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Winter warming causes widespread shifts in the metabolome and hinders supercooling in *Pieris rapae* butterflies

KEY WORDS: Cold tolerance, diapause, cryoprotectant, metabolomics

ABSTRACT

Global climate change has the potential to devastate 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, to our knowledge, no research has characterized the direct effects of winter warming on the biology 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 evidence of local adaptation of supercooling in *P. rapae*, as we observed significantly lower supercooling points in Vermont individuals relative to North Carolina individuals. 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

In the context of climate change, shifts in seasonality (i.e. later onset of winter and/or earlier onset of spring) and the increased frequency of temperature anomalies will expose organisms to unpredictable thermal environments to which they may not be adapted (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). Additionally, according to the latest IPCC special report, winter temperatures have shown continued patterns of increased variability with both hotter mean temperatures and a higher frequency of extreme temperatures and weather events (Allen et al., 2019). These winter-specific 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). 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.

Temperate species of insects that enter diapause in the winter may be vulnerable to winter warming, as environmental temperature and seasonality can impact their physiology (Colinet et al., 2015; Sinclair et al., 2016). 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).

Diapause is an overwintering strategy that 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, which describes the effect of temperature on the rate of chemical reactions, 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.

Pieris rapae, or the cabbage white butterfly, is a globally abundant species with populations found across five continents, providing a model system to study the effects of winter warming patterns on populations of temperate diapausing insect species (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, 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 the overwintering physiology of *P. rapae* butterflies. Specifically, 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 underlined by changes in metabolite abundances across the metabolome. Importantly, we characterized metabolomic profiles of pupae from which we also measured supercooling points, allowing us to directly correlate metabolomes to supercooling. 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). 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.

125

126 **Juvenile stage rearing and diapause induction**

127 Upon oviposition, eggs were removed and placed into plastic containers (35.6cm length x
128 20.3cm wide x 12.4cm height) and into incubators (Percival model DR-36VL) set to 24°C and
129 55% relative humidity, with approximately 20 eggs in each container. *Pieris rapae* diapause in
130 the pupal stage, with the larval stage as the sensitive stage or preparative stage (Richards 1940).
131 To ensure all individuals entered diapause, we subjected all individuals to short-day
132 photoperiods (8L:16D) starting at the embryonic stage. We replaced fresh organic kale leaves
133 every day. Upon pupation, roughly 14 days post oviposition, we placed individuals into one of
134 three winter warming treatments, ensuring that eggs from each female were represented in each
135 treatment.

136

137 **Winter warming treatments in diapausing pupae**

138 We determined the winter warming treatments based from the historic data records of a local
139 weather station in Burlington, VT (National Weather Service Forecast Office,
140 https://w2.weather.gov/climate/local_data.php?wfo=btv, accessed February 2020). These
141 patterns reflect historic, concurrent and predicted chronic and acute warming patterns observed
142 in Vermont winters (December-February). We isolated individual pupae into petri dishes (60 x
143 15mm) and kept them under one of the following conditions until Day 90 of pupal diapause or
144 supercooling analysis (measured on Days 25, 50 and 75). The three temperature conditions
145 consisted of a control treatment, chronic warming treatment, or acute warming treatment. We
146 kept control individuals under a temperature regime with daily fluctuating temperature 4°C-8°C,
147 representing autumn temperatures when individuals first enter diapause. We kept control
148 individuals under this regime for the entire experiment. We kept the chronic warming individuals
149 under a temperature regime of 7°C-11°C, representing a 3°C increase from the control. This
150 pattern reflects both the long-term degree of warming pattern seen in Vermont over the last 50
151 years, as well as the predicted, continued pattern we expect to see over the next 50 years
152 (National Weather Service Forecast Office,
153 https://w2.weather.gov/climate/local_data.php?wfo=btv, accessed February 2020). We kept the
154 acute warming individuals under the control conditions of daily fluctuating 4°C-8°C but with
155 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). We 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), and thus, we excluded weight as a factor in future analyses.

Supercooling point measurement

We took a final weight measurement of each individual prior to the internal freezing temperature analysis. 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°Cmin^{-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). We ran weight as a factor in the ANOVA for supercooling

point, but as stated above, it wasn't a significant factor, so we re-ran the ANOVA as a 2-way reduced model. We modeled treatment, or population, and days in diapause as fixed effects. 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. For this analysis, 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

We compared the normalized peak intensities, as a proxy for metabolite abundance, of all metabolite features identified by LC-MS in the control and chronic warmed pupae. We conducted a principal components analysis to describe the major axes of variation in the dataset. We 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

Winter warming caused shifts in supercooling point. 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 50 – control vs. acute warming, P=0.0070, control vs. chronic warming, P=0.0079). 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 little standard deviation, 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).

