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Local thermal environment and warming influence supercooling and drive widespread shifts in the metabolome of diapausing *Pieris rapae* butterflies

KEY WORDS: Cold tolerance, climate change, diapause, cryoprotectant, metabolomics, overwintering

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 know relatively little about how warming will alter the physiology of overwintering organisms. Here, we simulated future warming conditions by comparing diapausing *Pieris rapae* butterfly pupae collected from disparate thermal environments and by exposing *P. rapae* pupae to acute and chronic increases in temperature. First, we compared internal freezing temperatures (supercooling points) of diapausing pupae that were developed in common-garden conditions but whose parents were collected from northern Vermont, USA, or North Carolina, USA. Matching the warmer winter climate of North Carolina, North Carolina pupae had significantly higher supercooling points than Vermont pupae. Next, we measured the effects of acute and chronic warming exposure in Vermont pupae and found that warming induced higher supercooling points. We further characterized the effects of chronic warming by profiling the metabolomes of Vermont pupae via untargeted LC-MS metabolomics. Warming caused significant changes in abundance of hundreds of metabolites across the metabolome. Notably, there were warming-induced shifts in key biochemical pathways, such as pyruvate metabolism, fructose and mannose metabolism, and beta-alanine metabolism, suggesting shifts in energy metabolism and cryoprotection. These results suggest that warming affects various aspects of overwintering physiology in *P. rapae* and may be detrimental

depending on the frequency and variation of winter warming events. Future research is needed to ascertain the extent to which the effects of warming are felt among a broader set of populations of *P. rapae*, and among other species, in order to better predict how insects may 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 (Buckley and Kingsolver, 2011; García-Robledo et al., 2016; Sinclair et al., 2016; Somero, 2010; Somero et al., 2017). Mean atmospheric winter temperatures are increasing at a faster rate than any other season (Allen et al., 2019). According to the latest IPCC special report, winter temperatures have shown increased variability with both hotter mean temperatures and a lower frequency of days below freezing (Allen et al., 2019; Hayoe et al., 2018). Thus, it is imperative to characterize how overwintering organisms respond to warming conditions in order to predict how these species will respond to the future climate.

Diapause is an overwintering strategy for temperate insects, and relies on intrinsic physiological mechanisms (Ragland and Keep, 2017; Ragland et al., 2011) that depress metabolic activity, arrest development, and confer cold and stress tolerance (Koštál, 2006; Koštál et al., 2017). A key trait that underlies cold tolerance during diapause is supercooling (Lee, 2010; Sinclair et al., 2015), a freeze avoidance strategy in which body solutions can drop below 0°C without the formation of ice (Somero et al., 2017). Ice formation is detrimental, particularly when it occurs inside cells, due to the volume expansion of freezing aqueous solutions (Somero et al., 2017). Supercooling is achieved by various biochemical mechanisms that work in concert, such as cryoprotectant metabolites that reduce the freezing point via colligative properties (Koštál et al., 2007), ice binding proteins that hinder the formation and spread of ice crystals (Duman, 2015), and cryoprotective dehydration that decreases the potential for body water to freeze by increasing the concentration of cryoprotectant metabolites and ice binding proteins (Walters et al., 2011). For example, one of the lowest supercooling points was reported in larvae of the arctic beetle *Cucujus clavipes*, which can supercool to -58°C via the synthesis and

63 accumulation of high concentrations of glycerol (4-6 mol L^{-1}) (Sformo et al., 2010). Vitrification,
64 the conversion of intra- and extracellular water into solid-state vitreous water that does not
65 expand in volume, is yet another mechanism of freeze avoidance that enables *C. clavipes* larvae
66 to survive temperatures down to -100°C (Sformo et al., 2010).
67 In addition to the intrinsic physiological mechanisms that enable diapausing insects to
68 survive through months of extreme winter conditions, extrinsic factors such as temperature may
69 also influence overwintering. The Arrhenius relationship (i.e., the Q_{10} effect) predicts that
70 increases in temperature will lead to exponential increases in the rates of biochemical reactions
71 (Somero et al., 2017). Thus, if dormant animals rely on cold temperatures as a means to
72 extrinsically regulate and depress the rates of their physiological processes (Geiser, 2004; Hodek
73 and Hodková, 1988; Snapp and Heller, 1981; Storey et al., 2010), warming could lead to
74 increases in biochemical activity that alter development rates (Buckley et al., 2017), deplete
75 energy reserves (Sgolastra et al., 2011; Williams et al., 2012), and hinder cold tolerance
76 (Coleman et al., 2014; Sobek-Swant et al., 2012). Notably, cryoprotectants can be used as fuel
77 sources (Sinclair, 2015; Sinclair and Marshall, 2018; Storey and Storey, 1986), which could
78 directly impact cold tolerance as warming increases energetic demand. Furthermore, insects may
79 be particularly vulnerable to the increased thermal variability (Colinet et al., 2015; Sinclair et al.,
80 2016) that accompanies winter warming (Allen et al., 2018), as even subtle changes in
81 temperature can have adverse effects on diapause development and subsequent spring eclosion
82 success (Lehmann et al., 2018). As further evidence that winter warming could impact
83 overwintering physiology in insects, many studies have reported evidence of local adaptation of
84 cold tolerance that correlates with local thermal environments (Arambourou and Robby, 2015;
85 Bradshaw et al., 2004; Hoffmann et al., 2005; Kukal and Duman, 1989; Nguyen et al., 2019;
86 Preisser et al., 2008; Vrba et al., 2014), suggesting that warmer winters will lead to evolutionary
87 responses in overwintering physiological traits that support cold tolerance and ultimately
88 influence fitness.
89 While previous work has established connections between the thermal environment and
90 overwintering physiology in various insects, the extent to which changes in temperature
91 influence the molecular underpinnings of overwintering physiology, such as the metabolome, has
92 not been fully explored. Much of the work to uncover the molecular physiological basis of
93 overwintering physiology in insects has employed targeted metabolomics to profile candidate

94 molecules that are known to play a role in overwintering physiology, such as various
95 cryoprotectants (Colinet et al., 2016; Koštál et al., 2007; Koštál et al., 2011; Koštál et al., 2016;
96 Lehmann et al., 2018; Michaud and Denlinger, 2007). These candidate metabolomics studies
97 have contributed important discoveries that enhance our understanding of the molecular basis of
98 diapause and overwintering physiology in insects, but more studies are needed to expand upon
99 this work to include broader surveys that use untargeted metabolomics, such as (Chen et al.,
100 2021; MacMillan et al., 2016; Williams et al., 2014a). In particular, untargeted metabolomics can
101 be employed in order to limit the ascertainment bias that is inherent in candidate metabolite
102 studies (Feder and Walser, 2005) and ultimately provide a more comprehensive picture of
103 complex molecular physiological processes.
104 To address this gap in knowledge, we investigated thermal effects on supercooling and
105 the metabolome of North American *Pieris rapae* butterflies, which diapause and overwinter in
106 the pupal stage (Richards 1940). *P. rapae,* or the cabbage white butterfly, is a globally abundant
107 insect species that has a broad distribution that spans thermal gradients across five continents
108 (Ryan et al., 2019). Previous work has shown that populations of *P. rapae* from locations with
109 different winter climates have distinct cold tolerance strategies—i.e., freeze avoidance vs. freeze
110 tolerance (Li et al., 2020; Li and Averenskii, 2007; Sømme, 1982). *P. rapae* from North America
111 and Europe are freeze avoidant (Li et al., 2020; Sømme, 1982), whereas *P. rapae* from eastern
112 Siberia are freeze tolerant (Li et al., 2020, Li and Averenskii, 2007). This suggests that the
113 thermal environment influences various aspects of *P. rapae* overwintering physiology, but also
114 highlights the importance of considering ecological relevance when investigating the
115 physiological mechanisms that underlie cold tolerance. Thus, we first sought to confirm the
116 ecological relevance of supercooling in North American *P. rapae* by comparing the supercooling
117 points of diapausing *P. rapae* that were collected from disparate thermal environments in
118 northern Vermont and North Carolina, USA. Because North American *P. rapae* have been
119 shown to be freeze avoidant (Li et al., 2020), we predicted that the ability to supercool would
120 correlate with the local thermal environments in Vermont and North Carolina. We note that
121 supercooling is but one aspect of cold tolerance and may not fully describe fitness or survival in
122 response to cold stress (Lehmann et al., 2018). Nonetheless, supercooling is a critical trait for
123 freeze avoidant insects and, thus, we use it as a proxy for cold tolerance in the present study.
124 Next, we tested whether acute (hours) or chronic (weeks) increases in temperature influence

