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Thermal effects on metabolic rate in diapausing *Pieris rapae* butterflies

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ABSTRACT

As ectotherms, many insects spend the winter months in a state of suspended animation (i.e., diapause), lowering their metabolic rates to subsist on a limited store of energy reserves. The ability to lower metabolic rate during diapause relies, in part, on cold winter temperatures to intrinsically lower metabolic rate. Winter warming associated with global climate change may pose a challenge to diapausing insects by intrinsically increasing metabolic rate, potentially leading to the exhaustion of energetic reserves. We used stop-flow respirometry to measure oxygen consumption in response to temperatures representative of both acute and chronic winter warming scenarios in diapausing *Pieris rapae* pupae. Metabolic rate increased with increasing temperature in diapausing pupae, but metabolic rate depended on both pupal age and warming severity, with older pupae having lower metabolic rates overall. Despite the increases in metabolic rate, pupae recovered metabolic rate within 24-hours after short-term acute-warming exposure. In contrast, chronic exposure to warming over weeks and months led to significant decreases in metabolic rate later in diapause, as well as reductions in pupal mass. These results demonstrate that while respiration was thermally responsive, warming did not lead to sustained increases in metabolic rate. Instead, diapausing *P. rapae* appear to acclimate to higher temperature by lowering their metabolic rates in response to months of chronic warming. Overall, these patterns suggest that this species could be resilient to winter warming, at least in the context of energetics. However, the precise mechanisms underlying these responses remain to be characterized. Thus, future research—e.g., on the genetic underpinnings of energetics in the context of warming—could further elucidate the relative vulnerability of diapausing insects to future winter warming.

INTRODUCTION

The latest climate report from the UN's Intergovernmental Panel on Climate Change estimates a global 1.1 °C increase in average atmospheric temperatures since the industrial revolution and predicts an additional increase of 1.5 °C by the year 2040 if global warming continues at its current rate (Calvin et al., 2023). These increases in environmental temperatures may be particularly stressful for ectothermic organisms due to the temperature-dependency of their physiology (Deutsch et al., 2008; Huey et al., 2012; Somero, 2010). Acute increases in temperature within an organism's natural range can lead to two to threefold increases in rates of biochemical reactions (Schulte, 2015; Tattersall et al., 2012). To cope with thermal challenges induced by acute increases in temperature, changes in biological processes such as development, metabolism, and thermal tolerance have been observed in ectotherms (Colinet et al., 2015; García-Robledo et al., 2016; Robinet and Roques, 2010). These changes are perhaps not surprising, given that with each 1 °C increase in environmental temperature metabolism increases by approximately 8 % in ectotherms (Somero et al., 2017).

Both rates of environmental change and species vulnerability to warming are expected to vary by season (Bale and Hayward, 2010; Bradshaw and Holzapfel, 2010; Hahn and Denlinger, 2007). In fact, winters are warming faster than other seasons (Balling et al., 1998; Mikucki and Lockwood, 2021), with hotter mean temperatures, increased variability and a higher frequency of extreme-temperature anomalies (Calvin et al., 2023). Winter warming has the potential to negatively impact temperate ectothermic organisms, but this has only been explored in a few studies (Arambourou and Stoks, 2015; MacLean et al., 2017; Williams et al., 2012). Thus, there remains a large gap in our knowledge about the potential responses of insects to warmer winters.

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Many temperate insects survive the winter by entering diapause (Denlinger and Lee, 2010; Storey and Storey, 2012), a state of metabolic dormancy to conserve accumulated energetic reserves including lipids, carbohydrates, and amino acids (Hahn and Denlinger, 2011; Tomcala et al., 2006; Zhou and Miesfeld, 2009). Thus, most individuals face limited food availability in winter because they enter diapause with a fixed amount of energy reserves. Therefore, the energetic reserves that are present at the onset of dormancy must sustain insects not only through dormancy, but also post-dormancy, which for diapausing insects can include energetically costly processes such as metamorphosis, flight, and reproduction (Hahn and Denlinger, 2007; Ragland et al., 2009). Insects who enter diapause with fewer reserves or burn through reserves at a faster rate could end diapause early, die during or after diapause, or have fewer energetic reserves to support adult fitness (Hahn and Denlinger, 2011).

Hypometabolism during winter dormancy is sustained by low winter temperatures that lower metabolic rate via the Q_{10} effect (Hahn and Denlinger, 2007, 2011). For temperate species of insects, metabolic rates drop significantly while in diapause (Hoback and Stanley, 2001), but hotter temperatures may compromise this pattern by increasing metabolic rates (Angilletta, 2009; Dillon et al., 2010). Increased temperatures for both field and laboratory populations of diapausing *Eurosta solidaginis* larvae led to higher metabolic rates while in diapause, with decreased survival or decreased fecundity post-diapause (Hahn and Denlinger, 2007; Irwin and Lee, 2000, 2003). Longer, warmer prewinter temperatures paired with longer periods of winter led to decreased metabolic reserves and subsequent decreased survival in diapausing *Rhagoletis pomonella* pupae (Feder et al., 1997). Furthermore, increased variable temperatures in autumn led to higher metabolic rates and higher energy store loss in diapausing *Erynnis propertius* larvae (Williams et al., 2012). These studies provide evidence for the potential consequences of pre-winter warming on overwintering metabolic activity and energy metabolism.