Figure 1

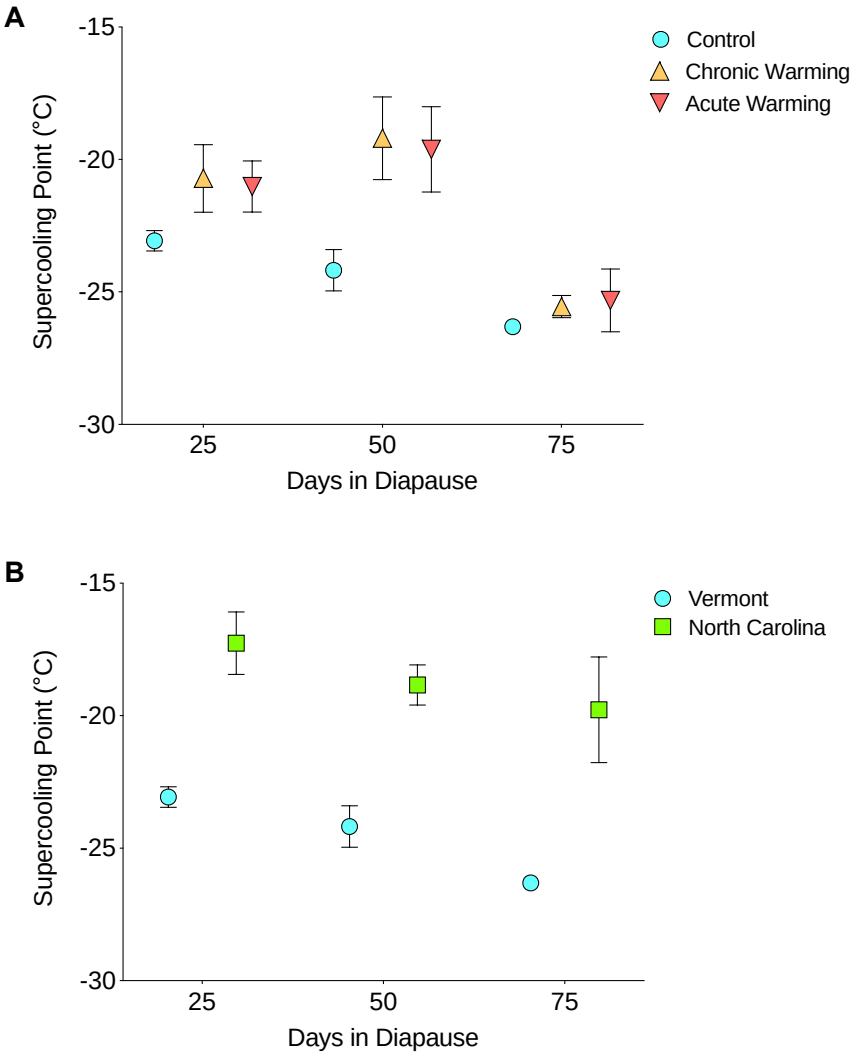


Figure 1. Supercooling points (SCP) of diapausing *Pieris rapae* pupae exposed to winter warming conditions and from Vermont and North Carolina populations. (A) SCPs (internal freezing temperature) were higher in pupae exposed to winter warming (2-way ANOVA; temperature factor, $F_{2,43}$=6.566, P=0.0032; 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 50 – control vs. acute warming (P=0.0070), control vs. chronic warming (P=0.0079). 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.

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). Interestingly, supercooling point reached an average of -26.3 ± 0.3°C among 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 (Fig. 1B, 2-way ANOVA, population factor, $F_{1, 26}$=67.35, P<0.0001).

