



Costs of averting or prematurely terminating diapause associated with slow decline of metabolic rates at low temperature

Jantina Toxopeus^{*,1}, Lahari Gadey, Lalitya Andaloori, Matin Sanaei, Gregory J. Ragland

Department of Integrative Biology, University of Colorado, Denver, 1151 Arapahoe St, Denver, CO, 80204, United States

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ABSTRACT

Diapause, a form of insect dormancy, generally facilitates overwintering by increasing cold tolerance and decreasing energy drain at high temperatures via metabolic rate suppression. Averting or terminating diapause prior to winter is generally assumed to be a lethal phenotype. However, low temperature acclimation can also increase cold tolerance and decrease metabolic rates. Here, we tested the hypothesis that non- and post-diapause individuals in a cold-induced quiescence can achieve a diapause-like phenotype, compensating for the potential costs of averting diapause. We tested this in the apple maggot fly *Rhagoletis pomonella*, which typically overwinters in the soil as a diapause pupa, but can avert diapause (non-diapause) or terminate diapause early ('weak diapause') when reared at warm temperatures. Metabolic rates were initially higher in non- and post-diapause than diapause pupae at high (25 °C) and low (4 °C) temperatures, but quiescent non- and post-diapause pupae achieved diapause-like metabolic rates slowly over time when incubated at 4 °C for several weeks. We found that diapause and quiescent pupae were freeze-avoidant and had similar tolerance of extreme low temperatures (cooling to c. -18 °C) following 8 weeks acclimation at 4 °C. Despite high tolerance of subzero temperatures, quiescent pupae did not survive well when chilled for prolonged periods (8 weeks or more) at 4 °C. We conclude that cold acclimation can only partially compensate for costs associated with aversion or premature termination of diapause, and that energy drain at low (not just high) temperatures likely contributes to chilling mortality in quiescent insects.

1. Introduction

Terrestrial organisms living in seasonal habitats encounter numerous challenges, including variation in abiotic factors (e.g. temperature) and biotic factors (e.g. resource availability) that affect survival. To persist in such habitats, many animals seasonally adjust their physiology in response to environmental cues and/or developmental programming (e.g. diapause). For example, many temperate insects increase their cold hardiness and suppress their metabolism during fall, increasing their likelihood of surviving the winter (Toxopeus and Sinclair, 2018; Sinclair, 2015). Cold-hardy species can survive subzero temperatures via one of two strategies: freeze-tolerant insects survive ice formation inside their bodies, while freeze-avoidant insects survive extreme low temperatures as long as ice formation does not occur (Lee, 2010). To prevent energy reserve depletion (i.e. use of stored carbohydrates, lipids, and proteins) when food resources are scarce, insects can achieve metabolic

rate suppression at high temperatures by decreasing the thermal sensitivity (Q_{10}) of metabolic rate, or across a range of temperatures by maintaining the same Q_{10} and decreasing their metabolic rate globally (Sinclair, 2015). Metabolic suppression is particularly important for energy reserve conservation during spring and fall, when high environmental temperatures can drive high metabolic rates (Sinclair, 2015; Williams et al., 2015b). Together, cold hardiness and metabolic rate suppression are key adaptations allowing persistence in seasonal habitats (Bradshaw and Holzapfel, 2008) and responses to changing climates (Marshall et al., 2020).

Diapause is a common strategy for survival in seasonal environments; this developmental dormancy program is associated with suppressed metabolism and enhanced stress tolerance (Hahn and Denlinger, 2011; Kostál, 2006). Within a species, diapause is usually associated with one life history stage and can be obligate, i.e. a necessary step in completion of the life cycle. Once the diapause program initiates, the

* Corresponding author.

E-mail addresses: jtoxopeu@stfx.ca (J. Toxopeus), lahari.gadey@ucdenver.edu (L. Gadey), lalitya.andaloori@cuanschutz.edu (L. Andaloori), matin.sanaei@ucdenver.edu (M. Sanaei), gregory.ragland@ucdenver.edu (G.J. Ragland).

¹ Present address: Department of Biology, St. Francis Xavier University, PO Box 5000, Antigonish NS, B2G 2 W5 Canada

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insect will remain dormant until appropriate internal or external signals allow diapause to terminate, after which development can resume under permissive conditions (Kostál, 2006). In many insect species, the diapause program is facultative and induced by specific environmental cues (e.g. short days, low temperatures associated with fall conditions) prior to periods of environmental stress (e.g. winter) (Kostál, 2006). Absence of those cues will result in direct (non-diapause) development through the life cycle. Diapause intensity (recalcitrance to terminate diapause) and diapause duration may vary among individuals of a species and can depend on the environmental conditions. Longer periods of mild chilling often result in faster completion of development (diapause + post-diapause morphogenesis) at warm temperatures (e.g. Lehmann et al., 2016). Diapause is distinct from quiescence – a form of dormancy in which development can be directly inhibited at any point in the life cycle by unfavourable environmental conditions but may resume immediately following return to benign conditions (Kostál, 2006).

Because diapause has clear benefits for overwintering survival, it is generally understood that there are dire fitness consequences for individuals that avert diapause or prematurely terminate diapause prior to the onset of winter. These fitness costs are clear when these individuals continue to develop. For example, insects may develop to a feeding stage when seasonal resources have disappeared and exhaust their stored energy reserves due to insufficient metabolic suppression at warm temperatures (Hahn and Denlinger, 2011; Sinclair, 2015). Even if resources are abundant, individuals may develop to a life stage that has low cold hardiness. However, if non-diapause or post-diapause individuals are able to enter quiescence, they may survive winter if they can achieve adequate levels of cold hardiness and metabolic suppression.

Although diapause is a common strategy for surviving harsh environments, there are other pathways to enhance stress tolerance and suppress metabolism. Low temperatures can induce an acclimation response, where exposure to mild low temperatures induces physiological changes that increase tolerance of extreme low temperatures (Lee, 2010; Toxopeus and Sinclair, 2018). Low temperatures directly inhibit metabolism via biophysical effects on enzymes and reaction rates (Toxopeus and Sinclair, 2018), and low temperature acclimation can also induce metabolic suppression over a range of temperatures in the absence of diapause (e.g. Toxopeus et al., 2019). Finally, low temperatures can induce quiescence: most temperate insects have a clear lower developmental threshold, below which they do not develop (Trudgill et al., 2005). Quiescence can occur in the absence of diapause (non-diapause quiescence) or following diapause (post-diapause quiescence). Indeed, many insects terminate diapause mid-winter, and survive the remaining harsh winter conditions in quiescence (Kostál, 2006).

