



## Diapause survival requires a temperature-sensitive preparatory period

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### ABSTRACT

Diapause is a form of internally-controlled dormancy that allows insects to avoid stressful conditions and periods of low food availability. Eastern spruce budworm (*Choristoneura fumiferana* Clemens), like many cold-adapted insects, enter diapause well in advance of winter conditions, thus exposing them to elevated temperatures during fall that can deplete energy stores and impact post-diapause survival. We explored the impact of fall conditions on *C. fumiferana* by manipulating the length of the fall period and exposure temperatures during the diapause initiation phase of second instar larvae in a factorial design. We exposed second instar larvae to four fall temperatures (10, 15, 20, and 25°C) and five exposure times (1, 2, 4, 6, and 10 weeks) prior to standardized diapause conditions. We measured metabolites (glycogen, glycerol, and protein) prior to and during diapause for a subset of individuals. We also measured post-diapause survival by quantifying emergence following diapause conditions for a subset of individuals. We found that long, warm fall conditions depleted glycogen content and lowered post-diapause survival. We also found that short, cool conditions impacted post-diapause survival, although glycogen content remained high. Our results showed that fall conditions have substantial fitness consequences to overwintering insects. Optimal fall conditions struck a balance between exposure time and temperature. Our findings point to a potentially adaptive reason for early diapause onset: that an undescribed, but temperature-sensitive process is occurring in *C. fumiferana* larvae during the diapause initiation period that is essential for overwintering survival and successful post-diapause emergence.

### Introduction

Dormancy allows ectotherms to avoid stressful environmental conditions and periods of low food availability (Danks, 1987). This period of inactivity can either be an internally controlled developmental arrest (i.e. diapause) or an immediate response to deteriorating environmental conditions (i.e. quiescence; (Denlinger, 2022)). Diapause is a distinct developmental stage that proceeds through a series of programmed physiological steps, defined as induction, preparation, initiation, maintenance, and termination (see Košťál, 2006). Each stage involves dynamic coordination of many interacting processes that must be initiated and completed for a diapausing insect to enter, maintain, and finally leave the dormant state and successfully resume development (Denlinger, 2022).

Entry into diapause, also called diapause initiation, typically precedes the onset of stressful winter conditions (Denlinger, 2022; Tauber et al., 1986; Taylor & Spalding, 1989). Diapause in a wide range of insect taxa is consistently initiated weeks, or even months, prior to onset of

cold temperatures (Joschinski & Bonte, 2021). For example, the apple maggot fly *Rhagoletis pomonella* enters diapause in August/September while daytime high temperatures are high (e.g. 20 - 26°C), and subzero temperatures are still two months away (Meyers et al., 2020). Early entry into diapause is often considered a protection against unpredictability in the arrival of lethal low temperatures, loss of resources, or predation avoidance (Taylor & Spalding, 1989).

Early entry into diapause, however, means that dormant, non-feeding insects are arresting development during the summer or fall season, thus exposing themselves to prolonged periods of elevated temperatures. Given that the metabolic rate of ectotherms is temperature sensitive (Hahn & Denlinger, 2011; Irwin & Lee, 2003), energetic drain is likely higher during the early stages of diapause when conditions are warm, but slows as temperatures decrease (Williams et al., 2012). Fall temperatures are known to drive energetic drain in dormant insects and can significantly impact their survival, post-diapause fitness, and population dynamics. For example, the green-veined white butterfly *Pieris napi* survives winter in a pupal diapause and experiences

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significant weight loss and elevated metabolic rates during long, warm falls, which results in lower post-diapause fitness (Nielsen et al., 2022). Similarly, in the Propertius duskywing butterfly *Erynnis propertius*, fall conditions have a disproportionate impact on energy consumption in overwintering larvae, highlighting the importance of this season on larval fitness (Williams et al., 2012). Ectotherms must therefore balance the advantage of diapause for improved stress tolerance with the energetic costs of entering diapause in warm conditions (Hahn & Denlinger, 2011). Thus, it is clear that fall conditions impact the fitness of diapausing ectotherms, but despite this impact the effect of changing fall conditions during this critical period of dormancy is relatively understudied compared with other seasons (e.g. spring; Gallinat et al., 2015).

Under climate change, fall is warming faster than summer (Marshall et al., 2020) and impacting a range of biotic processes. For example, climatic changes during the fall period are delaying plant senescence (Menzel et al., 2006), shifting timing of fall migration in migratory birds (Jenni & Kéry, 2003; Van Buskirk et al., 2009), and altering rates of insect development. For example, phenology, voltinism, and winter survival may all be impacted as insects are exposed to longer, hotter warm spells, with increased probability of extreme temperature events (Glazacow et al., 2016; Schebeck et al., 2017; Van Dyck et al., 2015). As well, a recent examination of western American butterflies showed that population declines across 450 butterfly species are driven by fall warming in particular, while warming summers drive population increases (Forister et al., 2021). This suggests that understanding fall processes may be particularly important for understanding the drivers of insect dynamics and population declines.