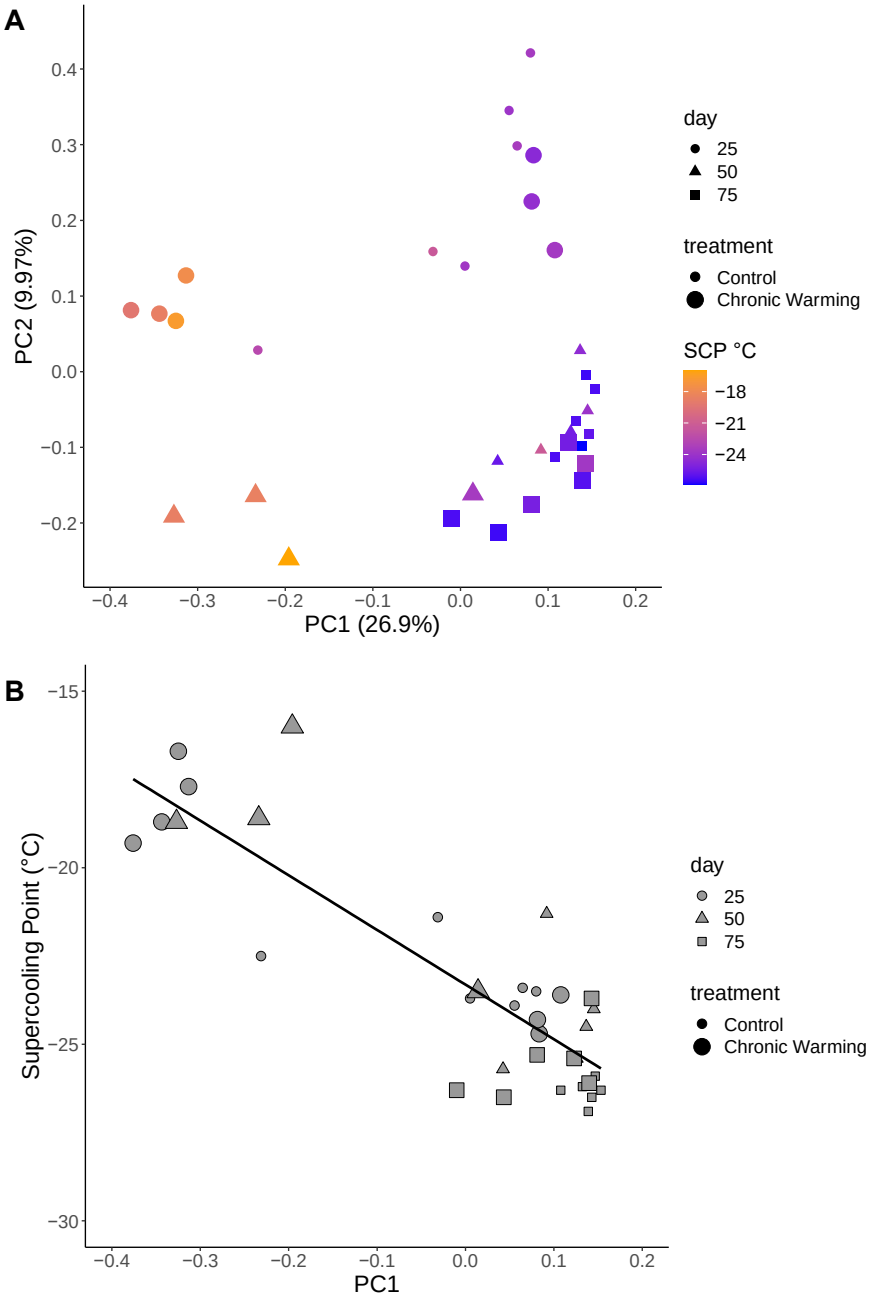
Responses of the metabolome to winter warming

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).

Metabolomes were strongly correlated to supercooling point (Fig. 2). A principal components analysis of metabolite feature abundances revealed that pupae cluster primarily by supercooling point, accounting for nearly 27% of the total variation in abundances of all 14,379 features among pupae (Fig. 2A). In addition, 10% of the total variation in metabolomes accounts for differences among pupae by days in diapause (Fig. 2A). Moreover, the variation among metabolomes, as described by PC1, was strongly correlated to supercooling point (Fig. 2B; Least-squares linear regression of PC1 on SCP, $y = -15.48x - 23.3$, $R^2=0.73$, $P<0.00001$).

Figure 2



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326 **Figure 2. Whole metabolomes cluster by supercooling point (SCP) and days in diapause.**

327 (A) Principal components analysis of normalized intensities of 14,379 metabolite features among

328 34 pupae. Each point represents the metabolome of an individual pupa, collapsed in principal

329 component space for the first two principal components that describe 37% of the variation