The cabbage white butterfly *Pieris rapae* is a cosmopolitan species of butterfly found across five continents in temperate and sub-tropical zones (Ryan et al., 2019). The overwintering physiology of *P. rapae*, including metabolic rate, cold tolerance, and metabolite levels, has been well described across various populations of the species (Kono, 1970; Li et al., 2020; Mikucki and Lockwood, 2021). *P. rapae* enters facultative diapause during the pupal stage, spending months in dormancy until spring eclosion (Richards, 1940). This dormancy period is facilitated by elevated cold and stress tolerance and depressed metabolic activity (Denlinger and Lee, 2010). Previously, we have shown that diapausing *P. rapae* pupae exposed to acute and chronic winter warming had compromised supercooling points, lowered abundances of cryoprotectant molecules, and shifted metabolomic profiles (Mikucki and Lockwood, 2021). These winter-warming-induced metabolomic profiles suggest that metabolic rate may also be disrupted by winter warming. While warming has been shown to increase metabolic rate in diapausing pupae of the sister species *Pieris napi* (Süess et al., 2023), to our knowledge the effects of winter warming on metabolic rate in *P. rapae* have not been previously described.

Because metabolic rate is intrinsically dependent on temperature, we predicted that metabolic rate of diapausing pupae would increase under winter warming scenarios and lead to sustained increases in metabolic rate following acute and chronic warming. To test this prediction, we used stop-flow respirometry to measure the effects of acute and chronic increases in temperature, representing winter warming scenarios, on oxygen consumption during and after exposure (i.e. recovery) to winter warming in diapausing *P. rapae* pupae. Our study provides insight into the potential metabolic challenges of coping with current and future winter warming conditions that will expose diapausing insects to novel thermal extremes.

METHODS

Adult butterfly collections and juvenile stage rearing

To generate diapausing *Pieris rapae* pupae to test for the effects of real-time warming on metabolic rate (Experimental Scenario 1 described below), we collected approximately 40 adult *P. rapae* butterflies from two locations around Burlington, Vermont (44°28′51.94″N, 73°11′34.57″W and 44°20′46.59″N, 73°06′15.15″W) in July-August 2019. To generate diapausing *P. rapae* pupae to test the effects of warming on metabolic rate recovery from acute warming (Experimental Scenario 2 described below) and after chronic warming (Experimental Scenario 3 described below), we collected approximately 40 adult *Pieris rapae* butterflies from two locations in northwestern Vermont (44°29′48.52″N, 73°1220.19″W and 44°17′10.07″N, 73°14′07.11″W) in late September 2018. We kept all adults in mesh containers (Carolina Biological Supply, 11″ diameter × 12″ height, $n = 10$ adults per container) under common garden conditions of 24 °C, 12L:12D photoperiod, 55 % relative humidity, and with direct access to sunlight to improve oviposition. We fed adults every 24 h with a diet of 10 % honey solution on a sponge. After 48 h, we isolated each female into an individual container with fresh, organic kale leaves for oviposition. We collected fertilized eggs every 24 h and placed them into plastic containers (35.6 cm length x 20.3 cm wide x 12.4 cm height; $n \approx 20$ eggs per container) in incubators (Percival model DR-36VL) with standard conditions of 24 °C, 8L:16D photoperiod to induce pupal diapause, and 55 % relative humidity. We fed larvae fresh organic kale leaves every day. Individuals were reared under these conditions until pupation, approximately 14 days post-oviposition. Upon pupation we placed all pupae into individual petri dishes (60 × 15 mm), and then we haphazardly moved them into temperature treatments, with offspring from each mother represented in each treatment. After one week of acclimation in the lab at the University of Vermont, all pupae were shipped to the University of Nebraska, Lincoln, where all metabolic rate measurements were conducted. We acquired all necessary permits for transportation of live *P. rapae* from Burlington, Vermont to Lincoln, Nebraska (USDA permit P526P-16–02,649). Over one day of travel, pupae were transported on ice, in coolers at 4 °C with a photoperiod of 8L:16D kept using LED lights on timers. Once in Nebraska, pupae were placed into their respective acclimation conditions (see below) and acclimated for at least 4 days prior to metabolic rate measurements.

Experimental scenarios for diapausing pupae

We subjected diapausing pupae to temperature treatments reflective of historic, current, and predicted future winter warming scenarios in Vermont, USA during the winter months (November-February). We note that these treatments were designed based on the winter weather in Vermont, USA in the winters of 2019 and 2020, which occurred prior to and following our butterfly collections. We determined all winter warming treatments from historic data records from a local weather station in Burlington Vermont (National Weather Service Forecast Office, https://w2.weather.gov/climate/local_data.php?wfo=btv, accessed 2020) and from global climate predictions (Calvin et al., 2023) (Table 1).

We exposed pupae to three distinct experimental scenarios, each of which was conducted on separate sets of individuals to avoid using pupae that had been exposed to another experimental condition that could impact their metabolic rate.

Experimental Scenario 1—Real-time Warming: We kept all pupae at 4 °C (in both Vermont and Nebraska) prior to metabolic rate measurement (Fig. S1A). Metabolic rates were then measured in real time at 6 °C, 12 °C, or 23 °C (see next section for details on metabolic rate measurements). Note that early diapausing pupae (< 30 days into diapause) were used in Experimental Scenario 1.