125 supercooling in Vermont *P. rapae* pupae. Lastly, we conducted untargeted LC-MS metabolomics
126 to characterize the metabolomic profiles of Vermont *P. rapae* pupae that were exposed to
127 chronic warming. We measured metabolomic profiles of Vermont pupae for which we also
128 measured supercooling points, allowing us to correlate metabolomes to supercooling.
129 Our experiments were designed to assess the relative effects of predicted near-term future
130 increases in temperature on overwintering physiology; the difference in winter temperatures in
131 Vermont and North Carolina, and the degree to which pupae were exposed to increased
132 temperature, approximate the extent of warming that is predicted to occur in Vermont in the next
133 century, if current trends continue (Fig.1). We predicted that (1) pupae would exhibit
134 supercooling points that reflected the thermal environment of their population of origin, (2)
135 higher temperature during diapause would adversely affect supercooling, and (3) warming would
136 induce changes in metabolite abundances across the metabolome that reflect larger shifts in
137 metabolism. Overall, our results suggest that overwintering physiology in diapausing *P. rapae* is
138 strongly influenced by temperature and that winter warming may have important consequences
139 for the ecological physiology of this species.
140
141 **MATERIALS AND METHODS**
142
143 **Adult butterfly collections and maintenance**
144 We collected approximately 40-50 male and female adult *Pieris rapae* butterflies in mid to late
145 September in 2017 at two locations in northwestern Vermont, at least 15 miles apart
146 (44°29’48.52”N, 73°12’20.19”W and 44°17’10.07”N, 73°14’07.11”W). Adult *P. rapae*
147 butterflies were collected from two locations in North Carolina (35°36’19.47”N, 82°20’07.25”W
148 and 35°36’31.57”N, 82°26’31.33”W). After collection, we kept adults in mesh containers
149 (Carolina Biological Supply, 11" diameter × 12" height) with 10 butterflies in each container
150 under common garden conditions of 24°C, 12:12 Light:Dark photoperiod, 55% relative
151 humidity, and with direct access to sunlight. We fed adults a diet of 10% honey solution on a
152 sponge every 24 hours. After 48 hours post-collection, we isolated females in individual mesh
153 containers, and gave them fresh organic kale leaves on which to oviposit. Fertilized eggs were
154 collected every 24 hours, and placed into common garden juvenile rearing conditions. We note

155 our egg collection methods were designed to collect as many eggs as possible over a short period
156 of time from a relatively limited sample of adults, and thus we allowed adults to mate en masse.
157
158 **Juvenile stage rearing and diapause induction**
159 *Pieris rapae* diapause in the pupal stage, with the larval stage as the sensitive, or preparative,
160 stage (Richards 1940). To ensure all individuals entered diapause, we subjected all individuals to
161 short-day photoperiods (8L:16D) starting at the embryonic stage. Upon oviposition, eggs were
162 collected and randomly assigned to treatment groups, and placed into plastic containers (35.6cm
163 length x 20.3cm wide x 12.4cm height) in incubators (Percival model DR-36VL) set to 24°C and
164 55% relative humidity, with approximately 20 eggs in each container. Due to space limitations
165 inside of our incubators, egg clutches were reared in groups of eggs from multiple females. Thus,
166 we did not keep siblings separate from non-siblings and cannot test for the effect of individual
167 female on SCP (see below). We reared larvae on fresh organic kale leaves that were replaced
168 daily.
169 Upon pupation, roughly 14 days post oviposition, we placed all individuals into petri
170 dishes (60 x 15mm) and in one of three temperature treatments (described below). We note the
171 following constraints of our experimental design: (1) We cannot determine whether among
172 population differences in supercooling point were due to genetic divergence or maternal effects
173 caused by the local environments from which adults were collected, and (2) we cannot account
174 for the parental source of genetic differences observed among individuals within an experimental
175 group, as individuals may or may not have been siblings—multiple female-male pairs were bred
176 in common garden. We also note, however, that adults were collected from outbred natural
177 populations, and F1 offspring were used in the experiment. Thus, there was no inbreeding over
178 generations in the lab, and likely only minimal lab adaptation. Importantly, all individuals in the
179 study possessed unique genomes, regardless of whether they were siblings, due to genetic
180 recombination.
181
182 **Warming treatments in diapausing pupae**
183 Trends in winter temperature (December, January, and February) from 1940 to 2020 in
184 Burlington, VT indicate that winters were 3°C warmer, on average, in 2021 than in 1940 (Fig. 1;
185 Least-squares linear regression; daily high temperatures, $R^2 = 0.11$, $P = 0.0013$, $y = 0.027x$ –

54.15; daily low temperatures, $R^2 = 0.22$, $P = 0.0000065$, $y = 0.046x - 101.8$). We calculated the average change in temperature from 1940 to 2020 by averaging the slopes in the regression fits of daily high and low temperatures and multiplying by 80 years (0.027°C per year + 0.046°C per year) ÷ 2 x 80 years = 2.9°C). We used these trends to determine ecologically relevant winter warming treatments consisting of a control treatment, chronic warming treatment, and acute warming treatment. We kept control individuals under a temperature regime with a daily fluctuating temperature of 4°C-8°C, which was established from day and nighttime averages in Vermont October representing autumn temperatures when individuals first enter diapause. We kept control individuals under this regime for the entire experiment. To assess population-level differences in diapause physiology, we only subjected individuals from North Carolina to this control treatment, thus, comparing them to the Vermont control treatment. Only pupae from Vermont were exposed to the warming treatments. We kept the chronic warming Vermont individuals under a temperature regime of 7°C-11°C, representing a 3°C increase from the control group, which is indicative of the winter warming pattern described above. 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, which reflect record high winter temperatures observed in Vermont. 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).

Figure 1

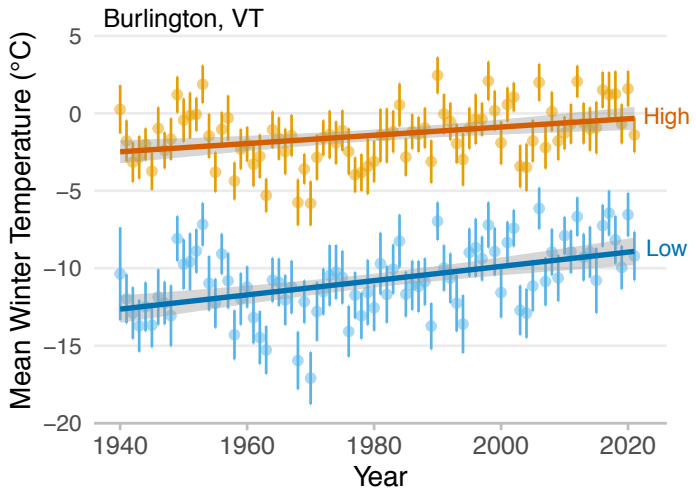

Figure 1. Winter temperature trends in Burlington, VT from 1940 to 2021. Plotted are mean daily high and low temperatures, averaged over December, January, and February for each year. Both daytime highs and lows are warmer now than they were 80 years ago (Least-squares linear regression; High, $R^2 = 0.11$, $P = 0.0013$, $y = 0.027x - 54.15$; Low, $R^2 = 0.22$, $P = 0.0000065$, $y = 0.046x - 101.8$). Error bars and error bands indicate 95% confidence intervals.

