05 |
| GO:0040029 | regulation of gene expression, epigenetic | 15 | 3.91E-04 |
| GO:0098727 | maintenance of cell number | 10 | 0.00106074 |
| GO:0009994 | oocyte differentiation | 12 | 0.00246491 |
| GO:0051129 | negative regulation of cellular component organization | 12 | 0.00246491 |
| GO:0048645 | animal organ formation | 7 | 0.00246491 |
| GO:0001655 | urogenital system development | 8 | 0.00246491 |
| GO:0007219 | Notch signaling pathway | 12 | 0.00246491 |
| GO:0009792 | embryo development ending in birth or egg hatching | 13 | 0.00261277 |
| GO:0070848 | response to growth factor | 9 | 0.00327613 |
| GO:0055123 | digestive system development | 9 | 0.00405373 |
| GO:0072359 | circulatory system development | 9 | 0.00433525 |
| GO:0007548 | sex differentiation | 9 | 0.00433688 |
| GO:0007224 | smoothened signaling pathway | 7 | 0.00465983 |
| GO:0007293 | germarium-derived egg chamber formation | 8 | 0.00793558 |
| GO:0034330 | cell junction organization | 8 | 0.00793558 |
| GO:0048017 | inositol lipid-mediated signaling | 4 | 0.00963224 |
| GO:0035218 | leg disc development | 8 | 0.01132949 |
| GO:0051338 | regulation of transferase activity | 10 | 0.01137875 |
| GO:0007264 | small GTPase mediated signal transduction | 11 | 0.01235541 |
| GO:0048732 | gland development | 11 | 0.01235541 |
| GO:1901698 | response to nitrogen compound | 10 | 0.01813 |
| GO:0030865 | cortical cytoskeleton organization | 6 | 0.02072046 |
| GO:0050803 | regulation of synapse structure or activity | 10 | 0.02072046 |

| | | | |
|---|---|---|---|
| GO:0097696 | STAT cascade | 4 | 0.02458354 |
| GO:0090130 | tissue migration | 10 | 0.02458354 |
| GO:0060537 | muscle tissue development | 4 | 0.02661408 |
| GO:0035272 | exocrine system development | 8 | 0.0279016 |
| GO:0007530 | sex determination | 4 | 0.0279016 |
| GO:0048859 | formation of anatomical boundary | 5 | 0.02881932 |
| GO:0050890 | cognition | 9 | 0.02964274 |
| GO:0007300 | ovarian nurse cell to oocyte transport | 4 | 0.03155821 |
| GO:0002164 | larval development | 9 | 0.03155821 |
| GO:0014070 | response to organic cyclic compound | 7 | 0.0338642 |
| GO:0061458 | reproductive system development | 6 | 0.03550884 |
| GO:0001763 | morphogenesis of a branching structure | 5 | 0.03550884 |
| GO:0008544 | epidermis development | 5 | 0.0393083 |
| GO:0031344 | regulation of cell projection organization | 8 | 0.0393083 |
| GO:0031098 | stress-activated protein kinase signaling cascade | 6 | 0.04481629 |
| GO:0019932 | second-messenger-mediated signaling | 7 | 0.05549979 |
| GO:0007626 | locomotory behavior | 9 | 0.06108758 |
| GO:0071103 | DNA conformation change | 8 | 0.06227176 |
| GO:0034248 | regulation of cellular amide metabolic process | 9 | 0.06227176 |
| GO:0030534 | adult behavior | 8 | 0.06756182 |
| GO:0098732 | macromolecule deacylation | 3 | 0.06757223 |
| GO:0007498 | mesoderm development | 6 | 0.07114646 |
| GO:0044770 | cell cycle phase transition | 7 | 0.07114646 |
| GO:0007349 | cellularization | 6 | 0.07548408 |
| GO:0016333 | morphogenesis of follicular epithelium | 4 | 0.07880888 |
| GO:0007164 | establishment of tissue polarity | 6 | 0.08240832 |
| GO:0007568 | aging | 8 | 0.08971864 |
| GO:0098876 | vesicle-mediated transport to the plasma membrane | 3 | 0.09466236 |
| GO:0051235 | maintenance of location | 6 | 0.09900418 |