The eastern spruce budworm, *Choristoneura fumiferana* (Clemens, 1865, Lepidoptera: Tortricidae) is a destructive forest insect that defoliates spruce and fir trees, and causes significant tree mortality throughout the boreal forest (Liu et al., 2019; Marshall & Roe, 2021). Overwintering survival is an important factor driving the dynamics of this pest (Gray, 2013), thus knowledge of its overwintering biology is foundational to predicting spatial and temporal changes in its population density. Carbohydrates stored as glycogen are the primary energy source for overwintering survival in *C. fumiferana* (Han & Bauce, 1998; Marshall & Sinclair, 2015). Glycogen, used to maintain critical metabolic functions during diapause, is converted to glycerol to facilitate cold tolerance, and then fuels post-diapause emergence in the spring (Marshall & Roe, 2021; Storey & Storey, 2012). Earlier work on *C. fumiferana* and related *Choristoneura* species has demonstrated that dormant larvae have a finite amount of energy to survive the winter period. Resource depletion (e.g. reduction in glycogen stores) is expected to contribute to mortality during diapause, particularly during fall before the onset of cooler winter conditions (Han & Bauce, 1998; Nealis & Régnière, 2016). In fact, conditions during early diapause are known to strongly influence larval survival and post-diapause fitness (Han & Bauce, 1997; McMorran 1973), highlighting the importance of this developmental stage.

The objective of this study was to assess the impact of climatic conditions on diapausing *C. fumiferana* during the fall prior to the onset of winter. We manipulated the length of fall and exposure temperatures experienced by diapausing second instar larvae. We hypothesized that elevated fall temperatures for longer periods of time would increase metabolic rates in diapausing *C. fumiferana*, causing greater energetic drain and leading to starvation during overwintering. Therefore, we predict that larvae exposed to warmer, longer fall conditions will have lower glycogen reserves and higher overwintering mortality than larvae exposed to short cool falls.

## Methods

### Experimental Design & Study Organisms

*Choristoneura fumiferana* has one generation per year, with adults laying eggs on host plant needles and new larvae hatching in late

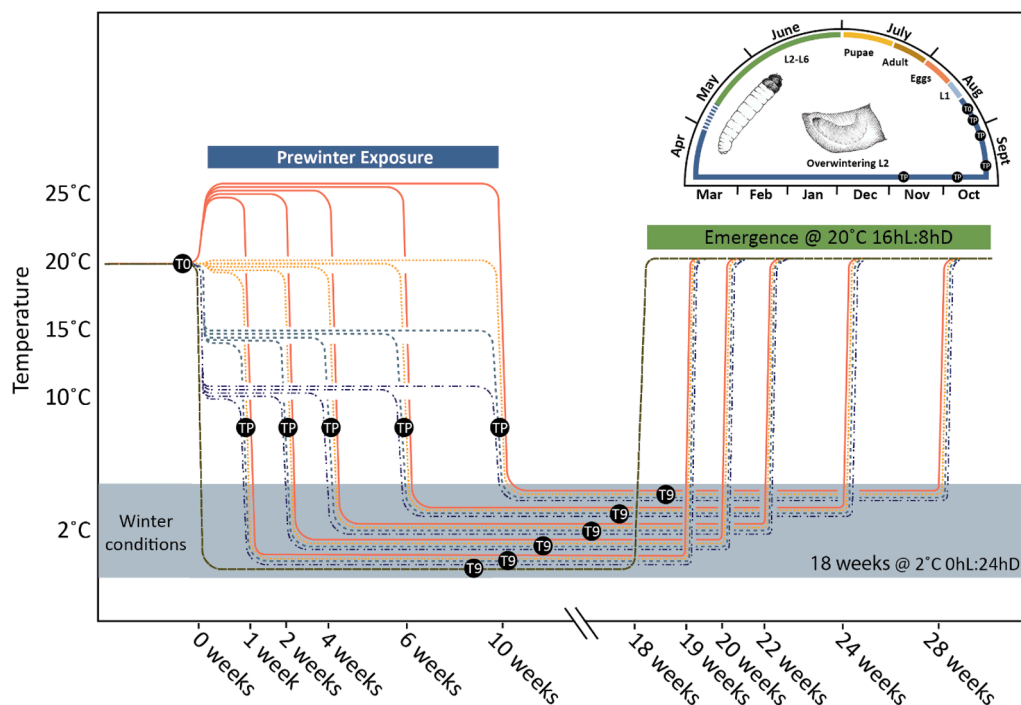
summer (late July - early August; (Candau et al., 2018; Marshall & Roe, 2021; Nealis & Régnière, 2016)). The larvae do not feed after hatching but migrate towards the lower crown (Eveleigh & Johns, 2014) to seek overwintering sites in bark crevices and lichen mats where they construct a silken overwintering structure called a hibernaculum (Nealis & Régnière, 2016). Larvae molt into a second instar within the hibernaculum by mid-August and enter dormancy several months prior to the onset of stressful winter conditions (Harvey, 1957; Marshall & Roe, 2021; Fig. 1). This dormant period is best described as a period of obligate diapause (Han & Bauce, 1993, 1998), and although phenotypic variation does exist (Marshall & Roe 2021), this is the most typical phenotype expressed in natural populations.