Supercooling point measurement

Diapause length is variable in *P. rapae* and differs by latitude (Saunders, 1982), and we did not measure *in situ* diapause length of the source populations in North Carolina and Vermont. But while pupae in North Carolina are likely to diapause for shorter lengths of time than pupae in Vermont, based on the winter temperatures at both of these sites (Dec., Jan., and Feb.; Figure 2B), we expect that diapause in nature lasts for at least 90 days. We measured the internal freezing temperature, or supercooling point (SCP), of the diapausing pupae based off of the protocols described in Boychuk et al. (2015) and Sinclair et al. (2015) (Boychuk et al., 2015; Sinclair et al., 2015). We weighed individual pupae every 2-4 days using a fine-scale balance at room temperature for no longer than 5 minutes 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). Thus,

238 we did not observe a significant loss of water throughout the experiment. Immediately prior to
239 supercooling point measurement, we weighed each pupa. To determine SCP, we placed pupae
240 attached to a type-K thermocouple wire (OMEGA Engineering) into individual 2-ml
241 microcentrifuge tubes, and then sealed them with parafilm. We equilibrated individuals in a
242 circulating water bath (Polyscience PP15R-30) with Polycool HC -50 anti-freeze liquid, then
243 kept individuals at 0°C for 10 minutes, and then cooled them from 0°C to -30°C at a rate of
244 0.5°Cmin^{-1}. We monitored body temperature using a thermometer and data logger program
245 (OMEGA Engineering HH806AW). SCP was defined as the temperature at which ice formed,
246 and was measured as the lowest temperature (°C) recorded before the detectable presence of an
247 exothermic reaction (ice formation) in the temperature trace. We analyzed individual internal
248 freezing temperatures on Days 25, 50 and 75 in the control (Vermont and North Carolina) and
249 chronic warmed individuals, and 24-hours post-warming in the acute warmed individuals. We
250 measured 4-7 individuals for each temperature by treatment combination. Immediately after SCP
251 analysis, we flash-froze Vermont control and chronic warmed individuals in liquid nitrogen and
252 preserved them at -80°C for metabolomics analysis.
253 We compared supercooling points of (1) Vermont vs. North Carolina populations (under
254 control conditions), (2) Vermont control vs. Vermont chronic warmed, and (3) Vermont control
255 vs. Vermont acute warmed using a linear model incorporating the factors day and treatment as
256 fixed effects and pupal mass as a covariate with a Type II analysis of variance (ANOVA) using
257 the "car" package in R. Pairwise differences between the control and warmed treatments were
258 assessed with Dunnett's multiple comparison post hoc test using the "lsmeans" package in R. We
259 also investigated trends in winter temperatures at the collection sites in NC and VT and
260 compared them to the observed SCP values for the NC and VT individuals by downloading
261 weather station data from NOAA using the R package "rnoaa" (Chamberlain 2021; NOAA,
262 https://www1.ncdc.noaa.gov/pub/data/ghcn/daily/all, accessed March 2021). The weather station
263 in NC is located at the Asheville Airport, 22 km from the NC collection site (weather station Id:
264 GHCND:USW00003812, 35° 25' 54.8394"N, 82° 32' 14.9994"W), and includes a complete daily
265 temperature record from 1964 until the present. The weather station in VT is located at the
266 Burlington Airport, 5 km from the VT collection site (weather station Id:
267 GHCND:USW00014742, 44° 28' 5.8794"N, 73° 8' 59.64"W), and includes a complete daily

268 temperature record from 1940 to the present. All supercooling point analyses were performed in
269 R version 4.0.0).
270
271 **Global metabolomics sample preparation**
272 We used individual pupae preserved from the supercooling point analysis for global, untargeted
273 metabolomics analysis. To determine the effect of chronic warming on metabolite abundance, we
274 only used control and chronic warmed Vermont individuals (n=17 pupae per group) with at least
275 4 individuals represented at each timepoint (Days 25, 50 and 75). All samples were sent to the
276 University of Florida Southeast Center for Integrated Metabolomics facility for analysis.
277 Samples were homogenized in 100 µL 5mM ammonium acetate and protein
278 concentration of each sample homogenate was measured. All samples were normalized to 500
279 µg/mL protein concentration prior to extraction. Note that because samples were normalized to
280 equal concentrations prior to metabolomics analysis, we did not normalize metabolite
281 abundances to pupal weights. Extraction was performed using protein precipitation. Briefly, 50
282 µL normalized homogenate was spiked with a mixture of internal standard. Proteins were
283 precipitated by adding 400 µL of 8:1:1 acetonitrile:methanol:acetone. After mixing, proteins
284 were allowed to precipitate for 15 min at 4°C. Supernatant from each sample was collected
285 following centrifugation at 20,000xg for 10 min and dried under a gentle stream of nitrogen at
286 30°C. Samples were reconstituted with 50 µL of reconstitution solution consisting of injection
287 standards and transferred to LC-vials for analysis.
288
289 **LC-MS analysis and data processing**
290 Untargeted metabolomics analysis was performed on a Thermo Q-Exactive Oribtrap mass
291 spectrometer with Dionex UHPLC and autosampler. All samples were analyzed in positive and
292 negative heated electrospray ionization with a mass resolution of 35,000 at m/z 200 as separate
293 injections. Separation was achieved on an ACE 18-pfp 100 x 2.1 mm, 2 µm column with mobile
294 phase A as 0.1% formic acid in water and mobile phase B as acetonitrile. The flow rate was 350
295 µL/min with a column temperature of 25°C. Injection volume was 2 µL.
296 MZmine 2.0 was used to identify features, deisotope, align features and perform gap
297 filling to fill in any features that may have been missed in the first alignment algorithm. All
298 adducts and complexes were identified and removed from the data set. This rendered a total of

299 14,379 features, which we analyzed for significant responses to warming (see below). We used
300 MetaboAnalyst 4.0 (Chong et al., 2019) to normalize the mass spec peak intensities of metabolite
301 features prior to statistical analyses. For each feature, peak intensity was log-transformed and
302 normalized to the sample median. The data were auto-scaled to facilitate comparison among
303 features.
304
305 **Statistical analysis of metabolomic data**
306 To test for differences in metabolite abundances between the Vermont control and chronic
307 warmed pupae, we compared the normalized peak intensities, as a proxy for metabolite
308 abundance, of all metabolite features identified by LC-MS. We conducted a principal
309 components analysis to describe the major axes of variation in the dataset, and then tested
310 whether the first principal component (PC1) significantly explained variation in supercooling
311 point among the samples via least-squares linear regression. We then measured the number of
312 metabolites with significantly different peak intensities via Type II ANOVA, with treatment and
313 days in diapause modeled as fixed effects. Features in the positive and negative ion modes were
314 analyzed separately. All p-values were corrected for false discovery via the Benjamini-Hochberg
315 method (Benjamini and Hochberg, 1995). All metabolite features with an FDR < 0.05 were
316 considered to have significantly different abundances. Unless otherwise indicated, we performed
317 all statistical analyses using R version 4.0.0.
318
319 **Metabolite annotation and pathway analysis**
320 We used the MS Peaks to Pathways module in MetaboAnalyst 4.0 (Chong et al., 2019) to
321 annotate metabolome features and to conduct pathway analysis. Accurate annotation of
322 untargeted metabolomics data is dependent upon a library of verified standards, which are often
323 incomplete and not representative of the focal species (Li et al., 2013). The MS Peaks to
324 Pathways approach subverts these shortcomings by identifying metabolite sets in the context of
325 KEGG pathways. Metabolite annotation of features is based upon the mass-to-charge ratios in
326 the context of pathways, whose compounds are found to respond in a coordinated manner to
327 experimental manipulation (i.e., warming). Because the goal of this study was to assess the
328 physiological consequences of warming, we focused our pathway analysis and metabolite
329 annotation on the features that were identified to change in abundance in response to chronic