**Table 4:** Gene ontology (GO) categories identified for the 316 significant transcripts that had lower abundance at 32°C relative to all other temperatures ("Down 32°C, up rest") (Webgestalt; Over-representation analysis (ORA) for biological processes; FDR < 0.1).

| GeneSet | Description | Number of Enriched Genes | FDR |
|---|---|---|---|
| GO:0031929 | TOR signaling | 9 | 0.0027036 |
| GO:0044770 | cell cycle phase transition | 10 | 0.03186213 |
| GO:0048193 | Golgi vesicle transport | 10 | 0.03186213 |
| GO:0009991 | response to extracellular stimulus | 11 | 0.03186213 |
| GO:0009798 | axis specification | 12 | 0.03186213 |
| GO:0009566 | fertilization | 5 | 0.03594662 |
| GO:0007349 | cellularization | 8 | 0.04612081 |
| GO:0071103 | DNA conformation change | 10 | 0.04612081 |
| GO:0051258 | protein polymerization | 7 | 0.06531665 |
| GO:0006352 | DNA-templated transcription, initiation | 7 | 0.06531665 |
| GO:0007277 | pole cell development | 4 | 0.06531665 |
| GO:0061919 | process utilizing autophagic mechanism | 10 | 0.06531665 |
| GO:0071824 | protein-DNA complex subunit organization | 8 | 0.09181977 |