Experimental Scenario 2—Acute Warming: In Vermont, we kept the

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

Month	Monthly Average (°C)	Deviation from 1981 to 2010 Average (°C)	Record High (°C)
Nov. 2019	0.5	−3.0	23.9
Dec. 2019	−2.5	+0.9	20.0
Jan. 2020	−3.3	+4.1	18.9
Feb. 2020	−3.9	+1.9	22.2

acute warmed pupae under a daily fluctuating temperature regime of 4 °C-8 °C; once in Nebraska, pupae were kept at a constant 4 °C prior to three 24-h warming exposures of 23 °C on Days 25, 50 and 75 (Figure S1B). This pattern reflects short-term warming events observed in Vermont during the winter months (Table 1). Metabolic rates were measured at 8 °C 24 h before and 24 h after the three acute 24-h warming exposures.

Experimental Scenario 3—Chronic Warming: In Vermont, we kept the control group pupae under a daily fluctuating temperature regime of 4 °C-8 °C, representing autumn temperatures when individuals first enter diapause. Once in Nebraska, these individuals were kept at a constant 4 °C (Figure S1B). In Vermont, we kept the chronic-warming group pupae under a temperature regime of 7 °C-11 °C, representing a 3 °C increase from the control. Once in Nebraska, the chronic warmed group was kept at a constant 8 °C (Figure S1C). This 3 °C increase, relative to controls, reflects both the long-term warming pattern seen in Vermont over the last 50 years, as well as the predicted, continued pattern of warming expected for the next 50 years (Calvin et al., 2023) (Table 1; National Weather Service Forecast Office, retrieved 2020). Metabolic rates were measured at 8 °C throughout diapause.

For Scenarios 2 and 3, we used pupae that were 25 to 90 days into diapause for metabolic rate measurements. We kept pupae used in all experiments under an 8L:16D photoperiod to maintain diapause.

Metabolic rate measurements

We used stop-flow respirometry (Lighton, 2018) to measure oxygen consumption (µl O_2/hr) as a proxy for metabolic rate. This approach maximized our ability to detect oxygen consumption in diapausing pupae, in which metabolic rates are characteristically low. We note, however, that stop-flow respirometry did not allow us to characterize potential discontinuous gas exchange (DGE), which has been observed in diapausing pupae of the sister species *Pieris napi* (Süess et al., 2023). Thus, DGE could have introduced a source of variation in metabolic rate that we could not account for in our analyses. Nonetheless, we believe that the data and conclusions we present herein are sound for two reasons. First, based on data reported for *P. napi* the duration of our stop-flow measurements was short (2 h) relative to the duration of DGE cycles (9–15 h), the vast majority of which are spent in the fluttering phase (Süess et al., 2023) when oxygen consumption is likely to be continuous (Chown et al., 2006). Thus, our data most likely represent the relatively low but consistent levels of oxygen consumption during the fluttering phase of DGE and are less likely to include infrequent short bursts of gas exchange during the open phase of DGE. Second, even if some of our measurements captured a short burst of gas exchange during the open phase of DGE, these occurrences did not obfuscate our data, as we observed consistent patterns that rendered statistically significant results. The goal of the present study was to characterize broadscale responses in metabolic rate to warming during diapause. Future work characterizing the effects of warming on DGE throughout diapause in *P. rapae* could help to further elucidate the relationship between metabolic rate and temperature in this species.

We used an RM8 Flow Multiplexer configured for stop-flow respirometry (Sable Systems International, Las Vegas, NV) to switch the airstream and sequentially flush a set of eight plastic 5mL-syringe chambers, seven of which contained individual pupae and one was an empty baseline chamber. This is the automated bolus integration with multiplexer approach as described in Lighton (2008). After an initial flush of the chambers, chambers were closed and pupae respired for a set amount of time (29 min at 23C; 104 min at colder temperatures) before we flushed the air in the chamber using dry CO_2-free air scrubbed using a combination of Drierite (calcium sulfate), Ascarite (sodium hydroxide) and soda lime (calcium hydroxide, sodium hydroxide, and potassium hydroxide) at a flow rate of 75 mL/min. In this flushed air, we measured the amount of oxygen consumed by the organism. The flushed air was scrubbed of CO_2 and water using Ascarite, soda lime, and magnesium perchlorate prior to O_2 measurement using the Oxzilla differential oxygen analyzer (Sable Systems International). Using the ExpeData software (Sable Systems International), O_2 data were baseline corrected, converted to rates, inverted to be on the positive scale, and integrated to obtain the area under the curve, which we then converted to a volume of O_2 (uL) consumed. To get an estimate of metabolic rate of the organism (uL/hr), we divided the volume of O_2 consumed by the time the organism was in the chamber in hours. During each respirometry run, each pupa was sampled three times. Note that Supplemental Figure S2 (Fig. S2) shows largely consistent estimates of metabolic rate for individuals across the three samples (0–2 h, 2–4 h, and 4–6 h samples) from the chronic warming experiment (Scenario 3 below). In our statistical analyses, we used the 2–4 h sample to estimate metabolic rate. We weighed all pupae prior to metabolic rate measurement, and pupal mass was incorporated into all analyses (see below).