330 warming. We conducted the GSEA algorithm in the MS Peaks to Pathways module of
331 MetaboAnalyst 4.0, which is a rank-based pathway enrichment test. Metabolite features were
332 ranked based on the F-value from the treatment main effect from the ANOVA (see above). We
333 used the *Drosophila melanogaster* KEGG pathway database, which is the only insect species for
334 which KEGG pathway information is available, to identify significantly enriched pathways and
335 metabolites in our dataset. Pathways with an FDR-corrected P-value less than 0.1 were
336 considered significant, following the recommendations of the authors of the analysis software.
337
338 **RESULTS**
339 **Effects of population of origin on supercooling**
340 Supercooling point was significantly lower (more negative) in Vermont pupae than North
341 Carolina pupae throughout 75 days of diapause (Fig. 2A). Supercooling point averaged -26.3 ±
342 0.3°C in Vermont pupae and -19.8 ± 4.0°C in North Carolina pupae at day 75, which
343 corresponds to the disparate extreme low temperatures in these two locations—average extreme
344 minimum temperatures in VT and NC are -29 to -26°C and -18 to -15°C, respectively (Fig. 2B).
345 SCP in both populations decreased through days in diapause.
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Figure 2

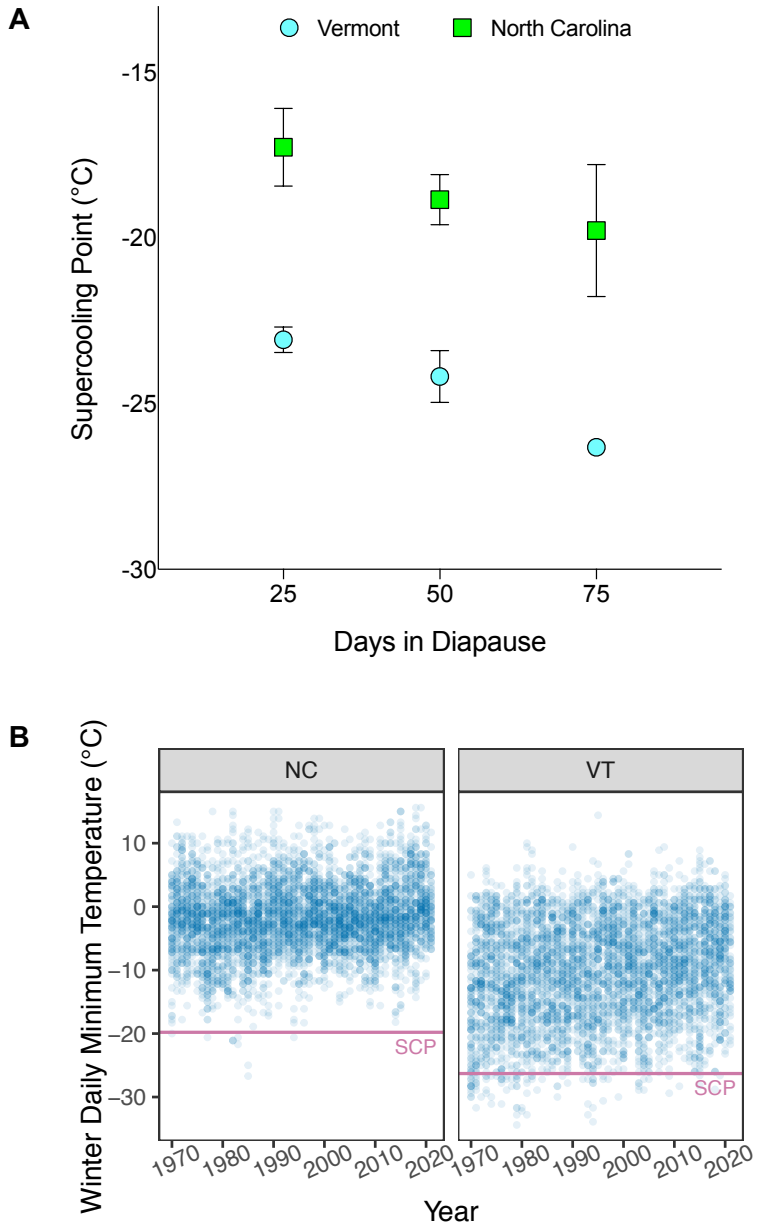

Figure 2. Supercooling points (SCP) of diapausing *Pieris rapae* pupae exposed to control conditions from Vermont and North Carolina populations. (A) SCPs (internal freezing temperatures) were higher in pupae from North Carolina (3-way ANOVA; population factor, $F_{1,24}$=60.056, P<0.0001; population x day interaction, $F_{1,24}$=0.382, P=0.543). SCPs decreased through the experiment in both Vermont and North Carolina pupae (3-way ANOVA; day factor, $F_{1,24}$=11.466, P=0.002). Pupal mass had no effect on supercooling point (3-way ANOVA; mass

covariate, $F_{1,24}$=0.677, P=0.419). Diapausing pupae were exposed to control temperature regime (4-8°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 are too small to be visible on the plot. (B) Winter daily minimum temperatures (daily lows) in December, January, and February from 1970 to 2021 in Asheville, NC (left panel) and Burlington, VT (right panel). Average supercooling points (SCP) of day 75 pupae collected from NC or VT are indicated by the horizontal pink lines.

393 **Effects of acute and chronic warming on supercooling in Vermont pupae**
394 Chronic and acute warming caused higher supercooling points in Vermont pupae, a pattern that
395 was driven by a marked difference in supercooling between control and warmed pupae at day 50
396 in diapause (Fig. 2). By day 75, warming had no effect on SCP (Fig. 3). There was an overall
397 decrease in supercooling point over the 75 days in diapause, regardless of treatment, with the
398 lowest average supercooling points on day 75 (Fig. 3).
399
400 **Effects of chronic warming on the metabolome in Vermont pupae**
401 Untargeted metabolomics identified a total of 14,379 metabolite features in all pupae from the
402 control and chronically warmed experimental groups. Of these, 1,370 showed significant
403 changes in abundance (normalized peak intensity) through diapause, irrespective of warming
404 treatment (2-way ANOVA, day factor, FDR < 0.01). 443 features showed significant changes in
405 abundance in response to chronic warming (2-way ANOVA, temperature factor, FDR < 0.01),
406 and 16 features showed significant changes in abundance through diapause and in response to
407 warming. No features had abundances that depended on the interaction between day and
408 treatment (2-way ANOVA, day x temperature interaction, all features had an FDR > 0.24).
409 Metabolite feature abundances representing individual metabolomes revealed that
410 individuals cluster primarily by supercooling point which accounted for nearly 27% of the total
411 variation in abundances of all 14,379 features among pupae (Fig. 4A). In addition, days in
412 diapause accounted for 10% of the total variation in metabolomic profiles (Fig. 4A). The
413 variation among metabolomes, as described by PC1, was strongly correlated to supercooling
414 point (Fig. 4B; Least-squares linear regression of PC1 on SCP, $y = -15.48x - 23.3$, $R^2=0.73$,
415 $P<0.00001$).
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Figure 3