Our experimental insects were obtained from the Insect Production and Quarantine Laboratory at the Great Lakes Forestry Centre (Sault Ste. Marie, Ontario, Canada). We used wild-type adults sourced from an outbreak population in Manic-cinq, Québec (GLFC:IPQL:CfumMQC01; Perrault et al., 2021) to create the diapausing larvae used in our treatments. The parental population was maintained in artificial rearing for four generations using standard rearing protocols (Ebling & Dedes, 2015) on the McMorran diet (Grisdale & McMorran, 1963; McMorran, 1965; Hervet et al., 2016). Adults (100 males/100 females) were mated in a large plastic bag under standard rearing conditions (20°C, 16h:8h Light:Dark, 60-70% relative humidity) to generate egg masses for the treatment population. Egg masses were placed in hatching pans with cheesecloth embedded in paraffin as an overwintering substrate. Egg hatch began after 2-5 days and first instar larvae were allowed to establish overwintering sites and molt into second instar larvae before beginning the experimental treatments. Following construction of the hibernaculum, larvae rapidly convert glycogen to glycerol and decrease their body water content (Han & Bauce, 1993). Based on these physiological changes, we believe that larvae were at the diapause initiation phase during our fall treatments (Koštal, 2006).

Cheesecloth containing overwintering larvae was cut into sections containing at least ten larvae each (hereafter called a patch). A single patch was placed in a snap Petri dish (Falcon® Petri Dishes, 50 × 9 mm, Tight-Fit Lid, Corning Inc. - Life Sciences, Durham NC USA) and 15 patches were haphazardly assigned to each temperature x time treatment. Upon completion of the fall treatment exposure (TP), five patches were frozen at -80°C for metabolite analysis and 10 patches were placed in winter conditions (18 weeks, 2°C 0h:24h Light:Dark) in a Conviron environmental chamber (Model C920 Controlled Environments Ltd., Winnipeg MB, Canada). After 9 weeks of winter conditions, five patches were frozen at -80°C for metabolite analysis (T9). The remaining patches (n=5) completed the obligate diapause period prior to fitness assessment. An additional set of control larvae (T0; n=10 patches) were preserved for metabolite analysis prior to treatments. Fitness assessments were conducted at GLFC after 18 weeks of exposure to winter conditions by placing Petri dishes containing individual patches in a Hotpack environmental chamber (Hotpack Canada Ltd., Waterloo ON, Canada) at standard rearing conditions. Emergence from the cheesecloth was monitored daily. We exposed groups of diapausing *C. fumiferana* to four fall exposure temperatures (10, 15, 20, and 25°C) and five fall time periods (1, 2, 4, 6, and 10 weeks) in a full factorial design (n=20 treatment conditions; Fig. 1) using a Conviron environmental chamber (Model 124L). Of these treatments, moderate temperatures (15-20°C) for intermediate durations (4-6 weeks) would be most similar to field conditions experienced by our source population (i.e. Manic-cinq, QC). We also note that standard rearing conditions for *C. fumiferana* laboratory colonies includes a two week “pre-diapause” treatment at 20°C (Ebling & Dedes, 2015).

### Metabolites

To complement the fitness assessment, patches of second instar *C. fumiferana* larvae (n=5) were shipped on dry ice from the Great Lakes Forestry Centre to the University of British Columbia (Vancouver,



**Fig. 1.** Overall factorial design manipulating temperatures and exposure duration during the fall diapause period for *Choristoneura fumiferana*. Life cycle of *C. fumiferana* shown as an inset. Treatments began after second instar larvae established hibernacula within the cheesecloth substrate indicating onset of diapause. Individuals were moved to overwintering conditions upon completion of their fall treatment and exposed for 18 weeks before being returned to rearing conditions for post-diapause emergence (standard rearing conditions at the Insect Production and Quarantine Laboratories). Sampling for metabolites occurred at T0 (prior to treatment), TP (prior to winter conditions), and T9 (9 weeks into diapause).

British Columbia, Canada) for metabolite analysis. Upon arrival, larvae were immediately stored at  $-80^{\circ}\text{C}$ . Prior to biochemical analyses, larvae that were alive prior to freezing in a  $-80^{\circ}\text{C}$  freezer were removed from their hibernacula and pooled from five patches into three 1.8 mL microcentrifuge tubes in sets of 20 larvae ( $n=3$  biological replicates per treatment). Pools of larvae were homogenized with approximately  $90 \times 0.5$  mm Zirconium oxide beads (Next Advance Inc., Averill Park, USA) in a Bullet Blender (Storm 24, Next Advance Inc., Averill Park, USA) for 2 minutes at the highest speed. We then added 50  $\mu\text{L}$  of 0.05% Tween 20 and repeated the homogenization step. An additional 250  $\mu\text{L}$  0.05% Tween 20 was added after and the sample was mixed using a vortexer (Vortex-Genie 2, Scientific Industries Inc., Bohemia, USA). Following mixing, we centrifuged each sample for 10 minutes at  $15,000 \times g$  (Allegra 64R, Beckman Coulter Canada Inc., Mississauga, Canada). Two aliquots of supernatant were removed and the samples were stored at  $-80^{\circ}\text{C}$  for later assays.