To measure metabolic rate during real-time warming (Scenario 1), we placed individual pupae into 5-mL syringes in a circulating water bath set to one of three temperatures, 6 °C, 12 °C and 23 °C, maintained to within ± 0.2 °C. These conditions were meant to span the thermal range of the acute- and chronic-warming treatments (Fig. S1). We did not observe condensation inside the syringes. We measured O_2 consumption as described above for $n = 10$, $n = 10$, and $n = 15$ pupae at 6 °C, 12 °C, and 23 °C, respectively. For the real-time warming experiment, each individual was measured once at a single temperature. Thus, different sets of individuals were measured at each temperature.

To measure metabolic rate in response to acute warming, chronic warming, and in control animals (Experimental Scenarios 2 and 3), we measured metabolic rate as described above, but we set the water bath temperature to 8 °C, which was a temperature shared by all three experimental warming scenarios: control, acute warmed, and chronic warmed. For the acute-warming treatment (Scenario 2), we measured metabolic rate 24 h before and 24 h after the three 24-hour warming treatments on Days 25, 50 and 75 to characterize the response of metabolic rate to the acute-warming event ($n = 28$ pupae). Following the acute 24-h exposures, pupae were returned to 4 °C for recovery for 24 h prior to metabolic rate measurement. For the control and chronic warmed pupae of Experimental Scenario 3, we measured metabolic rate at intervals over diapause, encompassing Day 30 to Day 90 ($n = 33$ and $n = 30$ pupae for the control and chronic-warming treatment, respectively). Since control and chronic warmed individuals were kept under constant conditions (i.e. no acute-warming events like the acute-warming treatment), we aimed to measure the response of metabolic rate over days in diapause, and not on set days like in the acute-warming treatment.

To account for individual and the potential effects of mass on metabolic rate, we included individual as a random effect and mass as a covariate in our mixed effects statistical models (see below). This also allowed us to implicitly account for repeated measures. Due to the

limitation of the respirometry equipment (we could only measure seven individual pupae per day) and the age of each pupa (many pupae had the same age), some individuals had repeated metabolic rate measurements over time while other individuals did not. In the case of the acute-warming experiment, all pupae were assayed before and after acute-warming exposure.

Statistical analyses

We first processed all respirometry data using the Expedata software package (v. 1.1.15; Sable Systems International, Las Vegas, NV). We used the O_2 values from the baseline chamber to drift-correct metabolic activity of each individual, and then converted all raw metabolic rate values from parts per million to μL/h (Hoekstra et al., 2018; Wat et al., 2020). We present metabolic rate data not normalized to mass. Instead, we incorporated mass as a covariate in our models. In nearly all cases, mass did not have a significant effect on metabolic rate, and we report the effects of mass on metabolic rate in the Results section.

For Experimental Scenario 1, we modelled the exponential relationship between temperature and metabolic rate during days 2–12 into diapause with non-linear least squares regression with the nls function in R (v. 4.4.0) (R Core Team, 2023). We restricted this analysis to days 2–12 into diapause because this was the range of pupal ages for which we assayed metabolic rate at all three temperatures (6 °C, 12 °C, and 23 °C). Because metabolic rates decreased over time (see below), we binned pupae into 2 separate age classes (2–5 days and 6–12 days into diapause) and fit separate exponential functions to compare thermal sensitivity over time (2–5 d vs. 6–12 d) in early diapause. We chose the age ranges to split the sample sizes of each age class relatively evenly, $n = 14$ and $n = 17$ for the 2–5 d and 6–12 d groups, respectively. We then estimated Q_{10} values from the two exponential line fits using the formula: $Q_{10} = e^{10*b}$, where b was the exponential coefficient extracted from the nls model. We estimated Q_{10} only for the real-time warming experiment, in which metabolic rates were measured at the warmed temperature.

The relationship between pupal age and metabolic rate followed an exponential decay across the first 2–3 weeks of diapause, which we fit with non-linear least-squares regression with the drm function in the "drc" package in R. To further explore whether metabolic rate changed in response to warming exposure, we used the lm function in R to fit a linear model with temperature, pupal age, and mass as fixed effects. We transformed the data with the natural logarithm prior to fitting the linear model. To test for main effects and interactions, we ran a type II ANCOVA with the Anova function in the "car" package.

To assess the effects of acute warming or chronic warming (Experimental Scenarios 2 and 3), we used the lme function from the "nlme" package in R to run mixed effects models with repeated measures. For the acute warming, we ran an ANCOVA with warming (pre- vs. post-warming) and pupal age as factors with fixed effects, mass as a covariate with fixed effects, and individual pupa as a factor with random effects. To assess significance of the main effects in the acute-warming experiment, we used the Anova function in the car package to compute a type II analysis of deviance.

For the chronic-warming experiment, we binned chronic-warming data into three pupal age ranges (i.e., 30–49 days, 50–65 days, and 66–90 days into diapause) and analyzed pupal age as a categorical factor. We did this to capture systematic differences in variance in metabolic rate across different age ranges. Each age range consisted of an equal number of pupae. We ran an ANCOVA with warming (control vs. warmed) and pupal age as factors with fixed effects, mass as a covariate with fixed effects, and individual pupa as a factor with random effects. Because of the presence of potential interactions, we assessed the significance of the main effects in the chronic-warming data using a type III analysis of deviance.

For both the acute-warming and chronic-warming datasets, to assess the significance of random effects, we used the log-likelihood ratio test with the anova function in the base R package to compare the fit of full models to models with the random effects removed. Note that both acute and chronic datasets were analyzed as repeated measures designs because individual pupae were assayed multiple times. The real-time warming dataset was not a repeated measures design because individual pupae were assayed once.