In this study we empirically evaluated whether quiescence could fully or partially compensate for the costs of overwintering in a non-diapause or post-diapause state in pupae of the apple maggot fly, *Rhagoletis pomonella* (Diptera:Tephritidae). This fly is a well-studied diapause model that exhibits inter- and intra-population variation in both diapause induction and diapause intensity, such that not all individuals overwinter in diapause. The fly larvae develop in apple (*Malus domestica*) or hawthorn (*Crataegus* spp.) fruits in the summer throughout North America, and spend most of their life history (including winter) as pupae in the soil beneath their host trees. Most of these pupae enter diapause, exhibit metabolic rate suppression at warm temperatures, and remain dormant for several months before resuming development the following summer. However, this diapause is facultative; when incubated at warm (e.g. 24 °C) temperatures, 10–20% of pupae do not enter diapause (non-diapause pupae) and develop directly into adults (Calvert et al., 2020; Dambroski and Feder, 2007). In addition, a small portion (10–20%) of *R. pomonella* pupae enter diapause but exhibit very low diapause intensity, only remaining dormant for a few weeks (rather than several months) if continuously held at warm temperatures (Calvert et al., 2020; Dambroski and Feder, 2007). Previous studies have called this phenotype ‘shallow’ diapause, though ‘weak’

diapause may be more accurate as it is characterized by low diapause intensity rather than depth of metabolic suppression. Diapause termination culminates in a rapid increase in metabolic rate over 48 h (Ragland et al., 2009), after which non-diapause and post-diapause individuals exhibit very similar changes in metabolic rate over time, as they complete metamorphosis and eclosion at warm temperatures (Calvert et al., 2020). Thus both non-diapause and ‘weak’ diapause phenotypes risk developing into adults during warm fall conditions, particularly in apple-infesting populations that experience relatively long, warm pre-winter conditions (Dambroski and Feder, 2007). However, at temperatures below their lower developmental threshold of about 6 °C (Reissig et al., 1979) non-diapause and post-diapause pupae can enter quiescence, and we have anecdotally observed that some can survive simulated overwintering.

Here we quantify fitness costs of overwintering in a quiescent (rather than diapause) state by assessing the thermal sensitivity of metabolism and survival of acute and chronic cold exposure in *R. pomonella* pupae. Previous studies have generally assumed that pupae not in diapause but quiescent at the onset of winter will die because of temperature stress or energy depletion (e.g., Dambroski and Feder, 2007), and that the phenotype is maintained by linked selection on other diapause developmental traits (Calvert et al., 2020). However, the metabolism and cold tolerance of quiescent pupae has not been previously measured. We tested 1) whether differences in thermal sensitivity of metabolism between diapause and quiescent pupae could yield comparable levels of metabolic suppression at low temperature, 2) whether acute cold hardiness as measured by supercooling point differed between diapause and quiescent pupae, and 3) whether quiescent pupae differed from diapause pupae in their ability to survive simulated winters of various lengths. Though it seems unlikely, we tested whether quiescent pupae could also demonstrate programmed developmental suppression (i.e. enter diapause), which we evaluated by measuring developmental delays of adult emergence following simulated winter.

2. Materials and methods

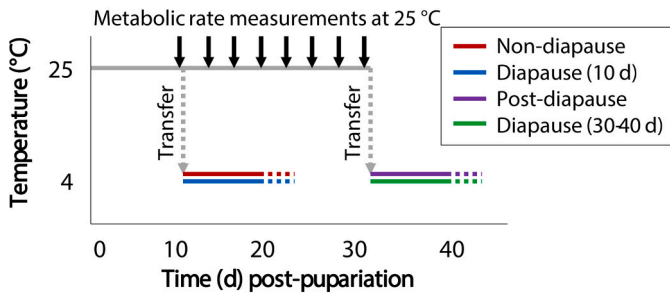
2.1. Insect collection

We collected apple fruits (*Malus domestica*) infested with *R. pomonella* pupae in August 2019 from a field site in Urbana, IL, USA. Fruits were transferred to our lab at the University of Colorado, Denver by ground transport at room temperature within 48 h of field collection, and *R. pomonella* were reared as previously described (Powell et al., 2020). Briefly, we placed fruits in wire mesh baskets suspended over plastic trays in an environmentally-controlled room (c. 22 °C). We collected newly formed pupae from plastic trays daily, and transferred them in petri dishes to a Percival DR-36VL incubator (Percival Scientific, Perry, IA) set to 25 °C, 14 L:10D, and 80% R.H. for 10 d prior to the start of metabolic rate measurements to determine diapause status (details below). Following incubation at 25 °C, all pupae were chilled at 4 °C in total darkness with c. 85% R.H. for simulated overwintering (Fig. 1A).

2.2. Determining diapause phenotype pre-chilling

To determine the diapause phenotype of *R. pomonella* pupae, we used stop-flow respirometry to measure the rate of CO₂ production ($\dot{V}CO_2$) at 25 °C as previously described (Ragland et al., 2009). Briefly, we weighed and transferred each pupa to an airtight syringe, and subsequently purged the syringe with CO₂-free air. We measured the amount of CO₂ produced in the syringe after 3 h incubation at 25 °C using a LiCor 7000 infrared CO₂ analyzer (Lincoln, NE) interfaced to Sable Systems International Expedata logging software (Las Vegas, NV) at room temperature (c. 22 °C). We transferred each pupa into a 0.2 ml PCR tube (with a hole for ventilation) and returned them to the 25 °C incubator following measurement. $\dot{V}CO_2$ was calculated with the manual bolus integration

A. Temperature treatments and determining diapause status



B. Time course for experiments (post-“Transfer” in A)

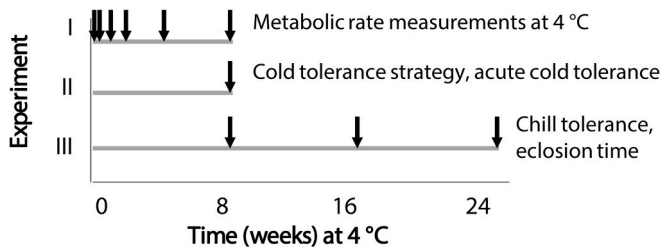


Fig. 1. Experimental design. (A) Non-diapause and diapause pupae were transferred to 4 °C after one metabolic rate measurement at 25 °C 10 d post-pupariation. Post-diapause and remaining diapause pupae were transferred to 4 °C 30–40 d post-pupariation, after 3 to 5 weeks of biweekly metabolic rate measurements at 25 °C (arrows). (B) Following transfer to 4 °C, all four groups of pupae were used in the three experiments. Measurements were taken at the time points indicated by arrows. Both non- and post-diapause pupae are referred to as ‘quiescent’ pupae throughout.

method (Lighton, 2018), normalized to control syringes that contained the same CO₂-free air but no pupae (Ragland et al., 2009).