We used spectrophotometric assays to measure glycerol, glycogen, and protein content for each of our pooled samples using a Spectra Max M2 spectrophotometer (Molecular Devices, San Jose, USA). We measured glycerol content using a glycerol standard (Fisher Scientific) and Free Glycerol Reagent (MAK117, Sigma-Aldrich Canada Co., Oakville, Canada). We loaded 30  $\mu\text{L}$  of each of the three pooled samples per treatment on a 96-well flat-bottomed microplate and 100  $\mu\text{L}$  Free Glycerol Reagent. Then we incubated the plates at  $37^{\circ}\text{C}$  for 5 minutes and measured absorbance at 540 nm. We measured glucose-based glycogen content using a Type II glycogen from oyster standard (Millipore Sigma) and a phenol-sulphuric acid method following (Masuko et al., 2005). Briefly, 10  $\mu\text{L}$  of each pooled sample per treatment was loaded on a 96-well microplate, and we added 40  $\mu\text{L}$  of 6.5% phenol and 200  $\mu\text{L}$  of sulphuric acid to each well. The plates were incubated at  $24^{\circ}\text{C}$  for 30 minutes and absorbance was measured at 490 nm. Finally, we measured protein content using a Bicinchoninic acid kit (BCA1, Sigma-Aldrich Canada Co.) and bovine serum albumin as a standard (Millipore Sigma). Briefly, we loaded 25  $\mu\text{L}$  of each pooled sample on a 96-well

microplate and added 200  $\mu\text{L}$  of reagent (50 parts bicinchoninic acid solution to 1 part 4%  $\text{CuSO}_4$ ). The plates were incubated at  $30^{\circ}\text{C}$  for 30 minutes and we measured absorbance at 562 nm.

#### Statistical Analyses

As *C. fumiferana* larvae significantly reduce water content throughout the fall (Han & Bauce 1993), we decided to report all metabolites on a per larva basis. Protein content is reported in  $\mu\text{g}$  per larva, glycogen content is reported in nmol of glucose per larva, and glycerol content is reported in nmol per larva. We started our analysis by examining whether protein content was significantly correlated with glycogen or glycerol content using linear models. As it was not ( $p > 0.1$  in both cases), we proceeded to examine experimental effects on metabolite content using linear models. We modelled metabolite content as a function of temperature exposure (10, 15, 20, or  $25^{\circ}\text{C}$ ), sampling time point (immediately following fall conditions or 9 weeks into diapause), time spent in fall conditions (1, 2, 4, 6, or 10 weeks), and their interactions, all modelled as numeric predictors. We then used the stepAIC function from the MASS package (Venables & Ripley, 2002) to simplify models based on Akaike's Information Criterion, and report the resulting outputs.

As survival to emergence was assessed in 5 replicates of  $28.01 \pm 16.12$  (mean  $\pm$  SD) larvae each, we used a logistic regression with replicate as a nested factor to measure the effect of fall duration and time on survival.

## Results

### Metabolites

There was no effect of protein content on the amount of any metabolite ( $p > 0.1$  in all cases), nor did any experimental manipulation impact protein content ( $p > 0.142$  in all cases). There was no effect of

sampling time point (either immediately following fall conditions or 9 weeks into overwintering conditions) on glycerol content, but the combination of fall duration and fall temperature significantly impacted glycerol content ( $t = -2.257$ ,  $df = 113$ ,  $p = 0.0259$ ). While the effect of fall duration was complex (Supplemental Fig. 1), generally higher temperatures led to lower glycerol content (Fig. 2; Supplemental Table 1).

Glycogen content was unaffected by sampling time point (either immediately following fall conditions or 9 weeks into overwintering conditions) but was strongly impacted by the interaction between fall duration and temperature ( $t = 2.749$ ,  $df = 113$ ,  $p = 0.007$ ) such that higher temperatures and longer durations of fall significantly decreased glycogen content (Fig. 3, Supplemental Fig. 2).

### Survival

We found that fall conditions were strong drivers of overwintering survival in *C. fumiferana*, with survival of replicates varying from 0 to 89% (Fig. 4). Logistic regression indicated that this was driven by the interaction between fall duration and temperature (deviance explained = 72.93,  $df = 99$ ,  $p < 0.001$ ) such that at high temperatures, survival is highest with short fall durations, and at low temperatures, survival is highest with long fall durations (Fig. 4).

Taken together, we show that glycogen content generally declines with increasing fall duration and temperature, while overwintering survival is lowest following either short, cool falls or long, warm falls (Fig. 5), with peak survival in long, cool fall conditions.