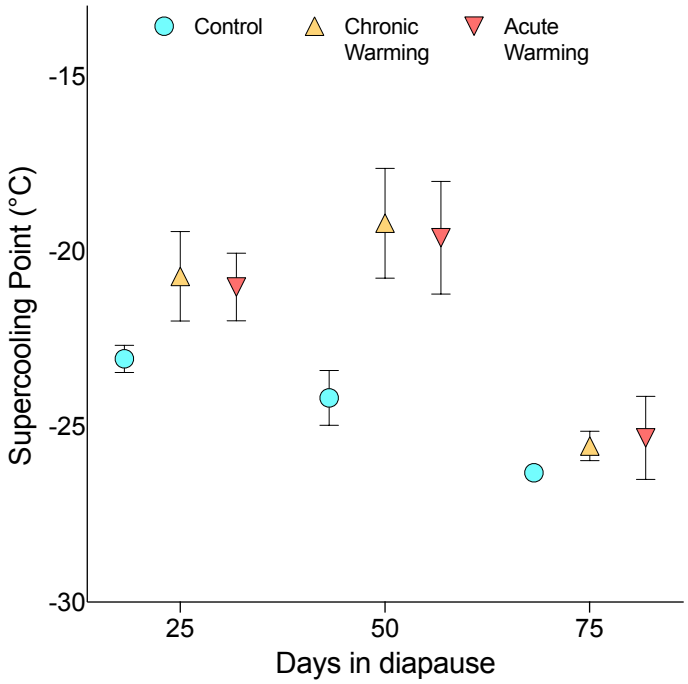


Figure 3. Acute and chronic warming caused higher supercooling points. SCPs (internal freezing temperatures) were higher in pupae exposed to both acute and chronic warming (3-way ANOVA; warming factor, $F_{2,40}$=4.928, P=0.012). SCP was significantly higher in warmed pupae at day 50 in diapause, but SCP trended higher in warmed pupae even by day 25 (Dunnett's multiple comparison test, df = 34; day 25 – control vs. acute warming, *t-ratio*=1.545, P=0.23, control vs. chronic warming, *t-ratio*=1.582, P=0.22; day 50 – control vs. acute warming, *t-ratio*=2.462, P=0.036, control vs. chronic warming, *t-ratio*=2.827, P=0.015). There was no difference in SCP between control and warmed pupae at day 75 (Dunnett's test, day 75 – control vs acute warming, *t-ratio*=0.726, P=0.69, control vs. chronic warming, *t-ratio*=0.59, P=0.81), and SCP decreased overall throughout the experiment (3-way ANOVA, day factor, $F_{1,40}$=18.93, P<0.0001, warming x day interaction, $F_{2,40}$=0.268, P=0.77). Mass had no effect on supercooling point (3-way ANOVA, mass covariate, $F_{1,40}$=0.829, P=0.368). Diapausing pupae were exposed to one of three temperature treatments: control (4-8°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 pupae. SCP is presented as mean freezing temperature (°C) ± standard error of the mean, error bars for control pupae at Day 75 are too small to be visible on the plot.

Figure 4

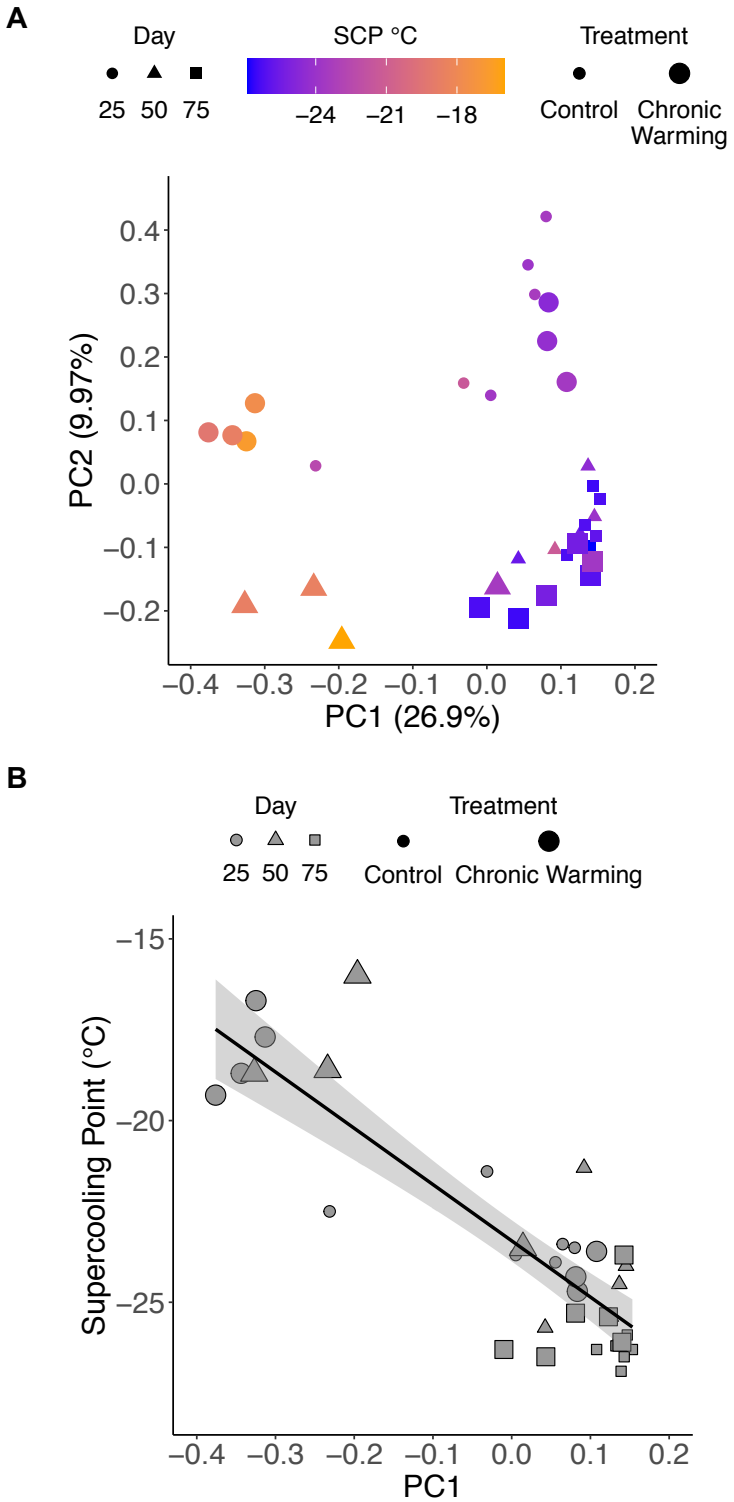
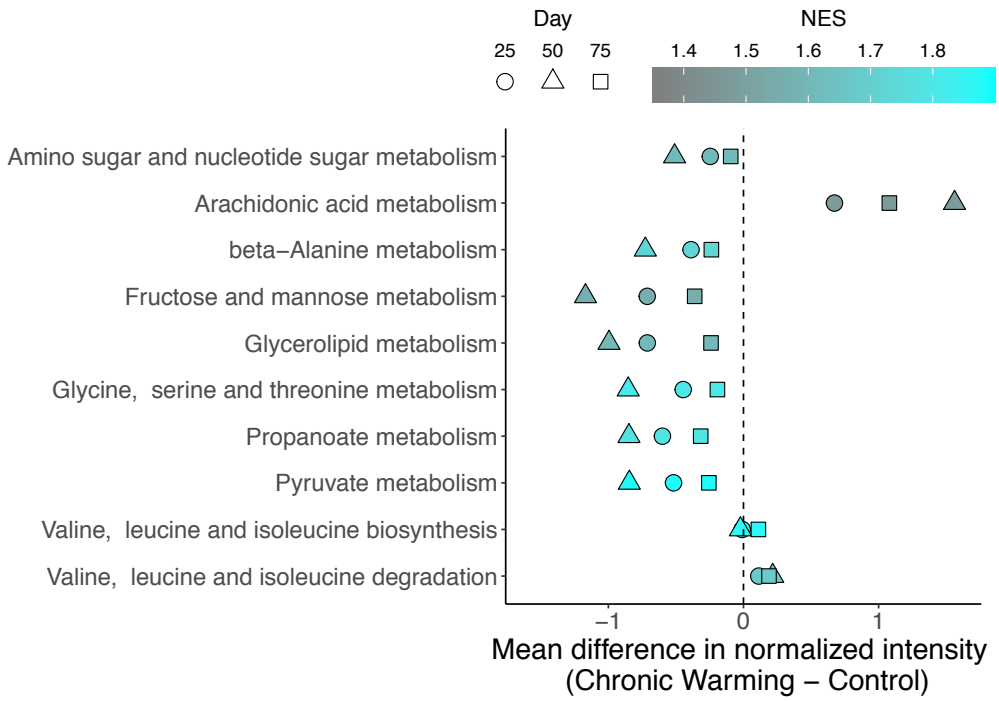