We measured metabolic rate at 10 d post-pupariation to initially distinguish diapause phenotypes; pupae in diapause at this time demonstrate clear metabolic depression compared to pupae that do not initiate diapause (Ragland et al., 2009). Pupae with high (> 50 µl/g/h) metabolic rates were scored as ‘non-diapause’, while pupae with low (<30 µl/g/h) metabolic rates were scored as ‘diapause’. We transferred equal numbers of diapause and non-diapause pupae of the same age (10 d post-pupariation) to 4 °C within 3 h of metabolic rate measurements (Fig. 1A) for use in the experiments below. We continued to measure metabolic rate on the remaining diapause pupae twice per week for the next 3 to 5 weeks to identify pupae that completed diapause without chilling. Approximately 20% of these pupae demonstrated an increase in metabolic rate (to >50 µl/g/h) during this time, indicating diapause completion; these were classified as ‘post-diapause’ pupae (the ‘shallow diapause’ phenotype in Calvert et al., 2020; Dambroski and Feder, 2007). Note that ‘post-diapause’ typically refers to individuals that have completed diapause after overwintering, whereas here the post-diapause phenotype has prematurely completed diapause without overwintering. Post-diapause pupae that maintained this increased metabolic rate for two consecutive measurements were transferred to 4 °C within 3 h of the second measurement (30–40 d post-pupariation) with an equal number of diapause pupae of the same age that continued to have low (< 30 µl/g/h; diapause) metabolic rates (Fig. 1A) for use in the experiments below. Below we refer to both non- and post-diapause pupae as ‘quiescent’ during the 4 °C treatment.

2.3. Experiment I: Thermal sensitivity and temporal changes in metabolic rate at 4 °C

We measured CO₂ production at 4 °C in a subset of quiescent non-

diapause, quiescent post-diapause, and age-matched diapause pupae ($N = 24\text{--}34$ per group) after 24 h, 48 h, 1 week, 2 weeks, 4 weeks, and 8 weeks of chilling (Experiment I; Fig. 1B). We used the same stop-flow respirometry as described above, except that pupae were kept in the syringes at 4 °C for 24 h between purging and measuring, and the syringes were kept on ice whenever they had to be handled outside of the 4 °C incubator (i.e. during purging, measuring).

All statistical analyses were conducted in R v3.6.3 (R Core Team, 2019). To compare metabolic rate of non-diapause vs. diapause and post-diapause vs. diapause pupae at 25 °C (the last measurement before chilling) and 4 °C (after 48 h of chilling), we used random intercepts linear mixed effects models on log-transformed $\dot{V}\text{CO}_2$ (Bates et al., 2015). $\log(\dot{V}\text{CO}_2)$ had an approximately normal distribution. The models included mass as a covariate, diapause status, temperature, and a diapause $\times$ temperature interaction as fixed effects, and pupa ID as a random (subject) effect. We did not sex pupae, and were unable to include this as a factor in any of our analyses. Best-fit models were selected to minimize Akaike’s Information Criterion (AIC) for pupae that began chilling 10 d and 30–40 d post-pupariation (two separate models). We used linear contrasts to determine point estimates and 95% confidence intervals for slopes of $\log(\dot{V}\text{CO}_2)$ as a function of temperature for each diapause class in these models, then used the slopes to calculate Q_{10} values: the fold-change increase in metabolic rate that occurs with a 10 °C increase in temperature (Toxopeus et al., 2019).

We compared the relationship between metabolic rate and time at 4 °C in non-diapause, post-diapause, and age-matched diapause pupae using nonlinear mixed effects models on $\dot{V}\text{CO}_2$ (Bates, 2014). We used the following exponential function as the nonlinear component to model exponential decay:

$$m = m_{\min} + (m_0 - m_{\min})e^{-\alpha t}$$

where m , the predicted metabolic rate, decays over time t at rate α from a metabolic rate at t_0 to $m_{\min}$, the asymptotic metabolic rate. We fit models for each diapause class separately (four models total), with random effects for all nonlinear model parameters and pupa ID as a grouping factor. In one case (post-diapause) the full model would not converge because of overfitting; in this case we included only random (pupa) effects for m_0 and α . We also compared $\log(\dot{V}\text{CO}_2)$ between diapause classes at each time point for pupae of the same age using repeated measures ANCOVAs with mass as a covariate and the interaction of diapause class and time as the fixed effect, followed by Tukey’s post-hoc tests.

2.4. Experiment II: Cold tolerance strategy and acute cold tolerance

We determined the cold tolerance strategy of quiescent non-diapause, quiescent post-diapause, and age-matched diapause pupae ($N = 22\text{--}30$ per group) after 8 weeks of chilling (Experiment II; Fig. 1B), using previously described methods (Li et al., 2020; Sinclair et al., 2015). Insects are classified as freeze-tolerant if they survive freezing of their body fluids, freeze-avoidant if they survive supercooling but not freezing, and chill-susceptible if they do not survive supercooling or freezing (Sinclair et al., 2015). To cool pupae, we put them individually into 1.5 ml microcentrifuge tubes, which we placed in a custom aluminum block cooled by 50% propylene glycol circulated by an Arctic A40 programmable recirculator (ThermoFisher, Waltham, MA). We recorded the temperature of each pupa with a 36-AWG type-T copper-constantan thermocouple (Omega Engineering, Norwalk, CT) interfaced with Picolog v6 software (Pico Technology, Cambridge, UK) via a Pico Technology TC-08 unit. Pupae were cooled at -0.25 °C/min from 4 °C to a temperature at which half of them froze while half of them remained supercooled. We then removed pupae from the aluminum block, incubated them at 4 °C for 24 h and then transferred them to 25 °C for 6 days to recover. We assessed survival 7 days post-cold treatment using stop-flow respirometry at 25 °C (as described above), followed by removal

of the puparium to check for visual signs of decay or decomposition. $\dot{V}CO_2$ at this time point is a reliable indicator of survival; dead pupae had a distribution of metabolic rates below and not overlapping with a distribution of metabolic rates for live pupae ($N = 10$ dead and 8 live pupae; Fig. S1).