### Discussion

Diapause is a complex life history trait that allows insects to survive months without feeding or developing, while simultaneously upregulating stress resistance (Denlinger, 2022). Yet surprisingly, insects often enter diapause months before the onset of winter conditions (Tauber et al., 1986). During this time, high temperatures elevate their metabolic rate, leading to energy drain that could impede overwintering survival and post-diapause development. We hypothesized that warmer and longer falls would lead to higher energy depletion during the early diapause phase of *C. fumiferana* larvae, reducing their ability to survive the overwintering period. While we found that these conditions did

deplete glycogen content and decrease survival, glycogen content was not a clear predictor of survival at all temperatures. We discovered that short, cool falls also resulted in poor overwintering survival, despite high concentrations of glycogen. In fact, optimal conditions for diapause survival in our study were intermediate conditions, striking a balance between exposure time and temperature. This finding points to a potentially adaptive reason for early diapause onset: that an undescribed, but temperature-sensitive process is occurring in *C. fumiferana* larvae during this early diapause period that is essential for overwintering survival and post-diapause emergence.

Fall temperatures in temperate and boreal climates can be warm and highly variable (Marshall et al., 2020). Since metabolic rate increases with body temperature in ectotherms like *C. fumiferana*, we expected that energy consumption would closely track environmental temperature (Hahn & Denlinger, 2011). We saw that warmer conditions during early diapause did cause greater depletion of energy reserves in *C. fumiferana* than cool temperatures (Figs. 3 and 5A), similar to previous observations for this species (Han & Bauce, 1998). We also observed that low post-winter survival in *C. fumiferana* was correlated with low glycogen concentrations (Fig. 5B), suggesting that glycogen depletion decreases post-diapause fitness. Our results coincide with earlier work on this species (Han & Bauce, 1997, 1998; McMorran, 1973). McMorran (1973) showed that pre-storage (=fall) conditions of *C. fumiferana* larvae were a strong predictor of diapause survival. They also observed that larvae from warm pre-storage conditions showed lower vigor, relative to individuals from cooler pre-storage conditions, which could indicate energy deficit issues. Early diapause conditions are known to cause the highest rate of energy consumption in a range of insects and elevated energy consumption is also known to negatively impact post-diapause fitness. In the fall webworm, *Hyphantria cunea*, glycogen content drops by 60% through the fall, and remains relatively stable for the rest of the winter (Zhao et al., 2022). Similarly, shifts in fall phenology in the Propertius duskywing *Erynnis propertius* can cause a 300% change in overwintering lipid consumption, while equivalent shifts in spring phenology only cause a 150% increase (Williams et al., 2012). Bosch et al. (2010) showed that pre-winter duration was positively correlated with fat body depletion in *Osmia lignaria*, and body weight dropped dramatically during the pre-winter period. Changes to diapause intensity was posed as an alternate hypothesis for lower post-diapause emergence in *C. fumiferana* larvae exposed to longer, warmer falls (Han & Bauce, 1998). Here, the authors suggest that extended exposures to elevated fall temperatures altered the diapause intensity of overwintering larvae such that post-diapause climate conditions could not meet the diapause requirements needed to trigger development in the spring. Diapause intensity is defined as the resistance to resuming development when conditions are favourable and can be assessed using metabolic rate or responsiveness to termination conditions (Denlinger, 2022). Since we did not assess the level of diapause suppression in overwintering *C. fumiferana* larvae, it is not possible to fully verify whether this or energetic deficit were the primary cause of post-emergence failure in our treatments. Even though our glycerol content data suggests that fall temperature impact overwintering cold hardiness, larvae exposed to warm falls produced as much glycerol as those in standard rearing conditions (Fig. 2), so warm falls do not cause complete ablation of the overwintering phenotype. Tracking development, metabolic rate and glycogen consumption over time would provide much needed insight to the factors dictating overwintering survival in this species.

Given the energetic costs of early diapause, it is interesting that insects forgo feeding to enter the diapause state during this warm season. As we have demonstrated, energetic costs of early diapause are well described among insects, hence there should be strong selection to delay diapause entry as long as possible to conserve resources for long periods of inactivity (Denlinger, 2022; Joschinski & Bonte, 2021). Early entry into diapause has traditionally been seen as a risk-avoidance strategy, in which it is better to overshoot the potential onset of winter and avoid the