A
Day
25 50 75
SCP °C
−24 −21 −18
Treatment
Control
Chronic Warming
PC2 (9.97%)
0.4
0.3
0.2
0.1
0.0
−0.1
−0.2
−0.4 −0.3 −0.2 −0.1 0.0 0.1 0.2
PC1 (26.9%)
B
Day
25 50 75
Treatment
Control
Chronic Warming
Supercooling Point (°C)
−15
−20
−25
−0.4 −0.3 −0.2 −0.1 0.0 0.1 0.2
PC1

Figure 4. Whole metabolomes cluster by supercooling point (SCP) and days in diapause for control and chronic-warmed Vermont pupae. (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 symbol shape, warming treatment is indicated by symbol size, and SCP is indicated by symbol 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 symbol shape and warming treatment is indicated by symbol size for both (A) and (B).

Figure 5



498

499 **Figure 5: Pathways significantly changed in response to warming.** Mean differences in
500 normalized intensity (Chronic warmed – Control), averaged among all features in a given
501 pathway and among pupae in a given day in diapause, for 10 pathways whose member KEGG
502 compounds showed significant differences in normalized intensity in warmed pupae.
503 Represented in the 10 pathways are 153 features that mapped to 81 annotated KEGG
504 compounds. Positive values (x-axis) indicate higher abundances of metabolites in warmed pupae,
505 and negative values indicate lower abundances in warmed pupae, relative to controls. Days in
506 diapause are indicated by the shapes, and normalized enrichment score (NES) is indicated by the
507 color scale. Pathways with higher NES reflect greater proportions of metabolites that were found
508 to be overrepresented in the pathway enrichment analysis. Pathways are listed in alphabetical
509 order.

510

511

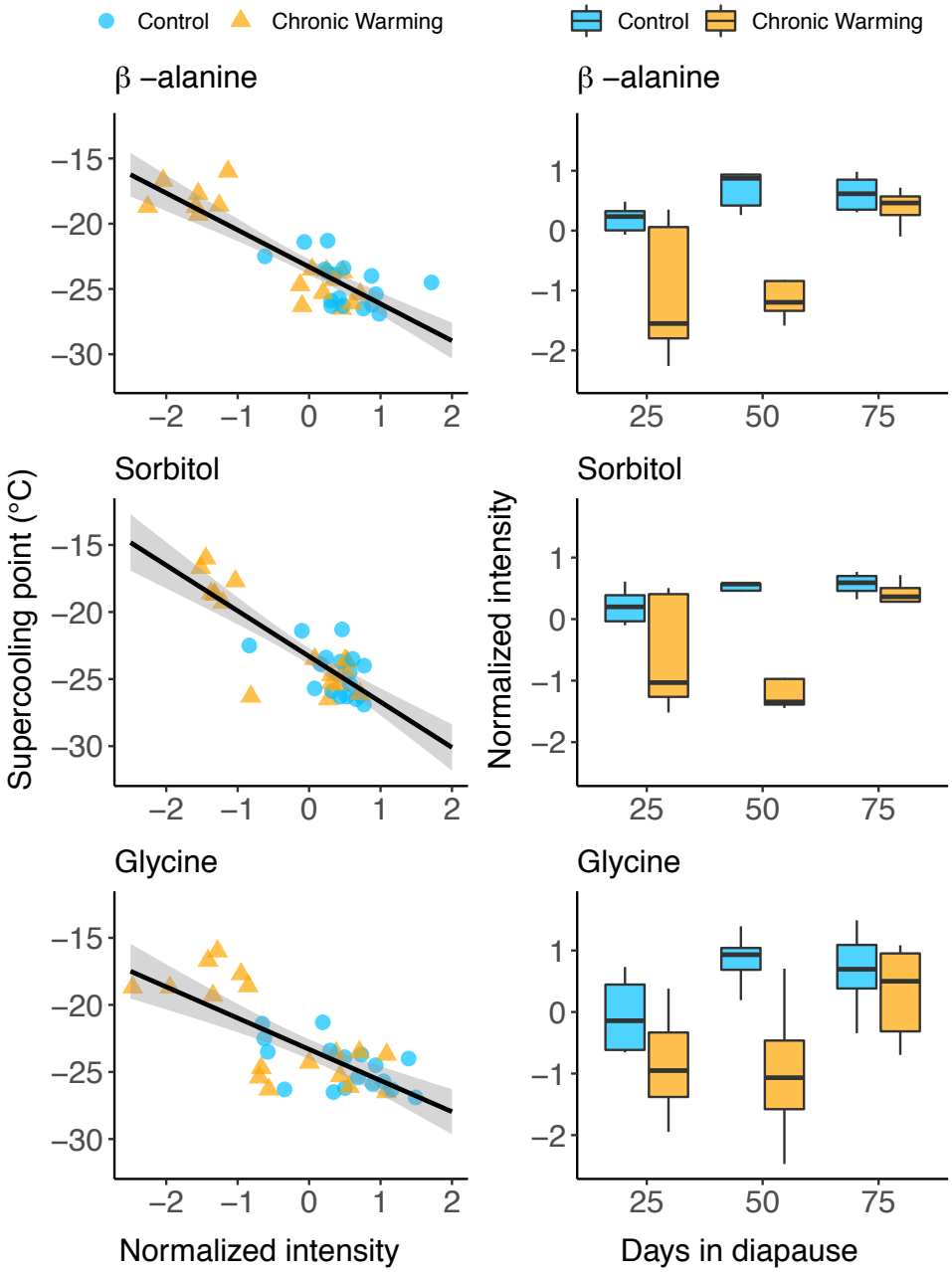
512

513

514

The coordinated changes in the metabolome that accompanied the chronic warming treatment constituted significant changes within 10 biochemical pathways (Fig. 5; Table S1). Chronic 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 warming-induced changes overall (Fig. 5). 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 (Hahn and Denlinger, 2011; Lee 2010; Michaud and Denlinger, 2007).

Figure 6



546

547 **Figure 6: Putative cryoprotectants correlated with SCP and were lower in abundance after**
548 **warming.** (A) β-alanine normalized intensity was negatively correlated with SCP (Least-squares
549 linear regression, $y = -2.83x - 23.31$, $R^2 = 0.72$, $P < 0.00001$). (B) β-alanine normalized intensity
550 was reduced after warming (2 way-ANOVA; temperature factor, $F_{1,30} = 17.16$, $P = 0.0003$; day
551 factor, $F_{1,30} = 12.00$, $P = 0.002$; temperature x day interaction, $F_{1,30} = 2.34$, $P = 0.14$). (C)