We measured supercooling point (SCP, temperature at which freezing begins) of quiescent non-diapause, quiescent post-diapause, and age-matched diapause pupae ($N = 18$ –20 per group) after 8 weeks of chilling to compare their acute cold tolerance, i.e. the ability to survive short exposures to extreme low temperatures (Experiment II; Fig. 1B). This is an appropriate metric for comparing acute cold tolerance of freeze-avoidant insects – the greater their supercooling ability, the lower the temperature they can survive (Sinclair et al., 2015). We cooled pupae (as described above) to a temperature at which all pupae froze, and determined the SCP values as the lowest temperature before the exotherm caused by the latent heat of crystallization (Sinclair et al., 2015). We compared SCP among diapause groups in R using an ANCOVA with mass and age at chilling (10 or 30–40 d post-pupariation) as covariates, and diapause class as the independent variable.

2.5. Experiment III: Tolerance of simulated winters and post-winter eclosion time

We compared the survival and development of quiescent non-diapause, quiescent post-diapause, and age-matched diapause pupae after prolonged chilling at 4 °C for 8, 16, or 24 weeks (Experiment III; Fig. 1B). A subset of these pupae were from the experiment that measured metabolic rate repeatedly over time at 4 °C, and were distributed evenly among the groups (5–7 pupae per diapause class per chill time). Following chilling, we transferred pupae to 25 °C for 100 d and checked for eclosion (exit of adults from the pupa case) weekly to determine the proportion eclosion in each group ($N = 30$ –45 pupae per diapause class per time point) and the approximate time to eclose post-chill. After 100 d at 25 °C, we removed pupa cases from all uneclosed individuals to determine their development and mortality status, classifying pupae into four categories. 1) Undeveloped, live pupae showed no signs of metamorphosis (Ragland et al., 2009) or decay – i.e. the pupae were pale and intact, similar to live pupae observed in the cold tolerance strategy experiment. 2) Undeveloped, dead pupae showed no signs of metamorphosis, but showed signs of death or decay, e.g. dehydration, discoloration (dark spots, browning), or mold growth. 3) Developed, dead pupae showed partial or complete development into the adult stage, but showed signs of death or decay. 4) Dead, undetermined pupae exhibited advanced decay that obscured the developmental stage (undeveloped/developed) at the time of death.

We compared the proportion of non-diapause vs. diapause and post-diapause vs. diapause pupae that survived after 8, 16, or 24 weeks of chilling at 4 °C using logistic regressions with a binomial error distribution and logit link function (Newman et al., 2017). Pupae that eclosed or remained undeveloped and alive within 100 d post-chilling were classified as ‘alive,’ while all other pupae were classified as ‘dead.’ Non-significant interaction terms were removed if they improved the fit (lower AIC). When the interaction term was significant, we used linear contrasts to determine which combinations of diapause classification and chill time had different proportions survival. To compare eclosion time post-chill of non-diapause vs. diapause post-diapause vs. diapause pupae after chilling, we used a pairwise Mann-Whitney U test at each time point (8, 16, or 24 weeks), with a Bonferroni correction for multiple testing.

3. Results

3.1. Metabolic rates of non-diapause and post-diapause individuals approach those of diapause individuals at cold but not warm temperatures

Diapausing pupae had lower metabolic rates than non-diapause pupae of the same age at both 4 °C and 25 °C, suggesting diapause pupae suppress their metabolic rate across environmental temperatures (Fig. 2A, Table S1). However, non-diapause metabolic rate was much more thermally-sensitive (significant diapause class $\times$ temperature interaction; 95% CI for Q_{10} of 3.45–4.13 for non-diapause compared to 1.95–2.26 for diapause). Thus, while metabolic rates were 90% lower for diapause compared to non-diapause pupae at 25 °C, they were only 65% lower at 4 °C (when non-diapause pupae are quiescent).

Likewise, metabolic rates of diapause pupae were suppressed but less thermally-sensitive compared to post-diapause pupae (that had ended diapause) of the same age after 30–40 days at 25 °C (significant diapause class $\times$ temperature interaction; 95% CI for Q_{10} of 3.23–4.00 for post-diapause compared to 2.09–2.58 for diapause; Fig. 2B, Table S1). Thus, metabolic rates of diapause pupae were substantially (91%) lower than post-diapause pupae at 25 °C, while metabolic rates were more similar at 4 °C. Note that Q_{10} estimates for non-diapause and post-diapause pupae were similar, suggesting that direct development through morphogenesis (non-diapause pupae) and post-diapause morphogenesis have similar thermal sensitivities when compared to pupae whose development is suppressed during diapause.

3.2. Metabolic rate decreases at a slow exponential rate over time at low temperatures

Mean metabolic rates of quiescent non-diapause and post-diapause pupae decayed exponentially over time after transfer from warm (25 °C) to cold (4 °C) temperatures, approaching the relatively low mean metabolic rates of diapausing pupae (Fig. 3; Tables S2, S3). This decrease in metabolic rate over time in quiescent pupae was not caused by mortality – most of these pupae were able to develop post-chill (Table S4). Quiescent individuals did not approach their minimum metabolic rate until about 4 weeks at 4 °C. In contrast, diapause pupae achieved relatively low mean metabolic rates throughout their time at 4 °C, with a slight linear decrease (shallow slope) over time (see substantial differences between non-/post-diapause and diapause fitted parameters for the exponential decay function; Table S2). The ANCOVA results suggested statistically significant (corrected $P < 0.001$) differences in metabolic rate between quiescent (non-diapause or post-diapause) and diapause pupae at all measured time points (Table S3), with differences most clearly-pronounced during the first two to three weeks of chilling. Note that not all quiescent individuals had exponential decreases in metabolic rate; a few individuals maintained relatively high metabolic rates throughout the 8 weeks of simulated winter (Fig. 3). The fates of these individuals with elevated metabolic rates varied – some developed post-chill while others died.

3.3. Diapause does not change cold tolerance strategy or supercooling point

Non-diapause, post-diapause, and diapause pupae were all freeze-avoidant after 8 weeks of chilling at 4 °C (Table 1). Mean SCP did not differ with diapause class, but was lower in pupae that were chilled following 30–40 d post-pupariation at 25 °C compared to those chilled following 10 d post-pupariation at 25 °C (Fig. 4; ANCOVA; Mass $F_{1,69} = 0.87$, $P = 0.35$; Age at Chill $F_{1,69} = 8.44$, $P = 0.005$; Diapause Class $F_{2,69} = 0.32$, $P = 0.724$).