To assess the effects of warming on mass for all three experimental scenarios, we fit linear models with the lm function in R with warming condition and pupal age as fixed effects. To assess significance, we ran type II ANCOVA with the Anova function in the "car" package.

RESULTS

Responses to warming in real time (Experimental scenario 1)

Warming caused real-time increases in metabolic rate during early diapause (Fig. 1A and B; ANCOVA; temperature effect, $F_{2,28} = 275.85$, $P < 2.2 \times 10^{-16}$), following an exponential relationship (Non-linear least-squares regression; 2–5 days into diapause: $y = 1.66e^{0.132x}$; 6–12 days: $y = 1.65e^{0.12x}$). There was also a subtle decrease in thermal sensitivity across early diapause (Fig. 1A), with Q_{10} values of 3.75 and 3.31 of pupae 2–5 days and 6–12 days into diapause, respectively. However, the overall effect of pupal age on thermal sensitivity was not significant (ANCOVA; pupal age x temperature interaction, $F_{2,28} = 0.27$, $P = 0.77$), even though metabolic rates decreased with time in diapause (ANCOVA; pupal age effect, $F_{1,28} = 12.497$, $P = 0.0014$), following an exponential decay (Fig. 1B; Non-linear least-squares regression; 6 °C: $y = 4.7e^{-\left(\frac{x}{15.2}\right)}$; 12 °C: $y = 9.7e^{-\left(\frac{x}{30.1}\right)}$; and 23 °C: $y = 39.5e^{-\left(\frac{x}{19.2}\right)}$). Although pupal mass decreased with time (Fig. 1C; ANCOVA; pupal age effect, $F_{2,29} = 14.96$, $P = 0.0006$), warming did not significantly affect pupal mass across early diapause (Fig. 1C; ANCOVA; temperature effect, $F_{2,29} = 2.53$, $P = 0.10$). Pupal mass had no effect on metabolic rate in the real-time warming experiment (ANCOVA, mass effect, $F_{1,28} = 1.91$, $P = 0.18$).

Recovery after acute warming (Experimental scenario 2)

Despite the effect of temperature on metabolic rate in real-time during early diapause, metabolic rates were not affected following 24 h of acute warming at 23 °C later in diapause (Fig. 2A; ANCOVA, temperature effect, $\chi^2 = 0.23$, $P = 0.63$). This pattern was consistent regardless of pupal age (ANCOVA, temperature x age interaction, $\chi^2 = 1.95$, $P = 0.16$). Similarly, acute warming had no effect on pupal mass (Fig. 2B; ANOVA, temperature effect, $F_{1,122} = 0.12$, $P = 0.73$), and pupal age also had no effect on mass (ANOVA, pupal age effect, $F_{2,122} = 1.31$, $P = 0.27$, temperature x age interaction, $F_{2,122} = 0.37$, $P = 0.69$). Overall, pupal mass had no effect on metabolic rate (ANCOVA, mass effect, $\chi^2 = 0.45$, $P = 0.50$). However, there was a significant interaction between the combined effects of pupal age and mass on metabolic rate (ANCOVA, age x mass interaction, $\chi^2 = 7.54$, $P = 0.006$), such that older pupae exhibited a negative relationship between mass and metabolic rate (Fig. S3). Importantly, this effect of age and mass on metabolic rate was present regardless of acute-warming exposure (ANCOVA, temperature x mass interaction, $\chi^2 = 0.21$, $P = 0.65$, temperature x age x mass interaction, $\chi^2 = 1.57$, $P = 0.21$). Thus, the lack of effect of acute warming on metabolic rate was not influenced by pupal mass.

Chronic responses to warming (Experimental scenario 3)

Chronic exposure to +3 °C warming led to decreases in metabolic rate, but this effect was not present until later in diapause (Fig. 3A; ANCOVA, temperature effect, $\chi^2 = 3.20$, $P = 0.07$, temperature x pupal age interaction, $\chi^2 = 6.19$, $P = 0.045$). Pupal age by itself had no effect on metabolic rate in the chronic-warming experiment (ANCOVA, pupal