552 Sorbitol normalized intensity was negatively correlated with SCP (Least-squares linear
553 regression, $y = -3.4x - 23.31$, $R^2 = 0.70$, $P < 0.00001$). (D) Sorbitol normalized intensity was
554 reduced after warming (2 way-ANOVA; temperature factor, $F_{1,30} = 11.11$, $P = 0.002$; day factor,
555 $F_{1,30} = 5.84$, $P = 0.02$; temperature x day interaction, $F_{1,30} = 0.10$, $P = 0.62$). (E) Glycine
556 normalized intensity was negatively correlated with SCP (Least-squares linear regression, $y = -$
557 $2.33x - 23.31$, $R^2 = 0.55$, $P < 0.00001$). (F) Glycine normalized intensity was reduced after
558 warming (2 way-ANOVA; temperature factor, $F_{1,30} = 11.36$, $P = 0.002$; day factor, $F_{1,30} = 4.52$,
559 $P = 0.02$; temperature x day interaction, $F_{1,30} = 2.30$, $P = 0.12$). Data represent mean normalized
560 intensity of all features that matched a given metabolite (β-alanine: 3 features, sorbitol: 11
561 features, glycine: 1 feature).
562
563 **Responses of putative cryoprotectants to warming**
564 Pupae with the lowest supercooling points had the highest abundances of three putative
565 cryoprotectants, β-alanine, sorbitol, and glycine, and SCP was negatively correlated with the
566 abundances of all of these metabolites (Fig. 6). Moreover, all three of these metabolites showed
567 significant decreases in abundance after chronic warming (Fig. 6).
568
569 **DISCUSSION**
570 A key question in the fields of ecological and evolutionary physiology is how populations and
571 species will respond to future climate change. Our data shed light on this question in two ways.
572 First, we report variation in supercooling among populations of *P. rapae* that matches the
573 historical winter thermal environments in Vermont and North Carolina, USA. Whether or not
574 this difference in supercooling ability is a fixed divergence that resulted from local adaptation is
575 an open question. But regardless of the cause, these data suggest that the natural thermal
576 environment influences overwintering physiology in *P. rapae*, which could have consequences
577 for this species in the future. If there is standing genetic variation in supercooling among
578 populations, then natural selection could act upon this variation and lead to evolutionary
579 responses in supercooling to future winter warming conditions. Second, we demonstrate that
580 warming modified the intrinsic physiological mechanisms that underlie freeze avoidance in
581 diapausing *Pieris rapae* pupae, as warming led to less negative supercooling points that were
582 accompanied by shifts in the metabolomic signatures of cryoprotection and other metabolic

583 pathways. Because supercooling is a key trait for the survival of freeze avoidant overwintering
584 insects, our results suggest that warming could threaten *P. rapae* by raising their supercooling
585 points.

586 While the present study cannot attribute differences in supercooling between populations
587 of *P. rapae* to a genetic basis, mounting evidence suggests that there is standing genetic variation
588 in various cold tolerance traits in insect populations. In *Drosophila melanogaster*, there is
589 evidence of clinal variation in chill coma recovery time (Hoffmann et al., 2003), and this trait has
590 been shown to evolve in response to laboratory selection (Williams et al., 2014a). In addition,
591 there is a substantial degree of standing genetic variation in whole-organism cold tolerance even
592 in a single population of *D. melanogaster* (Teets and Hahn, 2018). In the pitcher plant mosquito,
593 *Wyeomyia smithii*, classic work by Bradshaw and colleagues (Bradshaw et al., 2004) used
594 reciprocal transplants to demonstrate significant local adaptation in cold tolerance between
595 northern and southern populations. In *Colias* spp. of butterflies, there is evidence of local
596 adaptation in supercooling across elevational gradients that corresponds to winter climates in
597 lowland vs. mountain habitats in Europe (Vrba et al., 2014). Because cold tolerance has evolved
598 in the past, these patterns suggest that overwintering traits could evolve in response to future
599 winter warming; however, the presence of genetic variation in overwintering traits does not
600 necessarily mean that these traits will evolve, even if natural selection is predicted to act upon
601 these traits in a warmer world. It could be that maintaining robust cold tolerance traits, such as
602 supercooling, in itself does not pose a significant cost. Indeed, warmer winters may lead to
603 selection on photoperiodic cues that shorten diapause, rather than direct selection on cold
604 tolerance *per se* (Bradshaw and Holzapfel, 2010; Bradshaw et al., 2004). Ultimately, responses
605 of insect populations to future winter warming will depend not only on the degree of warming
606 but also on the level of thermal variation. Our data suggest that even short-term (24 h) warming
607 may compromise supercooling, which could have deleterious consequences if warm anomalies
608 are followed by extreme low temperatures (see below).

609 Our results suggest that warming could threaten the survival of *P. rapae* pupae in nature
610 by comprising supercooling ability. Although the observed supercooling points in warmed pupae
611 were relatively low, these supercooling points are within the range of winter temperatures in
612 Vermont. Thus, the warmed pupae from this study could have frozen to death in the wild. Other
613 studies have also shown that increased thermal variability leads to decreased cold hardiness in

614 diapausing insects, including the cabbage root fly, *Delia radicum* (Košťál and Šimek, 1995), the
615 emerald ash borer, *Agrilus planipenis* (Sobek-Swant, 2012), the hemlock looper, *Lambdina*
616 *fiscellaria* (Rochefort et al., 2011; Vallières et al., 2015), and the anise swallowtail butterfly,
617 *Papilio zelicaon* (Williams et al., 2014b).

618 The timing of winter warming, and subsequent cold challenges, may be an important
619 factor that determines the relative risk of *P. rapae* in warmer winters. Supercooling points
620 decreased throughout diapause in the present study, and many insect species have been shown to
621 follow this same trajectory through diapause (Bale, 2002; Hodek and Hodková, 1988; Marshall
622 and Sinclair, 2015; Pullin et al., 1991), which mirrors decreasing winter temperatures from
623 December through February. Meanwhile, in the present study, pupae at day 50 in diapause
624 showed the largest increase in supercooling points after warming treatment, yet day 75 pupae
625 maintained low supercooling points in spite of warming. The disparate responses of pupae to
626 warming at day 50 vs. day 75 could have been due to increased vulnerability to thermal
627 challenge at day 50 in diapause. Indeed, some stages of diapause may be more thermally
628 responsive than others, as has been observed during metamorphosis in *D. melanogaster* (Merkey
629 et al., 2011). Alternatively, it may not have been the vulnerability of day 50 pupae, but rather the
630 ability of pupae to physiologically acclimate to chronic warming by day 75, such that they could
631 regain low supercooling points. If this were the case, it would help explain the similarity in
632 metabolomes of all day 75 pupae, regardless of warming treatment (Fig. 3A). Thermal
633 acclimation to periodic warming has been previously shown to improve cold hardiness in locust
634 eggs, *Locusta migratoria* (Wang et al., 2006), and adult flies, *D. melanogaster* (Colinet et al.,
635 2016).

636 We note that extrapolating our results to what pupae experience in nature assumes the
637 direct exposure of diapausing pupae to changes in atmospheric temperatures, which may or may
638 not be a realistic assumption, depending on snow cover that could insulate insects against
639 thermal fluctuations (Boychuk et al., 2015; Sinclair, 2001). However, winter warming is
640 predicted to lead to loss or reduction in snow cover, which would subsequently expose
641 diapausing individuals to fluctuating temperatures and to a higher number of freeze-thaw events
642 (Bale and Hayward, 2010). We also acknowledge that supercooling is a mechanism of cold
643 tolerance that is most critical for species that are freeze avoidant—i.e., species that cannot
644 survive if they experience internal freezing. Supercooling may or may not be important for

645 overwintering survival in species that are freeze tolerant—i.e., species that can survive internal
646 freezing (Marshall and Sinclair, 2012). It remains to be determined whether freeze tolerant
647 species will be challenged by winter warming; however, thermal acclimation influences cold
648 tolerance in at least some freeze-tolerant insects (Li et al., 2020; Toxopeus et al., 2019),
649 suggesting that winter warming may also impact species that are freeze tolerant and not only
650 species that are freeze avoidant, as we have shown here.