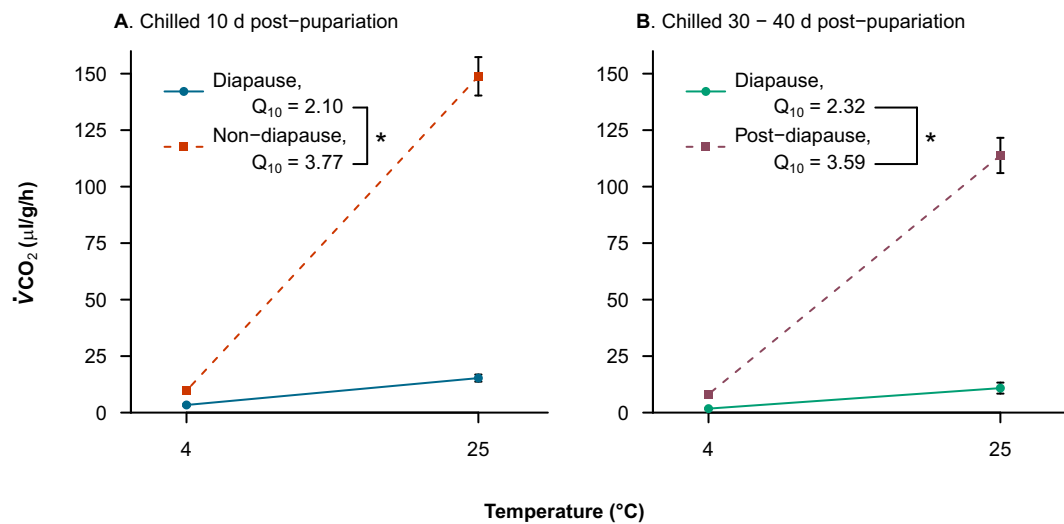


Fig. 2. Mean (± 2 s.e.m.) rates of CO_2 emission and thermal sensitivity of metabolic rate (Q_{10}) of *R. pomonella* pupae (A) 10 d and (B) 30–40 d post-pupariation. Small error bars are obscured by symbols. Asterisks indicate a significant difference in Q_{10} values (Table S1). $N = 24$ –34 per group.

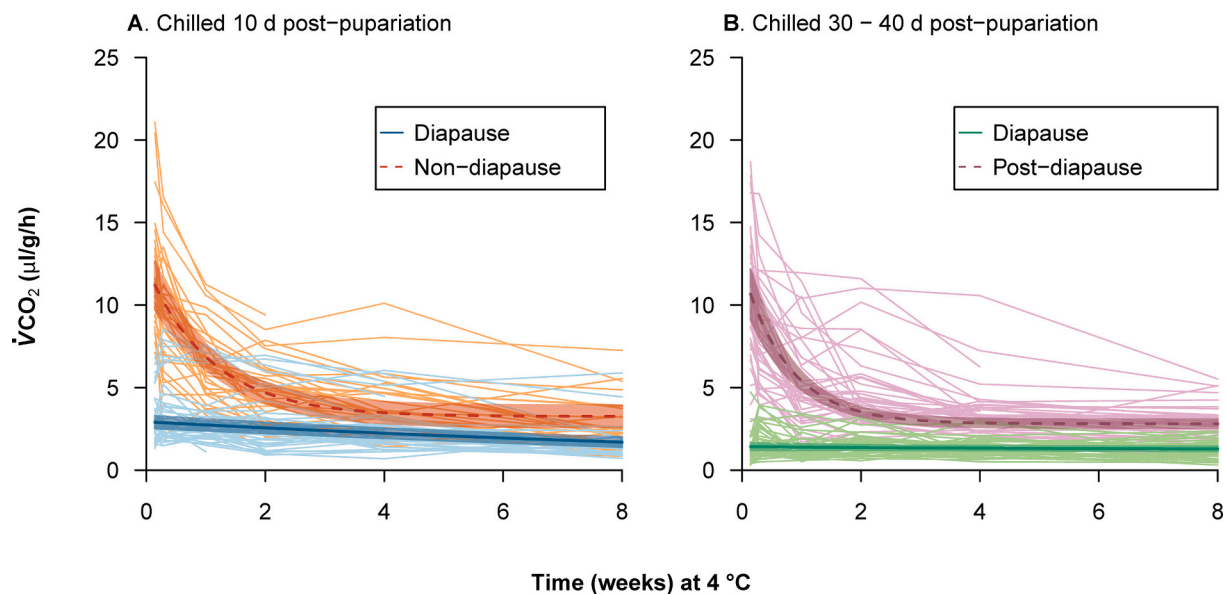


Fig. 3. Metabolic rate at 4 °C of *R. pomonella* pupae following transfer from 25 °C to 4 °C (A) 10 d post-pupariation or (B) 30–40 d post-pupariation. Each pale line represents one individual ($N = 24$ –34 per group); the thicker line and shading represent the fitted exponential model for a given group (Table S2) and the 95% confidence interval of the group mean response, respectively. There was a significant difference between diapause classes at all time points (Table S3).

3.4. Diapause conditionally, but not universally, increases survival of chronic chilling

Survival after prolonged chilling at 4 °C was higher in diapause than non-diapause pupae, and decreased with increased chill time in both groups (Fig. 5, Table S5). While non-diapause and age-matched diapause pupae had similar proportions of survivors that developed fully into adults ('Eclosed'), the diapause group had a higher proportion of unclosed but still alive pupae 100 d post-chill ('Undeveloped, alive'; Fig. 5A, B). Most of the unclosed non-diapause individuals died in the pupa case ('Developed, dead'; Fig. 5B). Note that we could not distinguish how much of the development in the non-diapause pupae occurred prior to versus following chilling. However most (c. 85%) of developed, dead non-diapause pupae exhibited substantial development (e.g. sclerotized bristles, wings) that would require longer than 10 d (pre-chill warming period) to develop (Ragland et al., 2009), suggesting some

development occurred post-chill. A small proportion of both diapause and non-diapause individuals died as pupae ('Undeveloped, dead'), or died with undetermined development status ('Dead, undetermined'; Fig. 5A, B). We note that proportion eclosion was generally lower in our study than similar studies on chilled *R. pomonella* (Feder et al., 1997a; Feder et al., 1997b), which we attribute to substantial handling (i.e. many metabolic rate measurements) of the pupae in this study.

The proportion of age-matched diapause and quiescent post-diapause pupae that survived prolonged chilling at 4 °C was similar following 8 or 16 weeks chilling, and was higher in diapause pupae after 24 weeks of chilling (Fig. 5, Table S5). Proportion eclosion was similarly low (< 25%) in both groups at all time points; this proportion decreased as chill time increased ('Eclosed'; Fig. 5C, D). Mortality was high in both groups: most diapause individuals died while still in diapause ('Undeveloped, dead'; Fig. 5C), while many post-diapause individuals developed into adults but failed to eclose ('Developed, dead'; Fig. 5D). As

Table 1

Cold tolerance strategy of *R. pomonella* pupae after 8 weeks at 4 °C, as determined by the proportion of pupae that survived freezing and supercooling to the same temperature. Survival post-cold stress was determined by respirometry and visual observations of decay. Chilling for 8 weeks at 4 °C started 10 d or 30–40 d post-pupariation. N, number of pupae.