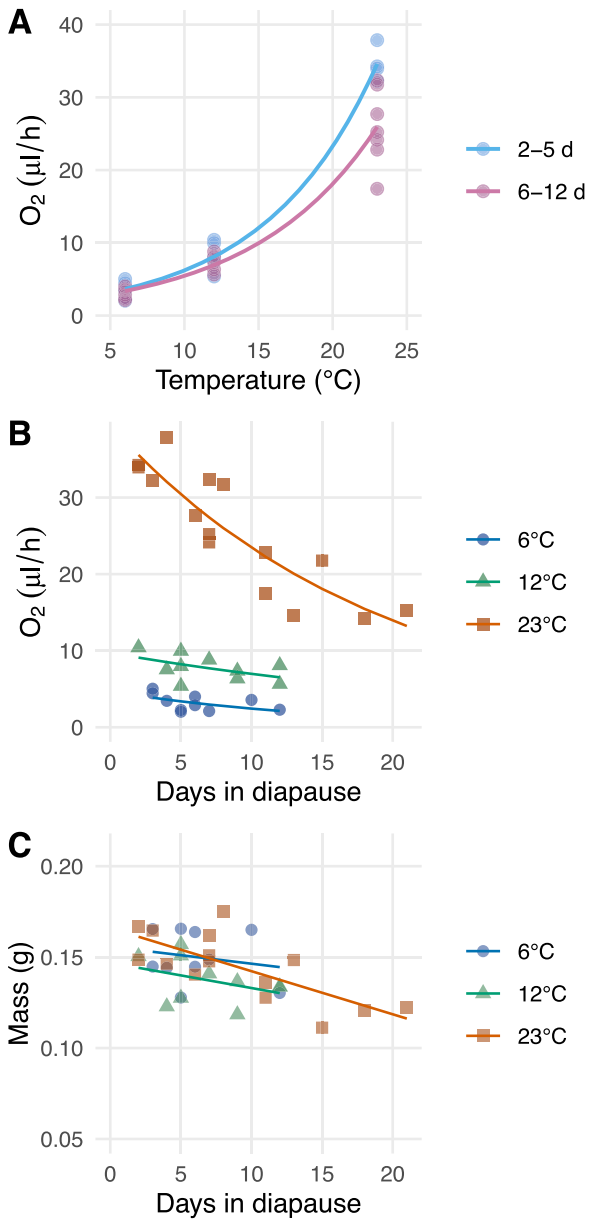

Fig. 1. Metabolic rate increased in response to warming and decreased across early diapause. (A) Oxygen consumption vs. assay temperature (6 °C, 12 °C, and 23 °C) 2–12 days into diapause. Each point represents the metabolic rate of an individual pupa, and each pupa was assayed once. Colors indicate range of days into diapause. Lines were fit with non-linear least-squares regression of an exponential function. (B) Oxygen consumption at 6 °C, 12 °C, and 23 °C assayed 2–21 days into diapause. Lines were fit with non-linear least-squares regression of an exponential decay function. (C) Pupal mass in response to warming 2–21 days into diapause. Lines were fit with linear least-squares regression.

age effect, $\chi^2 = 2.38$, $P = 0.30$). Chronic warming also led to decreases in pupal mass (Fig. 3B; ANOVA, temperature effect, $F_{1,59} = 19.95$, $P = 0.000037$). But mass did not have a significant effect on metabolic rate in the chronic-warming experiment (ANCOVA, $\chi^2 = 0.78$, $P = 0.38$).

In both the acute-warming and chronic-warming experiments, metabolic rates tended to be idiosyncratic, such that there were significant effects of individual on metabolic rate (Acute warming, LRT, $P < 0.0001$; Chronic warming, LRT, $P = 0.016$, Fig. S2). Hence, it was important to model individual as a random effect in our analyses.

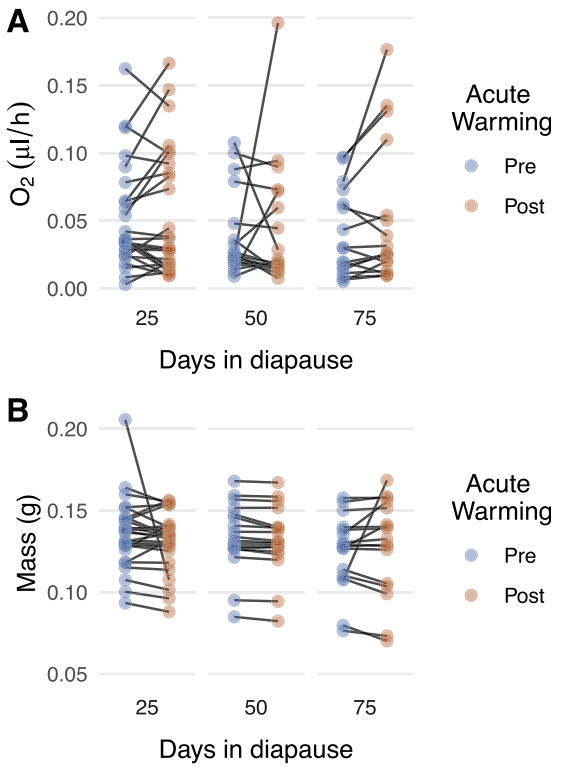

Fig. 2. Metabolic rate before and after acute warming at 23 °C. (A) Oxygen consumption pre- and post-acute warming. (B) Pupal mass pre- and post-acute warming. In both A and B, pupae were assayed at 25, 50, and 75 days into diapause, and black lines connect measurements on the same individuals before and after warming.

DISCUSSION

Here, we report that metabolic rate increased in response to real-time warming during the first 21 days of diapause in *Pieris rapae* pupae. However, later in diapause, pupae recovered metabolic rate within 24 h after acute-warming exposure, which suggests that *P. rapae* may be able to cope with short-duration winter warming events. Further, prolonged exposure to warming, for weeks to months, led to decreases in metabolic rate, which may signify an acclimatory capacity in *P. rapae* to adjust metabolic rate in response to higher average temperature during diapause.