330 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

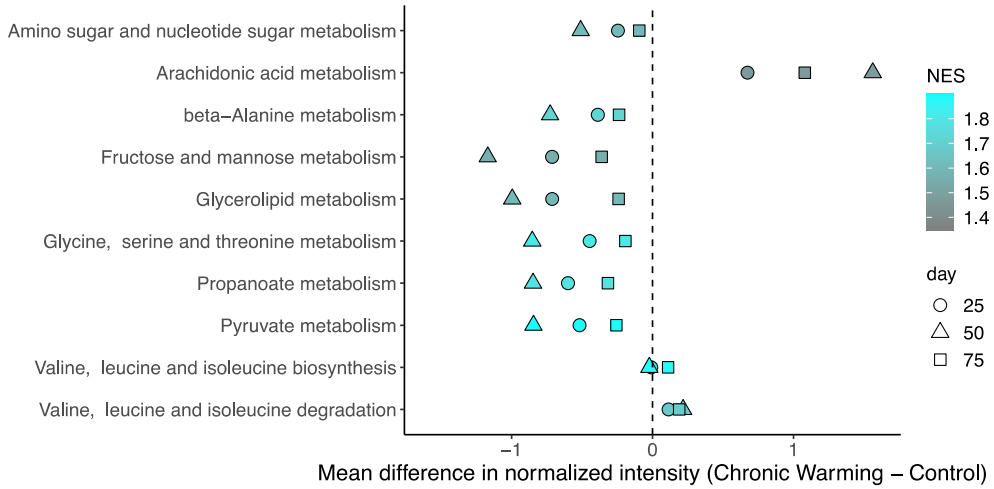
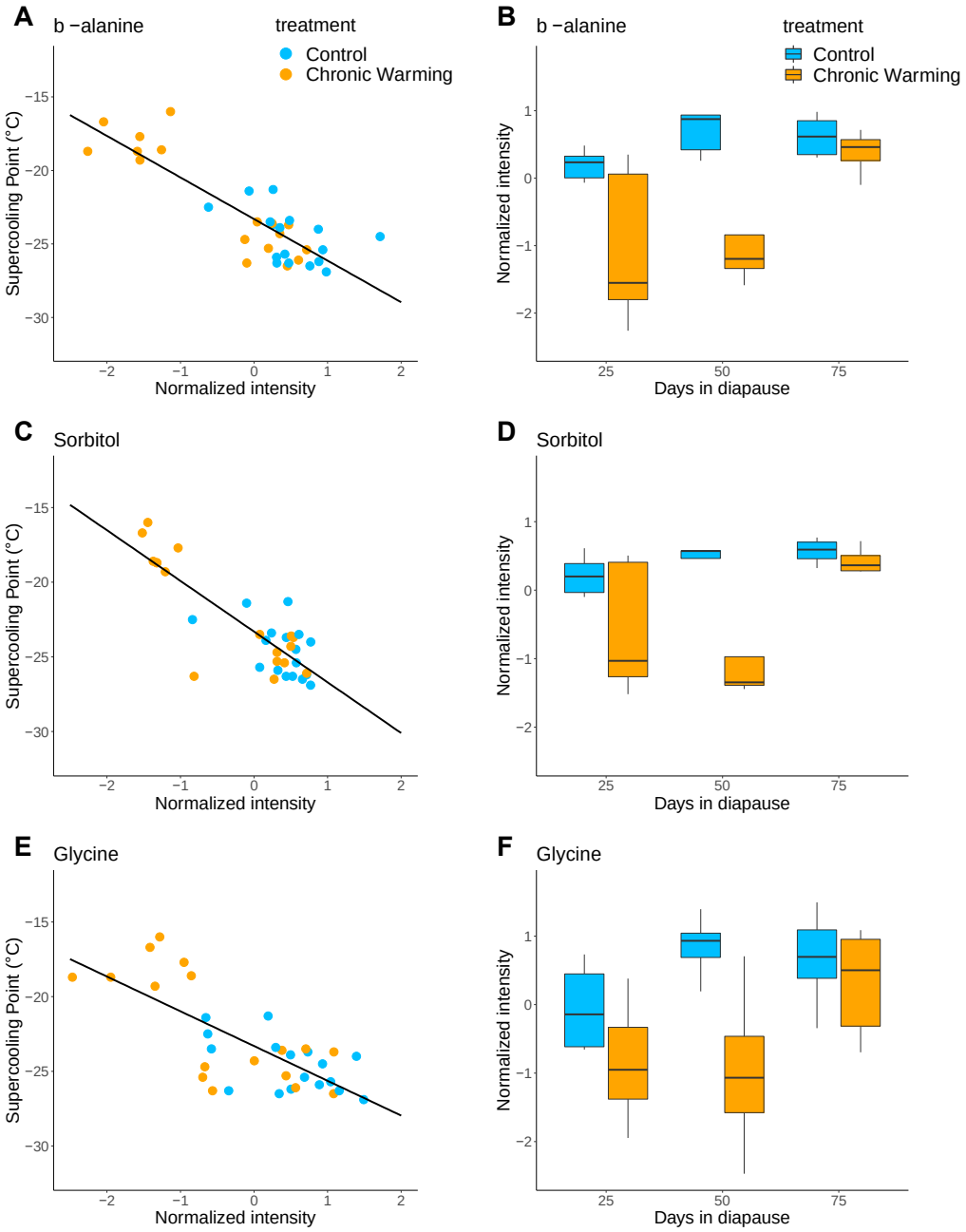


Figure 3: Pathways significantly changed in response to winter warming. Plotted are the mean differences in normalized intensity (Chronic warmed – Control), averaged among all features in a given pathway and among pupae in a given day in diapause, for 10 pathways whose member KEGG compounds showed significant 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.