	Mean mass $\pm$ s.e.m. (mg)	N frozen survived/N frozen	N supercooled survived/N supercooled	Cold tolerance strategy
Diapause (chill at 10 d)	6.78 $\pm$ 0.23	0/15	14/15	Freeze-avoidant
Non-diapause (chill at 10 d)	6.94 $\pm$ 0.48	0/15	12/15	Freeze-avoidant
Diapause (chill at 30–40 d)	6.36 $\pm$ 0.37	0/11	11/11	Freeze-avoidant
Post-diapause (chill at 30–40 d)	6.32 $\pm$ 0.32	0/11	11/11	Freeze-avoidant

above, we could not determine how much development occurred pre- or post-chilling, but most (c. 93%) of developed, dead post-diapause pupae exhibited substantial development, some of which likely occurred post-chilling.

3.5. Quiescent and diapause pupae remain developmentally distinct post-chilling

Despite apparent metabolic flexibility in quiescent pupae (Fig. 3), non-diapause and post-diapause pupae could not flexibly induce the same level of developmental suppression achieved by diapause pupae. Post-chill eclosion time was shorter (development was faster) for quiescent than diapause pupae after 8 weeks at 4 °C (Fig. 6). However, the mean emergence time for non-diapause pupae after 16 weeks of chilling was greater than emergence time at 8 weeks (Mann-Whitney *U* test, $P = 0.002$), suggesting some degree of developmental delay following prolonged chilling. This is also supported by the observation that the mean emergence times of 40 and 35 days (respectively) for non-diapause pupae after 16 and 24 weeks of chilling are equal to or longer than the maximum eclosion time of non-diapause pupae that are reared at 24 °C and never chilled (Dambroski and Feder, 2007). One non-diapause pupa (Fig. 6A) and one post-diapause pupa (Fig. 6B) had

very long eclosion times (>50 d) after 16 weeks of chilling, similar to diapause pupae. Thus, there may be variation in the degree of developmental delay within the population, though this constitutes relatively weak evidence. Chilling at 24 weeks for the non-diapause vs. diapause comparison and at 16 and 24 weeks for the post-diapause vs. diapause comparison resulted in very low eclosion proportion; thus, we refrain from highlighting any results for eclosion timing in these treatment combinations.

Overall, proportion emergence was lower than has been observed under comparable conditions in other studies (Feder et al., 1997a; Feder et al., 1997b). This was likely driven in part by repeated handling for respirometry, which did increase mortality compared to pupae that were handled less (Table S4). However, pupae handled multiple times for metabolic rate measurements were distributed equivalently across treatment combinations. Thus, handling does not explain differences between treatment groups.

4. Discussion

The fitness consequences of forgoing diapause are expected to be severe (Calvert et al., 2020; Dambroski and Feder, 2007; Feder et al., 1997b). We observed that quiescent non-diapause and post-diapause *R. pomonella* could partially achieve diapause-like phenotypes, exhibiting low metabolic rates and high tolerance of brief, extreme cold exposures. However, quiescent individuals did not survive prolonged simulated winter conditions well, demonstrating that there are costs to averting or prematurely terminating diapause, which we expand upon below.

4.1. Plasticity of metabolic rate and energetic costs of quiescence

Similar to other diapausing insects (Sinclair, 2015), diapausing *R. pomonella* pupae had a low thermal sensitivity (Q_{10}) of metabolic rate, facilitating active metabolic suppression at high and low temperatures relative to quiescent individuals. Non-diapause and post-diapause pupae that entered quiescence under simulated overwintering conditions were able to achieve diapause-like metabolic rates over time. However, the decline of metabolic rate at 4 °C in quiescent individuals was slow, resulting in relatively high metabolic costs over the first 4 weeks of chilling. Even after 8 weeks of chilling the mean metabolic rate at 4 °C was still higher in quiescent individuals. Metabolic costs can accumulate over the course of diapause at high (e.g. Irwin et al., 2001; Lindestad

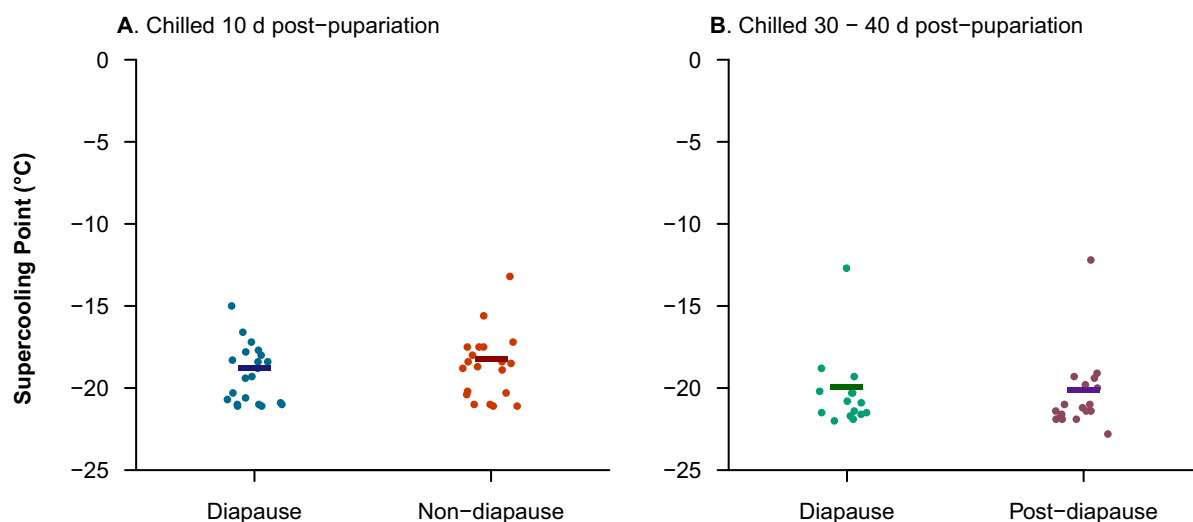


Fig. 4. Supercooling point (SCP) temperatures of *R. pomonella* pupae after 8 weeks at 4 °C; chilling at 4 °C started (A) 10 d post-pupariation or (B) 30–40 d post-pupariation. Each point represents one individual; the horizontal line represents the mean of those individuals ($N = 18$ –20 individuals per group). SCP did not differ with diapause class for pupae of the same age.