Because metabolic rate was thermally responsive early in diapause in *P. rapae* pupae, winter warming could pose short-term physiological challenges, including exhausting energy reserves; however, our results indicate that pupae can counteract these effects later in diapause and rapidly recover low metabolic rates after acute-warming events. During exposure to warming, we saw three to four-fold increases in oxygen consumption early in diapause, and such an exponential increase in respiration would require more fuel to meet the increased energetic demand. A depressed metabolic rate is a key trait for survival in insect species that diapause through the winter (Denlinger and Lee, 2010; Storey and Storey, 1988). Lower metabolic rates conserve limited energy reserves (Hahn and Denlinger, 2007, 2011), and low temperatures directly depress metabolic rates during diapause, via the Q_{10} effect. Metabolic rates can decrease by greater than 90 % during diapause, though the extent of metabolic suppression varies by species (Hoback and Stanley, 2001; Storey and Storey, 2012). Similar Q_{10} effects as those we report herein have been shown in a population of diapausing *P. rapae* pupae from London, Ontario (Li et al., 2020) and in pupae of the sister species *P. napi* in Sweden (Süess et al., 2023). Thus, the patterns that we report herein are likely to be indicative of a broadscale physiological response in this species and not specific to the population of *P. rapae* that

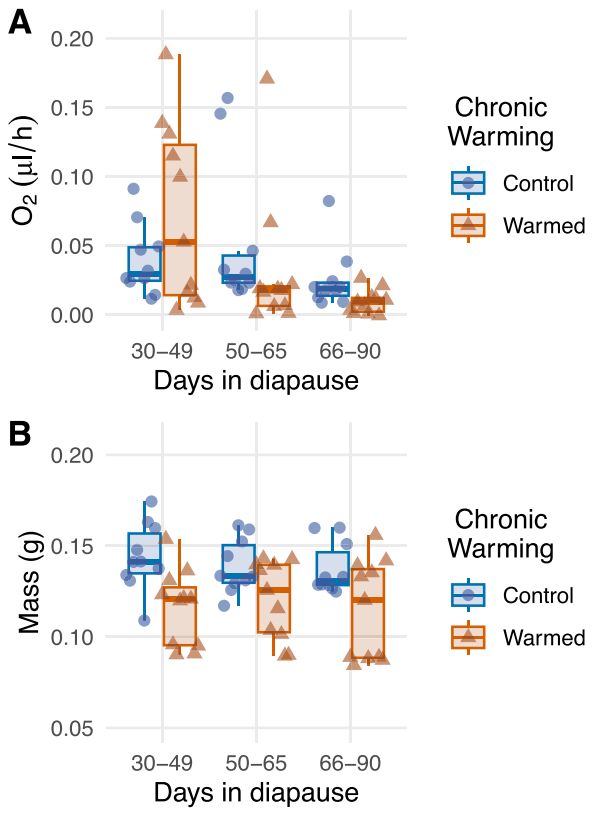

Fig. 3. Long-term effects of chronic warming of +3 °C. (A) Oxygen consumption and (B) pupal mass across days in diapause among control (4 °C) and chronically warmed (7 °C) pupae.

resides in Vermont. However, our data also indicate that metabolic rate decreased overall as pupae aged, regardless of warming. This pattern is consistent with previous data of other populations of *P. rapae* (Kono, 1970) and other insects, such as *Drosophila melanogaster* (Merkey et al., 2011). With lower metabolic rates in older pupae, even if the Q_{10} effect remains the same throughout diapause, the absolute cost of warming to the organism would be lower. This is perhaps why older pupae recovered metabolic rates following acute warming later in diapause (see below).

Pupal age strongly influenced metabolic rate, suggesting that the timing of warming in late fall and winter could impact vulnerability. Our results suggest that diapausing pupae are most thermally sensitive early in diapause. It has been hypothesized that insects are less sensitive to temperature early in diapause (i.e. autumn) to avoid development at heightened rates before temperatures drop in winter (Lehmann et al., 2016; Tauber and Tauber, 1976). In fact, a gradual increase in metabolic rate late in diapause has been observed in a beetle species (*Chrysomela aeneicollis)* but with no corresponding change in thermal sensitivity (Roberts and Williams, 2022). Our results contradict this observation and instead show that individuals early in diapause are susceptible to warming-induced changes in metabolic rate and have higher metabolic rates overall. Similarly, we previously found that the ability to supercool was adversely affected by acute and chronic warming in early but not late diapausing *P. rapae* pupae (Mikucki and Lockwood, 2021); thus, other aspects of overwintering physiology may be affected by warming in early diapause. With climate change, winters are arriving later, such that insects are exposed to pre-winter warming (Sgolastra et al., 2011). Diapausing insects that rely on photoperiodic cues for diapause induction, rather than low temperatures, may be subjected to hotter temperatures in the early stages of their diapause program, and might suffer downstream consequences later in diapause (Bale and Hayward, 2010). Previous work examining the effect of autumnal warming on diapausing *Erynnis propertius* larvae showed that warming early in diapause led to higher metabolic rate and energy store loss (Williams et al., 2012). Similar trends were shown for the butterfly *Pieris napi* (Nielsen et al., 2022) and for the eastern spruce budworm *Choristoneura fumiferana* (Roe et al., 2024). Furthermore, longer winter warming duration, starting at the onset of overwintering, was found to negatively impact survival and fat body depletion in *Osmia lignaria* bees (Bosch and Kemp, 2003). We note that because we only measured metabolic rates in real-time in early diapause (< 30 days), we do not know (1) if the pupae warmed in early diapause had any downstream effects on metabolic rate later in diapause, or (2) the extent to which pupae in later-stage diapause were responsive to increases in temperature.