The coordinated changes in the metabolome that accompanied the winter warming treatment were found to coincide with significant changes within 10 biochemical pathways (Fig. 3; Table

S1). Overwhelmingly, 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). Interestingly, 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).

Figure 4



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364 **Figure 4: Abundances of putative cryoprotectant metabolites were negatively correlated**

365 **with supercooling point and reduced after winter warming.**

366 (A) β-alanine normalized intensity was negatively correlated with SCP (Least-squares linear

367 regression, y = -2.83x – 23.31, $R2$ = 0.72, P < 0.00001). (B) β-alanine normalized intensity was

368 reduced after warming (2 way-ANOVA; temperature factor, $F_{1,30} = 17.16$, $P = 0.0003$; day
369 factor, $F_{1,30} = 12.00$, $P = 0.002$; temperature x day interaction, $F_{1,30} = 2.34$, $P = 0.14$). (C)
370 Sorbitol normalized intensity was negatively correlated with SCP (Least-squares linear
371 regression, $y = -3.4x - 23.31$, $R^2 = 0.70$, $P < 0.00001$). (D) Sorbitol normalized intensity was
372 reduced after warming (2 way-ANOVA; temperature factor, $F_{1,30} = 11.11$, $P = 0.002$; day factor,
373 $F_{1,30} = 5.84$, $P = 0.02$; temperature x day interaction, $F_{1,30} = 0.10$, $P = 0.62$). (E) Glycine
374 normalized intensity was negatively correlated with SCP (Least-squares linear regression, $y = -$
375 $2.33x - 23.31$, $R^2 = 0.55$, $P < 0.00001$). (F) Glycine normalized intensity was reduced after
376 warming (2 way-ANOVA; temperature factor, $F_{1,30} = 11.36$, $P = 0.002$; day factor, $F_{1,30} = 4.52$,
377 $P = 0.02$; temperature x day interaction, $F_{1,30} = 2.30$, $P = 0.12$). Data represent mean normalized
378 intensity of all features that matched a given metabolite (β-alanine: 3 features, sorbitol: 11
379 features, glycine: 1 feature).
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381 **Responses of putative cryoprotectants to winter warming**
382 Pupae with the lowest supercooling points had the highest abundances of three putative
383 cryoprotectants, β-alanine, sorbitol, and glycine, and SCP was negatively correlated with the
384 abundances of all of these metabolites (Fig. 4; Least-squares linear regression; β-alanine, $R^2 =$
385 0.72, $P < 0.00001$; sorbitol, $R^2 = 0.70$, $P < 0.00001$; and glycine, $R^2 = 0.55$, $P < 0.00001$).
386 Moreover, all three of these metabolites showed significant decreases in abundance after chronic
387 winter warming (Fig. 4; 2-way ANOVA, temperature main effect; β-alanine, $F_{1,30} = 17.16$, $P =$
388 0.0003; sorbitol, $F_{1,30} = 11.11$, $P = 0.002$; and glycine, $F_{1,30} = 11.36$, $P = 0.002$).
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Supplemental Tables

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

DISCUSSION

Here, we measured key overwintering physiological traits in *Pieris rapae* pupae exposed to acute and chronic winter warming. We support the prediction that winter warming modifies the intrinsic physiological mechanisms that underlie supercooling, as our experimental results demonstrated warming-induced shifts in supercooling point 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 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.

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). But warmed pupae broke with this trend on day 50 in diapause, which could signify a warming-induced mismatch between physiology and the environment. Particularly in the case of acute warming events, 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, 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.

Based on previous work (Li et al., 2020) and the results presented herein, it appears that North American *P. rapae* rely on supercooling as the 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 appears to be underlined 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 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 drastic 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 α, 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.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

E.E.M conducted the experiment. E.E.M and B.L.L performed data analysis and wrote the manuscript.

REFERENCES

Allen, M., Antwi-Agyei, P., Aragon-Durand, F., Babiker, M., Bertoldi, P., Bind, M., Brown, S., Buckeridge, M., Camilloni, I. and Cartwright, A. (2019). Technical Summary: Global Warming of 1.5° C. An IPCC Special Report on the Impacts of Global Warming of 1.5° C above Pre-Industrial Levels and Related Global Greenhouse Gas Emission Pathways, in the Context of Strengthening the Global Response to the Threat of Climate Change, Sustainable Development, and Efforts to Eradicate Poverty). Intergovernmental Panel on Climate Change (in Press).