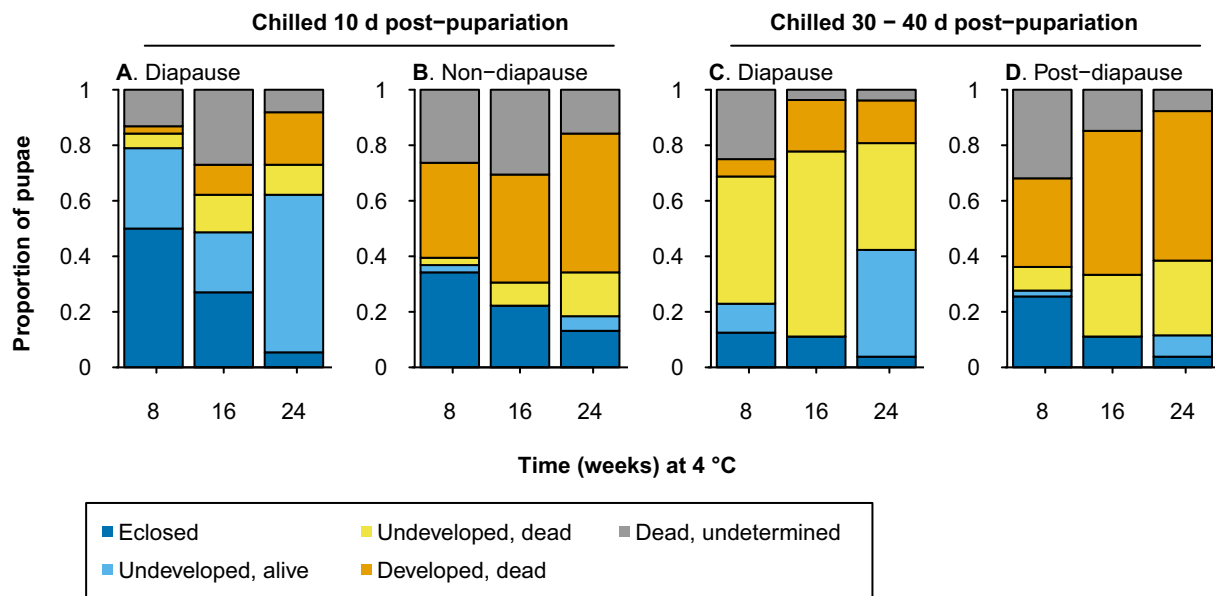


Fig. 5. Proportion of *R. pomonella* pupae that survived and/or developed after 8, 16 or 24 weeks at 4 °C; chilling at 4 °C started (A, B) 10 d post-pupariation or (C, D) 30–40 d post-pupariation. $N = 30\text{--}45$ pupae in each diapause class at each time point.

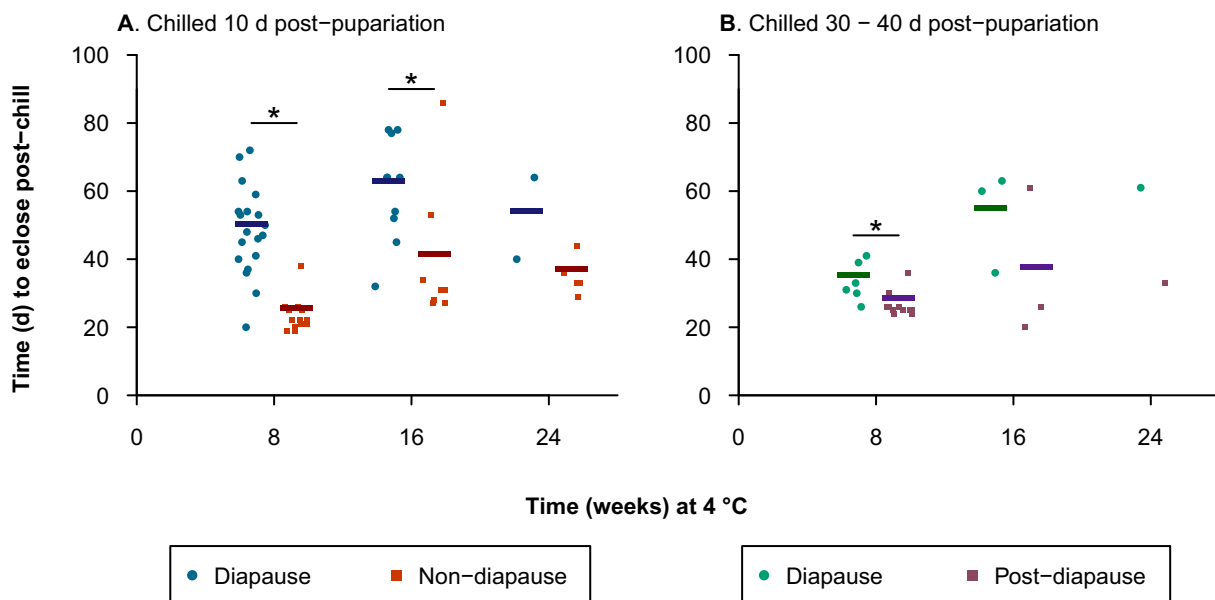


Fig. 6. Time to eclose at 25 °C after 8, 16, or 24 weeks at 4 °C; chilling at 4 °C started (A) 10 d post-pupariation or (B) 30–40 d post-pupariation. Each point represents one individual; the horizontal line represents the mean of those individuals. Asterisk indicates a significant difference in mean eclosion time between the two groups at each chilling time point (Mann-Whitney U test, $P < 0.05$); no statistical test was performed for diapause vs. post-diapause pupae after 24 weeks winter (only one data point in each group).

et al., 2020; Powell et al., 2020; Ragland et al., 2009; Sgolastra et al., 2010) and low temperatures (e.g. Irwin and Lee, 2002; Lehmann et al., 2016; Lester and Irwin, 2012) and are strongly associated with mortality during or after diapause, even when metabolic rates are relatively suppressed (Williams et al., 2015a; Williams et al., 2012).

We thus expect that even at low temperatures, time lags to achieve metabolic depression in quiescent individuals will result in burning through metabolic fuel needed for energetically expensive developmental processes (in this case, metamorphic morphogenesis), increasing mortality prior to adult emergence. When pre-chill warming was relatively brief, survival rates were lower for quiescent compared to diapause pupae. Most quiescent pupae exhibited partial completion of

metamorphosis before dying, suggesting that mortality occurred post-chill at temperatures permissive for development (see Reissig et al., 1979). We also note that particularly at 24 weeks overwintering, many live diapause pupae did not eclose in the experimental time window. We think it is likely that brief exposures to higher temperatures during the repeated transfers necessary for metabolic rate measurements influenced emergence, as elevated temperatures during diapause tend to delay post-winter emergence (Ragland et al., 2012). Finally, mortality was universally high for diapausing and quiescent pupae exposed to long, warm pre-winter conditions, likely reflecting the higher energetic costs of elevated metabolic rate and frequent handling during the pre-chill period. Thus, our results generally support a relationship

between elevated metabolic rate and elevated mortality during or after simulated winter.