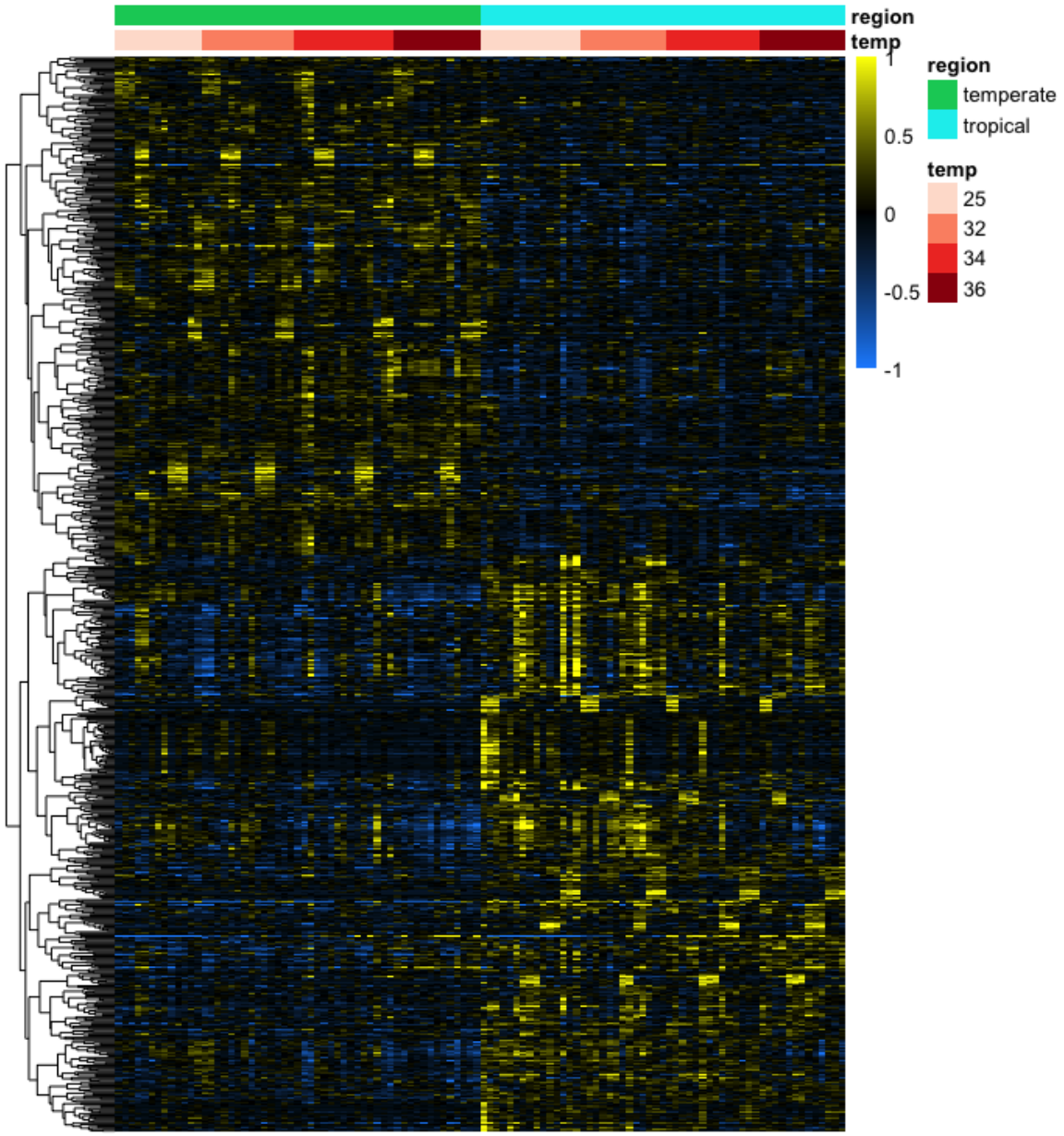


**Figure 6: Tropical versus temperate early *D. melanogaster* embryos (0-1hr) had differential transcript abundance by region across all four heat shock temperatures.** Heat map of the normalized transcript abundance counts ($log_2$-ratio) of 828 transcripts (rows) that were significantly differentially abundant with region across the 110 embryonic samples (columns) (LRT, region factor, FDR <0.05). Transcripts are clustered using a hierarchical clustering algorithm. The gradient scale bar indicates the deviation

from row means across all 110 samples, with blue indicating down-regulation in abundance, yellow indicating up-regulation in abundance, and black no change in abundance from the mean. Each sample is represented by region of origin and heat shock temperature.