Bale, J. S. (2002). Insects at low temperature: from molecular biology to distributions and abundance. *J. Insect Physiol.* **33,** 899-908.

Bale, J. S. and Hayward, S. A. L. (2010). Insect overwintering in a changing climate. *J. Exp. Biol.* **213,** 980-994.

Bartomeus, I., Acher, J. S., Wagner, D., Danforth, B. N., Colla, S., Kornbluth, S. and Winfree, R. (2011). Climate-associated phenological advances in bee pollinators and bee-pollinated plants. *Proc. Natl. Acad. Sci.* USA. **108,** 20645- 20649.

Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal statistical society: series B (Methodological)* **57(1)**, 289-300.

Bosch, J. and Kemp, W.P. (2003). Effect of wintering duration and temperature on survival and emergence time in males of the orchard pollinator *Osmia lignaria* (Hymenoptera: Megachilidae). *Environ. Entomol.* **32,** 711- 716.

Boychuk, E. C., Smiley, J. Y., Dahlahoff, E. P., Bernard, M.A., Rank, N. E. and Sinclair, B. J. (2015). Cold tolerance of the montane Sierra leaf beetle, *Chrysomela aeneicollis*. J. *Insect. Physiol.* **81,** 157-166.

Bradshaw, W. E. and Holzapfel, C. M. (2010). Insects at not so low temperature: climate change in the temperate zone and its biotic consequences. In *Low Temperature Biology of Insects* (eds. D. L. Denlinger and R. E. Lee, Jr), pp. 242-275. Cambridge, New York Cambridge University Press.

Buck, M. J., Squire, T. L. and Andrews, M. T. (2002). Coordinate expression of the PDK4 gene: a means of regulating fuel selection in a hibernating mammal. *Physiol. Genomics* **8**, 5–13.

Buckley, L.B., Arakaki, A.J., Cannistra, A.F., Kharouba, H.M. and Kingsolver, J.G. (2017). Insect Development, Thermal Plasticity and Fitness Implications in Changing, Seasonal Environments. *Int. Comp. Biol.* **57,** 988–998.

Chong, J., Wishart, D.S. and Xia, J. (2019). Using MetaboAnalyst 4.0 for comprehensive and integrative metabolomics data analysis. Current Protocols in Bioinformatics **68,** e86.

Coleman P. C., Bale, J. S. and Hayward, S. A. L. (2014). Cross-generation plasticity in cold hardiness is associated with diapause, but not the non-diapause developmental pathway, in the blow fly *Calliphora vicina*. *J. Exp. Biol.* **217,** 1454–1461.

Colinet, H., Sinclair, B. J., Vernon, P. and Renault D. (2015). Insects in fluctuating thermal environments. *Annu. Rev. Entomol.* **60,** 123-140.

Crozier, L. (2003). Winter warming facilitates range expansion: cold tolerance of the butterfly *Atalopedes campestris. Oecologia* **135,** 648-656.

Denlinger, D. L. (2002). Regulation of diapause. *Ann. Rev. Entomol*. **47,** 93-122.

Denlinger, D. L. and Lee, R. E. J. (2010). Low Temperature Biology of Insect. Cambridge: Cambridge University Press.

García-Robledo, C., Kuprewicz, E. K., Staines, C. L., Erwin, T. L. and Kress, W. J. (2016). Limited tolerance by insects to high temperatures across tropical elevational gradients and the implications of global warming for extinction. *PNAS* **113(3),** 680-685.

Hahn, D. A. and Denlinger, D. L. (2007). Meeting the demands of insect diapause: Nutrient storage and utilization. *J. Insect. Physiol.* **53,** 760-773.

Hahn, D. A. and Denlinger, D. L. (2011). Energetics of insect diapause. *Annu. Rev. Entomol.* **56,** 103-121.

Hodek, I. and Hodková, M. (1988). Multiple roles of temperature during insect diapause a review. *Entomologia Experimentalis et Applicata.* **49,** 153-165.

Kingsolver, J. G., Woods, H. A., Buckley, L. B., Potter, K. A., MacLean, H. and Higgins, J. K. (2011). Complex life cycles and the responses of insects to climate change. *Int. and Comp. Biol.* **51,** 719-732.

Koštál, **V. and Šimek, P.** (1995). Dynamics of cold hardiness, supercooling and cryoprotectants in diapausing and non-diapausing pupae of the cabbage root fly, *Delia radicum* L. *J. Insect Physiol.* **41,** 627-634

Koštál, **V., Zahradnıckova, H., Šimek P. and Zeleny, J.** (2007). Multiple component system of sugars and polyols in the overwintering spruce bark beetle, *Ips typographus*. *J. Insect Physiol.* **53,** 580–586.