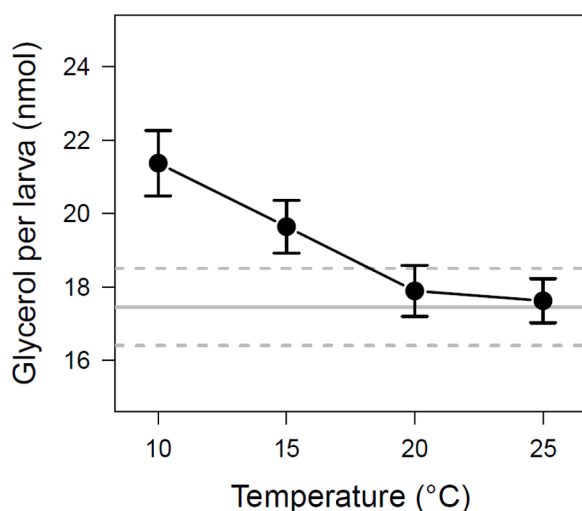
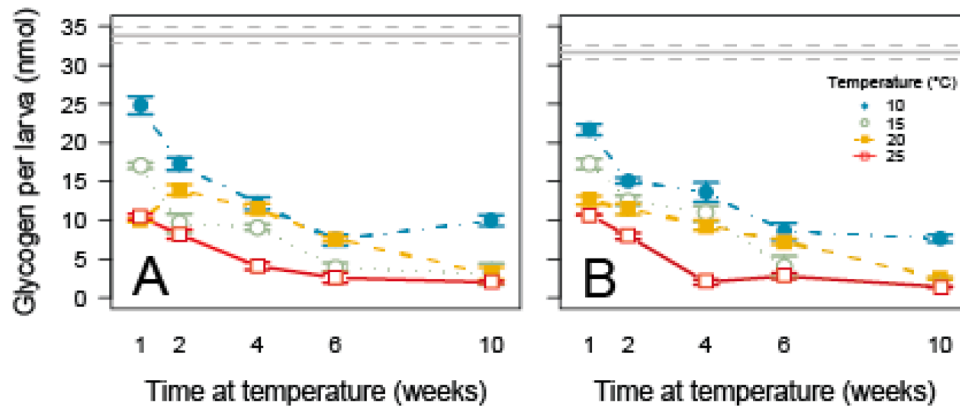
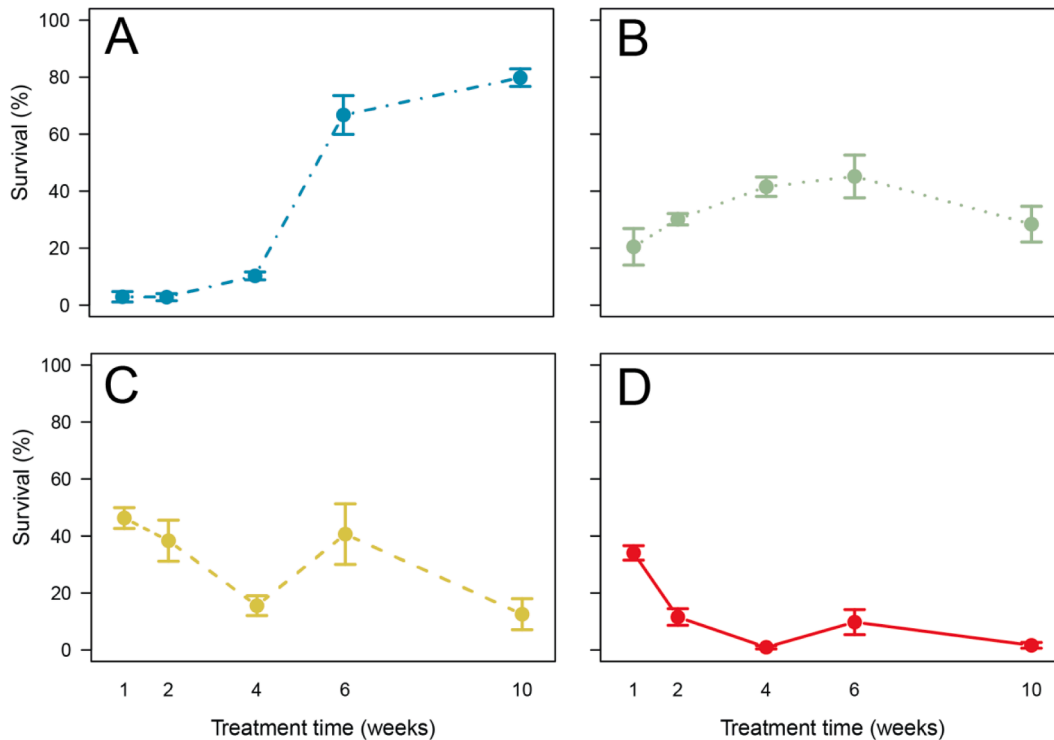


Fig. 2. Glycerol content (nmol per larva) in *C. fumiferana* was impacted by an interaction between fall duration and fall temperature ( $t = -2.257$ ,  $df = 113$ ,  $p = 0.0259$ ); (Supplemental Fig. 1), but the strongest effect was that higher temperatures generally led to lower glycerol content.  $N = 27 - 30$  groups of 10 larvae per temperature, pooled across treatments. Grey lines indicate the mean  $\pm$  SE for control larvae (T0, see details in methods).