We cannot, from our experimental design, determine the mechanism by which *P. rapae* lowered metabolic rates as diapause progressed. However, a recent study on metabolic rate during diapause in the sister species *P. napi* may provide a clue (Süess et al., 2023). In *P. napi*, pupae switch to discontinuous gas exchange (DGE) approx. 6–8 days into diapause, which leads to reductions in metabolic rate of greater than 50 % (Süess et al., 2023). DGE is a phenomenon where insects close their spiracles for extended periods and intermittently reopen spiracles for bursts of gas exchange (Quinlan and Gibbs, 2006). Our data in *P. rapae* show decreases in metabolic rate throughout the first 3 weeks into diapause, which is consistent with the switch to DGE in *P. napi*. However, we cannot assess the extent to which DGE contributed to this trend in the present study because our metabolic rate measurements were conducted using stop-flow respirometry, which measures the total amount of oxygen consumed over time and not the continuous profile of oxygen consumption. In the context of DGE, it is noteworthy that we observed a subtle decrease in thermal sensitivity in pupae that were 6–12 days into diapause as compared to pupae that were 2–5 days into diapause. Süess et al. (2023) found that thermal increases in metabolic rates, similar to those we report herein, were driven by the increased frequency of bouts of gas exchange. Thus, it is possible that modification of the frequency of gas exchange through diapause could serve as a mechanism for changes in thermal sensitivity. But the extent to which DGE duration and frequency changes throughout diapause in *P. rapae* is unknown.

While the acute increases in metabolic rate were pronounced early in diapause, older pupae did not show evidence of metabolic rate changes following acute warming to 23 °C. This is likely due to the depression of metabolic rate in later stages of diapause. Consider that the average oxygen consumption measured in the acute-warming experiment was 0.047 µl/ml at 8 °C (Fig. 2). Assuming a Q_{10} of 3.31 (as estimated from the real-time warming data), these later stage pupae would have increased their metabolic rates to an average of 0.28 µl/ml when exposed to 23 °C, which is approx. 90-fold lower than the average metabolic rate measured at 23 °C and 11-fold lower than the average metabolic rate measured at 6 °C during early diapause. Thus, we predict that hypometabolism later in diapause buffers *P. rapae* pupae against severe increases in metabolic rate that would otherwise be caused by short-duration winter warming.

Although hypometabolism may be an effective strategy for subverting the potentially dangerous effects of short-term winter warming, our data indicate that long-term chronic warming may still impose metabolic costs. Indeed, chronic warming caused decreases in pupal mass, which may have been driven by depletion of energy reserves (Bosch et al., 2010; Nielsen et al., 2022), or desiccation (Chown et al., 2011), or both (Gibbs et al., 1997). As a potentially adaptive mechanism to offset these metabolic costs, chronically warmed pupae lowered their metabolic rates even further. This response may constitute thermal compensation by effectively lowering the metabolic cost of maintaining diapause in a warmer environment. Thermal compensation has been observed in a vast array of physiological traits, including metabolic rate (Glanville and Seebacher, 2006; Zhu et al., 2016), enzyme activity (Kawall et al., 2002; Zhu et al., 2016), and lipid membrane remodeling (Cossins et al., 1977). Thermal compensation responses have often been

observed in the context of beneficial cold acclimation, where increased physiological rates offset the Q_{10} effect (i.e., lower levels of kinetic energy at colder temperatures) (Bullock, 1955; Fry, 1958). However, on the other side of the coin, decreases in physiological rates are beneficial at higher temperatures by offsetting the Q_{10} effect in the other direction, and thereby lowering the metabolic burden on the organism (Belhadj Slimen et al., 2014; Somero et al., 2017). In fact, the oriental tobacco budworm (*Helicoverpa assulta*) and the cotton bollworm (*Helicoverpa armigera*) enter hypometabolic diapause in the summer, induced by hot temperature, presumably to conserve energy and avoid heat stress (Liu et al., 2006, 2016). More broadly, diapause can be viewed as a strategy to cope with an array of environmental stressors, such as desiccation, hypoxia, and UV light (Hand et al., 2016; Wagner and Podrabsky, 2015; Wagner et al., 2019). A key feature of diapause is the ability to reduce metabolic rate, and we interpret the ability of *P. rapae* diapausing pupae to lower metabolic rate in response to chronic warming as a potentially adaptive plastic mechanism for conserving energy stores while overwintering.

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

Diapause is a complex dormancy program linking metabolic rate and metabolism to other key traits like cryoprotectant synthesis and cold tolerance (Lee and Denlinger, 2010; Sinclair, 2015). Considering previous studies demonstrating thermal sensitivity of diapausing insects to warming, we expected to see lasting effects of warming on metabolic rate in *P. rapae*. But while metabolic rate was thermally responsive in real time early in diapause, our results suggest that *P. rapae* may exhibit resiliency to acute and chronic winter warming. Future studies should address metabolic challenges experienced by overwintering species that diapause in other life stages, including egg, larval and adult stages, to better understand if these stages experience differential metabolic responses to winter warming. Furthermore, future studies should focus on understanding the effects of winter warming on the metabolic rate of diapausing species that enter dormancy for prolonged periods of time (> 1 year). Filling these gaps in knowledge could help us better predict which species may be most vulnerable to physiological challenges in the face of global climate change.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2025.100111.

Data availability

Data and code are available as supplementary files.

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