Koštál, V., Dolezal, P., Rozsypal, J., Moravcova, M., Zahradnıckova, H. and Šimek P. (2011). Physiological and biochemical analysis of overwintering and cold tolerance in two Central European populations of the spruce bark beetle, *Ips typographus*. *J. Insect Physiol.* **57,** 1136–1146.

Kukal, O., Denlinger, D.L. and Lee, R.E. (1991). Developmental and metabolic changes induced by anoxia in diapausing and nondiapausing flesh fly pupae. *J. Comp. Physiol.* **160,** 683–8967.

Li, S., Park, Y., Duraisingham, S., Strobel F. H., Khan N., Soltow, Q.A., Jones, D.P. and Pulendran, B. (2013) Predicting Network Activity from High Throughput Metabolomics. *PLoS Comput Bio*l **9(7),** e1003123.

Li, N. G., Toxopeus, J., Moos, M., Sørensen, J. G. and Sinclair, B. J. (2020). A comparison of low temperature biology of *Pieris rapae* from Ontario, Canada, and Yakutia, Far Eastern Russia. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **242**, 110649.

Lee, R. E. (2010). A primer on insect cold tolerance. In: Denlinger, D. L. and Lee, R. E. J. (Eds.), Low Temperature Biology of Insects. Cambridge University Press, New York, 3-34.

Lehmann, P., Pruisscher, P., Posledovich, D., Carlsson, M., Käkelä, R., Tang, P., Nylin, S., Wheat, C. W., Wiklund, C. and Gotthard, K. (2016). Energy and lipid metabolism during direct and diapause development in a pierid butterfly. *J. Exp. Biol.* **219,** 3049-3060.

Lehmann, P., Pruisscher, P., Koštál, V., Moos, N., Šimek, P. Nylin, S., Agren, R., Väremo, L., Wiklund, C., Wheat, C. W. and Gotthard, K. (2018). Metabolome dynamics of diapause in the butterfly *Pieris napi*: distinguishing maintenance, termination, and post-diapause phases. *J. Exp. Biol.* **221,** 1-9.

Marshall, K. E. and Sinclair, B. J. (2015). The relative importance of number, duration and intensity of cold stress events in determining survival and energetics of an overwintering insect. *Funct. Ecol.* **29,** 357-366.

Michaud, M. R. and Denlinger, D. L. (2006). Oleic acid is elevated in cell membranes during rapid cold-hardening and pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *J. Insect Physiol.* **52**, 1073–1082.

Michaud, M. R. and Denlinger, D. L. (2007). Shifts in carbohydrate, polyol, and amino acid pools during rapid cold-hardening and diapause associated cold-hardening in flesh flies (*Sarcophaga crassipalpis*): a metabolomic comparison. *J. Comp. Physiol.* **177,** 753-763.

Michaud, M. R., Benoit, J. B., Lopez-Martinez, G., Elnitsky, M.A., Lee, R. E. and Denlinger, D. L. (2008). Metabolomics reveals unique and shared metabolic changes in response to heat shock, freezing, and dessication in the Antarctic midge, *Belgica antartica. J. Insect Phsyiol.* **54,** 645-655.

Ogg, C. L., Howard, R. W. and Stanley-Samuelson, D. W. (1991). Fatty acid composition and incorporation of arachidonic acid into phospholipids of hemocytes from the tobacco hornworm *Manduca sexta*. *Insect Biochem.* **21**, 809–814.

Posledovich, D., Toftegaard, T., Wiklund, W., Ehrlén, J. and Gotthard, K. (2015). Latitudinal variation in diapause duration and post-winter development in two pierid butterflies in relation to phenological specialization. *Oecologia* **177,** 181-190.

Pullin, A. S., Bale, J. S. and Fontaine, X. L. R. (1991). Physiological aspects of diapause and cold tolerance during overwintering in *Pieris brassica*. *Physiol. Entomol.* **16,** 447-456.

Ryan, S. F., Lombaert, E., Espeset, A., Vila, R., Talavera, G., Dinca, V., Doellman, M. M., Renshaw, M. A., Eng, M. W. and Hornett E. A. (2019). Global invasion history of the agricultural pest butterfly Pieris rapae revelaed with genomics and citizen science. *Proc. Natl. Acad. Sci.* U. S.A. **116,** 20015-20024.

Salt, R. W. (1961). Principles of insect cold hardiness. *Ann. Rev. Entomol.* **6:** 55-74.

Saunders, D. S. (1971). The temperature-compensated photoperiodic clock "programming" development and pupal diapause in the flesh-fly, *Sarcophaga argyrostoma*. *J. Insect Physiol.* **17,** 801-812.