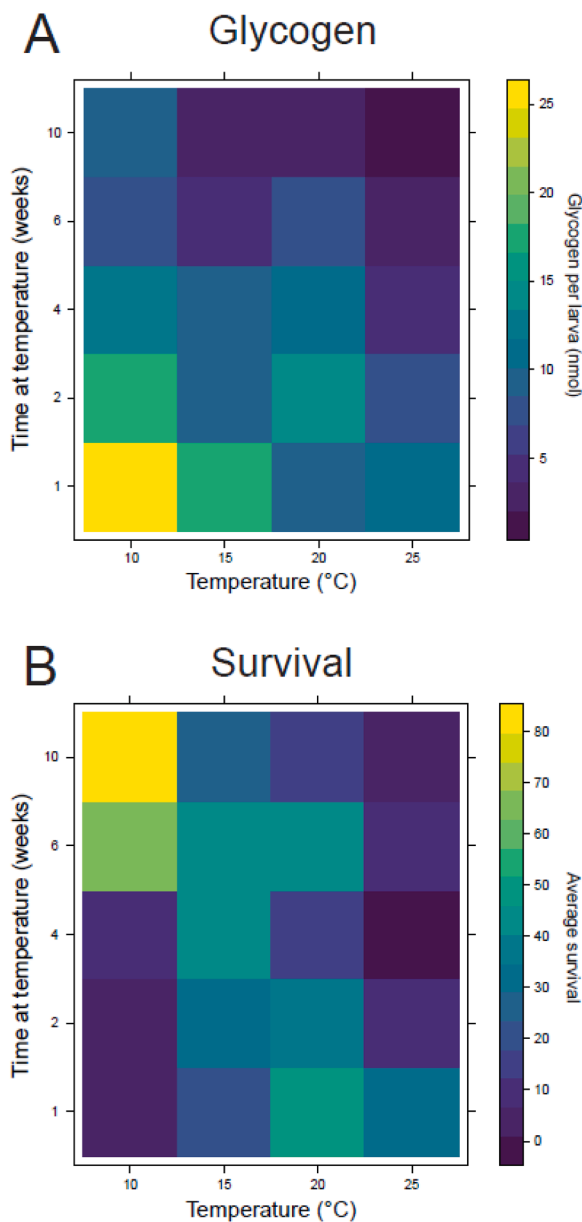
**Fig. 3.** Glycogen content (*nmol* per larva) in overwintering *C. fumiferana* is driven by the interaction of fall duration and fall temperature ( $t = 2.749$ ,  $df = 113$ ,  $p = 0.007$ ). Larvae were either sampled immediately after fall conditions ended (A) or after 9 weeks in overwintering conditions (B), but this sampling time difference had no impact on glycogen content.  $N = 3$  replicates of 10 larvae each. The larvae kept at 15°C for 10 weeks were not sampled due to the onset of the COVID-19 pandemic. Error bars represent standard error of the mean. Grey lines indicate the mean  $\pm$  SE for control larvae (T0, see details in methods).



**Fig. 4.** Overwintering survival of *C. fumiferana* is strongly driven by the interaction between fall duration and fall temperature (deviance explained = 72.93,  $df = 99$ ,  $p < 0.001$ ). A) Survival following fall temperatures of 10°C for durations from 1 - 10 weeks, B) 15°C, C) 20°C, and D) 25°C. Each point represents the mean survival of 5 replicates of 8 - 115 larvae, error bars represent standard error of the mean.

risk of being caught unprepared by cold conditions, despite the depletion of energy resources. However, empirical evidence for this evolutionary strategy is elusive (Joschinski & Bonte, 2021). If early diapause initiation is entirely driven by risk avoidance, then we would expect the highest survival in conditions that mimic short, cool falls, which would help conserve the limited energy reserves of a diapausing individual. Our results clearly contradict this expectation. While it was clear that long, warm falls impacted post-diapause fitness, we also observed extremely low emergence following a short, cool fall when glycogen was still plentiful (Fig. 5). Post-diapause emergence was instead highest when glycogen stores were partially depleted and when larvae were exposed to moderate temperatures prior to chill. Generally, it appeared that the thermal optimum for survival shifted to lower temperatures as fall duration increased: 10°C during a 10 or 6 week fall, 15°C during a 4

week fall, and 20°C during a 1 or 2 week fall (Figs. 4 and 5B). Han and Bauce (1997) observed a similar phenomenon and suggested that diapause development in *C. fumiferana* consists of two phases. They suggest that there is an early diapause window with specific temperature requirements that are needed to complete diapause. The importance of the pre-diapause period was also noted while developing and optimizing mass rearing protocols for *C. fumiferana*. Stehr (1954) and McMorran (1973) found that exposure to moderate temperatures prior to chill was needed to ensure maximal survival. In fact, Stehr noted that two to four weeks at 21.5°C was required prior to cold storage to “allow certain developmental processes to be completed” (Stehr, 1954), although at the time they did not speculate on what those processes were. Their results are in line with our own observations, adding additional evidence to the existence of key developmental processes occurring during diapause



**Fig. 5.** Glycogen content (A) and survival until spring (B) of overwintering *C. fumiferana* are driven by the interaction between fall duration and temperature, but in opposite ways. Glycogen content generally declines with increasing fall duration and temperature, while overwintering survival is lowest following either short, cool falls or long, warm falls.

initiation in this species. Taken together, we propose that an unknown, but temperature-sensitive process is occurring during the fall to prepare this species for a period of developmental arrest.