651 Our untargeted metabolomic analysis identified more that 14,000 metabolite features, and
652 the results reveal three major findings. First, supercooling in *P. rapae* diapausing pupae may be
653 influenced by the abundances of thousands of metabolites, as a large proportion of the variance
654 (27%) in abundances of metabolites across the whole metabolome and among pupae
655 significantly correlated to supercooling point. Previous work has established a solid paradigm for
656 interpreting the relationship between cold tolerance traits and metabolite abundance.
657 Overwintering insects accumulate higher concentrations of key metabolites, or cryoprotectants,
658 to lower the freezing point of intra- and extra- cellular solutions (Bale, 2002; Storey and Storey,
659 1988; Storey and Storey, 1990). Thus, it is perhaps not surprising to see correlations between
660 metabolite abundance and supercooling, particularly among putative cryoprotectants, such as β-
661 alanine, sorbitol, and glycine (Michaud and Denlinger, 2007; Michaud et al., 2008; Storey and
662 Storey, 1990). In addition, sorbitol and glycine have been shown to stabilize macromolecular
663 structures like proteins (Street et al., 2006; Yancey et al., 1982), suggesting other potential
664 benefits of these compounds in addition to freezing point depression. Targeted metabolomics
665 studies that measure tens-to-hundreds of metabolites corroborate the relationship between
666 metabolite abundance and various cold tolerance traits, including supercooling (Koštál et al.,
667 2007; Koštál et al., 2011; Lehmann et al., 2018; Michaud and Denlinger, 2007). Our data provide
668 a broadscale perspective on the potential role of the metabolome in setting lower thermal limits,
669 which extends previous work to implicate the involvement of a wide array of molecular players.

670 Alternatively, warming-induced changes in supercooling point and metabolite
671 abundances, including cryoprotectants, could have occurred independently from one another. It
672 is important to note that the present study does not fully account for the diversity of potential
673 mechanisms that underlie supercooling. A critical factor that influences the supercooling point is
674 osmolality, which we did not measure in this study. Dissolved solutes in an insect's hemolymph
675 colligatively lower their freezing point by 1.86°C per osmole of solute (Denlinger and Lee,

676 2010), but other non-colligative mechanisms, such as ice-binding proteins, can contribute to the
677 lowering of supercooling point as well (Meister et al., 2013). If non-colligative mechanisms
678 contribute to supercooling in diapausing *P. rapae* pupae, then the metabolomic data we present
679 herein would not fully describe the mechanisms that underlie the shifts in supercooling point that
680 occurred in response to warming. Nonetheless, the correlation between the metabolome and
681 supercooling is noteworthy and deserves further investigation. Future studies should measure
682 osmolality (indicative of colligative mechanisms) and thermal hysteresis (indicative of non-
683 colligative mechanisms) in response to warming to provide further insights into the connection
684 between putative cryoprotectant metabolites and supercooling in overwintering species.
685 The second major finding of the metabolomics screen is that chronic warming (+3°C)
686 caused shifts in core metabolic pathways, suggesting that even subtle changes in temperature
687 cause changes in metabolism during diapause. Overwhelmingly, warming caused metabolite
688 abundances to decrease; for example, metabolites within the fructose and mannose metabolism
689 pathway, glycerolipid metabolism pathway, and pyruvate metabolism pathway were all
690 significantly higher in control individuals (Fig. 3). Previous work has shown that metabolomes
691 are dynamic, shift throughout diapause, and respond to temperature (Koštál et al., 2007; Koštál et
692 al., 2011; Lehmann et al., 2018; Michaud and Denlinger, 2007). We noted shifts in the
693 metabolome through time, as the second main axis (PC 2), which accounted for approx. 10% of
694 the variation in metabolomic profiles among pupae, separated day 25 pupae from day 50 and day
695 75 pupae. But regardless of these metabolomic shifts that occurred through diapause, many of
696 the changes in metabolomic profiles were induced by warming, particularly at day 50 in
697 diapause. Moreover, many of the pathways that shifted in response to warming are involved in
698 energy metabolism, such as pyruvate metabolism. Warming-induced decreases in the metabolites
699 involved in pyruvate metabolism could indicate alterations in glycolysis or glycogenolysis,
700 suggesting that ATP generating pathways could respond to winter warming exposure (Denlinger
701 and Lee, 2010). This is of particular concern because diapausing pupae should be metabolically
702 quiescent and able to maintain stable metabolism throughout diapause. Yet, if warming increases
703 energy metabolism, then this could lead diapausing pupae to deplete their energy reserves. In
704 addition, the maintenance of cold tolerance during diapause is dependent on the availability of
705 energy reserves, as fuel sources (lipids, carbohydrates, and amino acids) are also used as anti-
706 freezing cryoprotectants (Denlinger, 2002; Hahn and Denlinger, 2011; Storey and Storey, 2012).

707 We did not assay total lipid or sugar content, nor did we measure metabolic rates; thus, the
708 significance of these findings remains unresolved. Nevertheless, the characterization of
709 energetics in the context of warming in diapause is likely to be a worthwhile avenue of future
710 research.
711 Third, untargeted metabolomics elucidated patterns of metabolite abundances that we
712 would not have otherwise seen if we had taken a targeted metabolomics approach. For example,
713 the arachidonic acid metabolism pathway was the only pathway in which metabolites exhibited
714 increased abundances in warmed pupae (Fig. 3). The specific function of arachidonic acid in
715 diapausing *P. rapae* pupae remains to be determined, but based on what is known about the role
716 of arachidonic acid in hibernating mammals, this result suggests that winter warming may impact
717 the utilization of energy stores. Arachidonic acid is a long-chain fatty acid that has been shown
718 to regulate the activity of peroxisome proliferator-activated receptor α, a protein involved in
719 mobilizing lipid stores (Wu et al., 2001) and a key regulator of lipid metabolism upon entrance
720 into hibernation in ground squirrels, *Spermophilus tridecemlineatus* (Buck et al., 2002).
721 Functional data on arachidonic acid in insects is lacking; thus, the potential role of arachidonic
722 acid in regulating energetic processes during diapause remains obscure. It has been shown that
723 arachidonic acid is a key polyunsaturated fatty acid in the cellular membrane phospholipids of
724 *Manduca sexta* (Ogg et al., 1991) and arachidonic acid is down-regulated in diapausing pupae of
725 the flesh fly (*Sarcophaga crassipalpis*) following acute cold stress (Michaud and Denlinger,
726 2006). But future study is needed to unravel the potential role of arachidonic acid in the context
727 of diapause and environmental change in insects.
728
729 **Winter warming: good or bad?**
730 Whether winter warming will benefit or hinder overwintering organisms is currently under
731 debate. Some research argues that warmer winter temperatures will result in beneficial effects on
732 temperate species, as these warmer patterns could lead to increased survival, decreased cold-
733 induced stress, and the ability to expand geographic ranges (Crozier, 2003). If cold stress lowers
734 survival in ectothermic organisms, then the predicted 1°C-5°C increase in winter temperatures
735 could increase survival through winter (Bale and Hayward, 2010). Although this prediction may
736 be true for some species, based upon the data we present herein, not all overwintering organisms
737 will benefit from winter warming. Indeed, previous research on *Pieris napi* 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., 2018; Posledovich et al., 2015). Thus, warming could disrupt the transition into post-diapause development, leading to a longer diapause state or decreased eclosion success. An additional consequence of winter warming may be the earlier spring emergence of insects, including many butterfly and bee species (Bartomeus et al., 2011; Bosch and Kemp, 2003). This phenomenon has potentially negative effects if it causes asynchrony with insects' host plants or if individuals experience severe environmental conditions post-emergence. Warming may also lead to 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 Hodková, 1988). Thus, at least for insects that have evolved to overwinter in a dormant state, winter warming may pose a significant environmental challenge (Stuhldreher et al., 2014), despite the presence of conditions that are seemingly less harsh.

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 winter temperatures increase and fluctuate with climate change. Future research exploring the effects of 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 after eclosion. Furthermore, research should also focus on comparing populations, ideally in a reciprocal transplant experimental design, to better understand population-level responses and assess the degree to which overwintering traits are locally adapted. This will allow us to better predict the adaptive potential of overwintering traits in the face of winter warming.

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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.

Supplemental Tables

Table S1. KEGG pathways significantly enriched in the metabolomic response to 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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