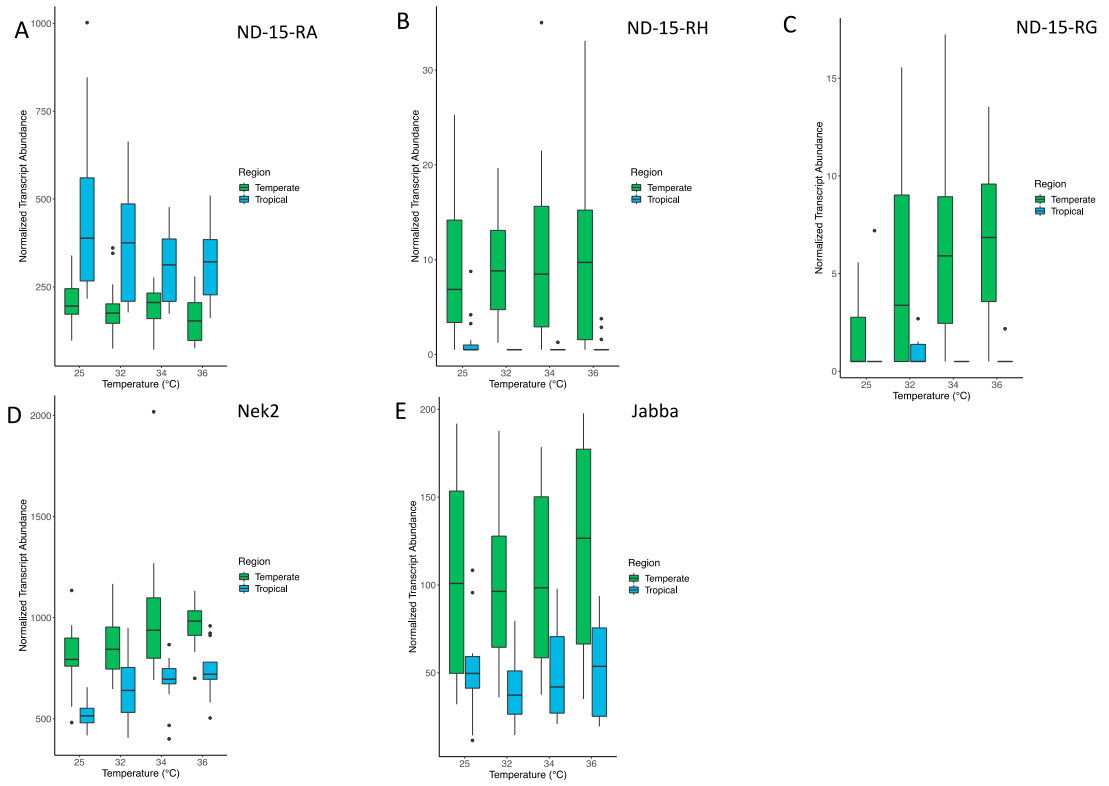


**Figure 7: Top significantly abundant gene transcripts across regions had, overall, low abundance across individuals.** (A) Normalized transcript abundances for *ND-15-RA*, NADH dehydrogenase, for early temperate and tropical *D. melanogaster* embryos (0-1hr) across heat shock temperatures (LRT; region factor, FDR = 9.66E-17). (B) Normalized transcript abundances for *ND-15-RH*, NADH dehydrogenase, for early temperate and tropical embryos (0-1hr) across heat shock temperatures (LRT, region factor, FDR = 9.66E-17). (C) Normalized transcript abundances for *ND-15-RG*, NADH dehydrogenase, for early temperate and tropical embryos (0-1hr) across heat shock temperatures (LRT, region factor, FDR = 1.25E-26). (D) Normalized transcript abundances for *Nek2*, which encodes a kinase that regulates mitosis, for early temperate and tropical embryos (0-1hr) across heat shock temperatures (LRT, region factor, FDR =

5.74E-16). (E) Normalized transcript abundances for *Jabba*, which promotes pre-cellular embryonic development, for early temperate and tropical embryos (0-1hr) across heat shock temperatures (LRT, region factor, FDR = 1.02E-14). Note that y-axis scales are different for each plot.

**Table 5:** Gene ontology (GO) categories enriched for the 473 transcripts that were significantly more abundant in the tropical region (Webgestalt; Over-representation analysis (ORA) for biological processes; FDR < 0.1).

| GeneSet | Description | Number of Enriched Genes | FDR |
|---|---|---|---|
| GO:0006979 | response to oxidative stress | 13 | 0.00981012 |
| GO:0006040 | amino sugar metabolic process | 11 | 0.07417656 |
| GO:0006839 | mitochondrial transport | 9 | 0.07417656 |
| GO:0044282 | small molecule catabolic process | 11 | 0.07606442 |
| GO:0032101 | regulation of response to external stimulus | 11 | 0.07606442 |
| GO:0006022 | aminoglycan metabolic process | 11 | 0.07606442 |
| GO:0009611 | response to wounding | 10 | 0.07606442 |
| GO:0016042 | lipid catabolic process | 10 | 0.07606442 |
| GO:0009991 | response to extracellular stimulus | 11 | 0.09577223 |

**Table 6:** Gene ontology (GO) categories enriched for the 355 transcripts that were significantly more abundant in in the temperate region (Webgestalt; Over-representation analysis (ORA) for biological processes; FDR < 0.1).

| GeneSet | Description | Number of Enriched Genes | FDR |
|---|---|---|---|
| GO:0071826 | ribonucleoprotein complex subunit organization | 13 | 0.01210658 |
| GO:0043543 | protein acylation | 10 | 0.03374253 |
| GO:0072657 | protein localization to membrane | 9 | 0.09092037 |