4.2. Physiological vs. developmental plasticity during quiescence

The relatively low thermal sensitivity of diapause metabolic rate suggests that most metabolic suppression during diapause is caused by diapause-induced physiological changes rather than thermal plasticity and/or passive responses of enzyme kinetics to temperature. Thus, the relatively high capacity of non-diapause and post-diapause individuals to plastically attain similar levels of metabolic suppression at low temperatures raises an interesting question; does thermal acclimation in quiescent individuals down-regulate metabolism via the same mechanism employed during diapause? Diapausing insects often substantially remodel metabolism (Hahn and Denlinger, 2011). Specific pathways involved may vary depending on diapause life stage and taxon, though some have been repeatedly identified across life stages and taxa, e.g., increases in abundance of glycolysis/gluconeogenesis metabolites and gene products (Bao and Xu, 2011; Michaud and Denlinger, 2007; Zhang et al., 2019). Quiescent insects could plastically employ similar mechanisms, though diapausing insects often also stockpile different amounts and ratios of fuel, e.g., diapausing mosquitoes dramatically increase lipid storage during the preparation phase (Batz and Armbruster, 2018). Depending on such diapause-specific metabolic preparation, individuals flexibly entering quiescence rather than diapause may not be capable of sustaining the same fuel burning strategies. We were unable to identify any studies that have investigated metabolic plasticity during winter quiescence, but this could be a powerful comparison to understand how thermal acclimation may impact metabolism beyond passive Q_{10} effects on metabolic enzymes (Havird et al., 2020).

Despite achieving diapause-like metabolic rates during quiescence, we found little evidence for non-diapause or post-diapause pupae switching to diapause-like development patterns. Quiescent pupae generally eclosed faster than diapause pupae post-chill, consistent with earlier studies on non-diapause and post-diapause ('weak diapause') individuals that are never chilled (Dambroski and Feder, 2007). However, the mean eclosion time of non-diapause pupae increased as chill time increased, suggesting that prolonged chilling does delay development slightly. Though it is possible that quiescent non-diapause individuals may actively suppress development, it is equally plausible that this delay arises from a need to repair chilling injury or to reverse chilling-induced metabolic quiescence. Chilling (e.g. temperatures near 0 °C) can cause loss of ion homeostasis and accumulation of cellular damage (Overgaard and MacMillan, 2017). Restoration of homeostasis after a cold-stress can be energetically-costly and take time (e.g. El-Saadi et al., 2020; Štětina et al., 2018), resulting in developmental delays post-cold stress (e.g. Košťál et al., 2019).

We did observe two quiescent pupae with markedly delayed post-winter development similar to that observed for diapause pupae. Thus, we cannot rule out segregating genetic variance or unobserved environmental variance within the sampled population for developmental delays induced in quiescence. Natural populations of *R. pomonella* harbor substantial, polygenic variation for both diapause duration (Ragland et al., 2017) and diapause intensity (Calvert et al., 2020; Dambroski and Feder, 2007). Though we did not measure genetic variance in this study, we did observe substantial phenotypic variation in, e.g., the level of metabolic suppression achieved by quiescent pupae. If genetic variance accounts for some or all of this phenotypic variance, metabolism and developmental modulation during quiescence could evolve as a direct response to selection, or as a correlated response to selection on variants that also affect diapause phenotypes (Calvert et al., 2020). Future studies of the evolutionary potential of alternative strategies like quiescence vs. diapause may reveal additional ways that populations can respond to changing environments.

4.3. Diapause status does not affect tolerance of extreme low temperatures

Most *R. pomonella* pupae survived cooling to temperatures just above the SCP (the temperature at which freezing begins), indicating that they are freeze-avoidant and survive short low temperature exposures well whether they are in diapause or quiescence. SCP did not vary with diapause status, similar to the codling moth *Cydia pomonella* (Neven, 1999) and the flesh fly *Sarcophaga crassipalpis* (Lee and Denlinger, 1985). SCP may also vary little with geography in *R. pomonella*; pupae in our study (from the American Midwest) froze at temperatures close to -20 °C, as seen in Eastern Canada populations (MacPhee, 1964). There may be limited selection pressure for increased supercooling in diapause because *R. pomonella* pupae are unlikely to experience extreme cold (c. -20 °C) temperatures in their overwintering habitat. Soil temperatures at our field site rarely drop below -5 °C during winter (<https://www.weather.gov>) and are unlikely to deviate substantially from 0 °C when covered with snow (Marshall and Sinclair, 2012). We did observe a decrease in SCP associated with longer pre-chill warming, and speculate that pupae held at warm temperatures for longer periods lost some water weight, resulting in higher hemolymph osmolality that would promote supercooling. This relationship between dehydration and SCP depression has been observed in several insects, e.g. *C. pomonella* (Rozsypal et al., 2013).

Though diapause did not appear to confer higher acute cold tolerance compared to quiescence, it may be important for protecting against deleterious effects of chronic exposure to mild chilling. Delayed mortality after cold stress has been observed in several insects and can often be attributed to accumulation of sublethal cold injuries or indirect chilling injury (Košťál et al., 2019; Marshall and Sinclair, 2011; Marshall and Sinclair, 2015; Štětina et al., 2018). Thus, mortality of quiescent pupae following long-term exposure to mild low temperatures may be caused by a combination of elevated metabolic rates and accumulation of chilling injury. Screening for early developmental abnormalities immediately following winter could in principle provide evidence for delayed cold injuries if they are present.

5. Conclusions

As winters warm (Marshall et al., 2020) it is increasingly important to understand how temperature affects the phenology and physiology of insects. While warm temperatures can induce changes in the *R. pomonella* diapause program – inducing non-diapause or premature diapause termination pathways – low temperature quiescence cannot reverse these transitions. Diapause does not appear to be important for extreme low temperature tolerance in *R. pomonella*, but differences in metabolic rate suppression (including those at low temperatures) may drive differences in chill tolerance. Despite flexibility in the metabolic rate of quiescent pupae at low temperatures, averting or prematurely terminating diapause incurs significant costs, likely impeding overwintering survival in the field.

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Data availability

All data collected in this study are available in SupplementaryData. xlsx and our analysis code is available on GitHub at <https://github.com/jtoxopeus/diapause-aversion/blob/master/Rcode>.

Declaration of Competing Interest

None. The authors have no competing interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cbpa.2021.110920>.

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