Sformo, T., Walters, K., Jeannet, K., Wowk, B., Fahy, G. M., Barnes, B. M. and Duman, J. G. (2010). Deep supercooling, vitrification and limited survival to -100°C in the Alaskan beetle *Cucujus clavipes puniceus* (Coleoptera: Cucujidae) larvae. *J. Exp. Biol.* **213**, 502–509.

Sgolastra, F., Kemp, W. P., Buckner, J. S., Pitts-Singer, T. L., Maini, S. and Bosch, J. (2011). The long-summer: Pre-wintering temperatures affect metabolic expenditure and winter survival in a solitary bee. *J. Insect Physiol.* **57,** 1651-1659.

Sinclair, B. J. (2001). Field ecology of freeze tolerance: interannual variation in cooling rates, freeze-thaw and thermal stress in the microhabitat of the alpine cockroach *Celatoblatta quinquemaculata*. *Oikos* **93**, 286–293.

Sinclair, B. J., Vernon, P, Klok, C. J. and Chown, S. L. (2003). Insects at low temperatures: an ecological perspective. *Trends in Ecology & Evolution* **18,** 257–262.

Sinclair, B. J. (2015). Linking energetics and overwintering in temperate insects. *J. Thermal Biol.* **54,** 5-11.

Sinclair, B. J., Coello Alvarado, L. E. and Ferguson, L. V. (2015). An invitation to measure insect cold tolerance: Methods, approaches, and workflow. J. Thermal Biol. **43:** 180-197.

Sinclair, B. J., Marshall, K. E., Sewell, M. A., Levesque, D. L., Willett, C. S., Slotsbo, S., Dong, Y., Harley, C. D. G., Marshall, D. J., Helmuth, B. S. and Huey, R. B. (2016). Can we predict ectotherm responses to climate change using thermal performance curves and body temperatures? *Ecology Letters.* **19,** 1372-1385.

Somero, G. N. (2010). The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. *J. Exp. Biol*, **213(6),** 912–920.

Somero, G. N., Lockwood, B. L. and Tomanek, L. (2017). Temperature. In *Biochemical adaptation: Response to environmental challenges from life's origins to the Anthropocene*. pp.298. Sunderland, MA. Sinauer Associates.

Storey, K. B. and Storey, J. M. (1988). Freeze tolerance in animals. *Physiol. Rev.* **68**, 27–84.

Storey, K. B. and Storey, J. M. (1990). Biochemistry of cryoprotectants. *In Insects at Low Temperature* (ed. R. E. J. Lee and D. L. Denlinger), pp. 64-94. New York: Chapman and Hall.

Storey, K. B. Heldmaier, G. and Rider, M. H. (2010). Mammalian Hibernation: Physiology, Cell Signaling, and Gene Controls on Metabolic Rate Depression. In *Dormancy and Resistance in Harsh Environments* (ed. Lubzens, E.), Cerda, J.), and Clark, M.), pp. 227–252. Berlin, Heidelberg: Springer Berlin Heidelberg.

Storey, K. B. and Storey, J. M. (2012). Insect cold hardiness: Metabolic, gene, and protein adaptation. *Can. J. Zool.* **90,** 456-475.

Street, T. O. Bolen, D. W., and Rose, G. D. (2006). A molecular mechanism for osmolyte-induced protein stability. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 13997–14002.

Tauber, M. J. and Tauber, C. A. (1978). Evolution of phenological strategies in insects: A comparative approach with eco-physiological and genetic considerations. In *Evolution of insect migration and diapause* (ed. H. Dingle). pp. 53-71. Springer-Verlag, New York.

Toxopeus, J., Koštál, V. and Sinclair, B. J. (2019). Evidence for non-colligative function of small cryoprotectants in a freeze-tolerant insect. *Proc. Roy. Soc. B. Biol. Sci.* **286**, 20190050.

Williams, C. M., **Marshall**, **K. E., MacMillan**, **H. A., Dzurisin**, **J. D. K., Hellmann**, **J. and Sinclair, B. J**. (2012). Thermal variability increases the impact of autumnal warming and drives metabolic depression in an overwintering butterfly. *PLOS ONE* **7(3),** e34470.

Wu, P., Peters, J. M. and Harris, R. A. (2001). Adaptive increase in pyruvate dehydrogenase kinase 4 during starvation is mediated by peroxisome proliferator-activated receptor alpha. *Biochem. Biophys. Res. Commun.* **287**, 391–396.

Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D. and Somero, G. N. (1982). Living with water stress: evolution of osmolyte systems. *Science* **217**, 1214–1222.