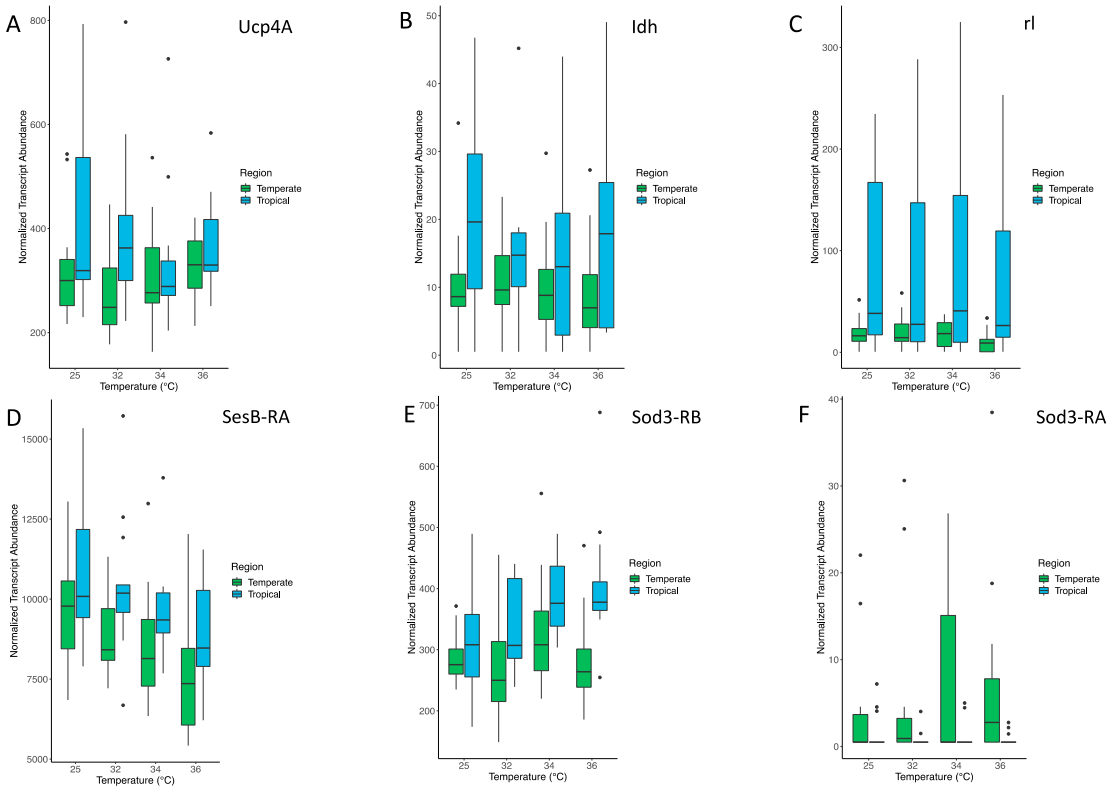


**Figure 8: Tropical embryos had significantly higher abundance of transcripts within the oxidative stress pathway.** (A) Normalized transcript abundances for *Ucp4A*, an uncoupling protein, for early temperate and tropical *D. melanogaster* embryos (0-1hr) across heat shock temperatures (LRT, region factor, FDR = 0.021). (B) Normalized transcript abundances for *Idh*, isocitrate-dehydrogenase, for early temperate and tropical embryos (0-1hr) across heat shock temperatures (LRT, region factor, FDR = 0.021). (C) Normalized transcript abundances for rolled (*rl*), a gene within the MAP-K cell signaling pathway, for early temperate and tropical embryos (0-1hr) across heat shock temperatures (LRT, region factor, FDR = 1.47E-06). (D) Normalized transcript abundances for *sesB-RA*, stress-sensitive B, for early temperate and tropical embryos (0-1hr) across heat shock temperatures (LRT, region factor, FDR = 0.02). (E)

Normalized transcript abundances for *Sod3-RB*, superoxide dismutase 3, for early temperate and tropical (0-1hr) across heat shock temperatures (LRT, region factor, FDR = 1.47E-06). (F) Normalized transcript abundances for *Sod3-RA*, superoxide dismutase 3, for early temperate and tropical embryos (0-1hr) across heat shock temperatures (LRT, region factor, FDR = 0.002). Note that y-axis scales are different for each plot.

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