Entry into diapause requires a series of dynamic, coordinated steps to prepare an organism for a period of extended inactivity and environmental stress. Diapause initiation occurs once the appropriate life stage is reached, and growth or development ceases. Insects need to shift many internal processes away from direct development into an endogenously controlled developmental arrest. Kostal et al. (2017) show that developmental hormone signaling is inhibited during diapause initiation, leading to significant metabolic restructuring and cessation of cell division. The conditions required for diapause initiation are highly variable between species, however conditions during this phase can impact diapause intensity and survival (Kostál 2006). In fact, it has been suggested that there is a diapause development rate or thermal

optimum, wherein physiological, cellular, and molecular changes are occurring that are critical to diapause completion (Andrewartha, 1952; Johnsen et al. 1997; Toxopeus et al., 2023). For example, in *R. pomonella* there are at least two development rates (=diapause timers) with different thermal sensitivities (Toxopeus et al., 2023). We believe that *C. fumiferana* also has a temperature-sensitive diapause development rate and individuals that failed to complete diapause development during the fall cannot survive diapause.

The process of diapause initiation in *C. fumiferana* raises several intriguing questions on the physiological and molecular processes occurring during this critical physiological window. First, we know that metabolic suppression and controlled developmental arrest are key traits of diapause initiation (Denlinger, 2022), and that entry into diapause is not instantaneous (Shingleton et al., 2003; Toxopeus et al., 2023). Processes that control metabolic suppression and developmental arrest may be temperature sensitive and must be completed for *C. fumiferana* to survive extended inactivity. In *Osmia lignaria*, pupae that experienced short pre-winter treatments were unable to reach full metabolic suppression and suffered low winter survival (Bosch et al., 2010). We predict that *C. fumiferana* larvae will rapidly suppress their metabolic rate as they transition through the diapause initiation period into diapause maintenance (Stuess et al. 2023). Quantifying seasonal changes in metabolic rate in *C. fumiferana* will provide key evidence to the patterns of metabolic suppression occurring during diapause and pinpoint when suppression begins and ends. Second, we also know that *C. fumiferana* experiences dramatic changes in water content in the early weeks of diapause initiation (Han & Bauce, 1993). Reduced water content can lower the risk of freezing, as well as increase hemolymph osmolality and cryoprotectant concentrations (Storey & Storey, 2012; Teets et al., 2023). Furthermore, *C. fumiferana* prepares for the onset of cold weather by accumulating a range of cryoprotectants (Han & Bauce, 1995; Marshall & Roe, 2021) as do many other insects (Teets et al., 2023). While our treatment groups were not exposed to stressful lower temperatures (i.e. below the lower thermal limits of this life stage, see Butterson et al., 2021; Han & Bauce, 1995; Marshall & Sinclair, 2015), reducing body water and increasing cryoprotectants may help protect proteins and membranes thus allowing *C. fumiferana* to survive long periods of inactivity at low temperatures (Storey & Storey, 2012). Regulatory pathways, such as insulin or Wnt signalling (Denlinger et al. 2012; Denlinger 2022; Ragland & Keep 2017), as well as histone acetylation (Teets et al. 2023), may serve critical roles during diapause initiation in *C. fumiferana*. However, detailed speculation on the specific regulatory or transcriptional processes that may control the diapause initiation phase of this species is beyond the scope of this article, particularly given the diversity of diapause strategies and their underlying molecular mechanisms expressed within insects, as well as the limited availability of time series data over this critical diapause phase (Denlinger et al. 2012; Ragland & Keep 2017). Thus, further examination of the physiological, molecular, and regulatory processes that are functioning during the diapause initiation period of *C. fumiferana* are needed to fill this important knowledge gap.

Diapause initiation appears to be a critical period for *C. fumiferana* to prepare for the challenges of overwintering and inactivity. With our increasing understanding of *C. fumiferana* physiology, this species will be a valuable model for untangling how temperature-sensitive processes contribute to diapause and cold tolerance, as well as post-diapause fitness in a cold-adapted species. Fall temperatures impact development and diapause initiation in first and second instar larvae (Han et al., 2000) and may significantly impact winter survival, timing of emergence, and post-winter development (Han & Bauce, 1997, 1998; McMorran, 1973). Understanding the impact of fall temperatures on *C. fumiferana* fitness is essential for predicting winter survival and spring phenology in response to a changing climate. Fall conditions may have important implications on modeling post-diapause development in *C. fumiferana* and planning control operations. For example, Blais (1958) documented significant population reductions in the Shickshock

Mountains in 1957 following a cool summer and fall in the preceding year. Blais hypothesized that these cool conditions impacted the ability for *C. fumiferana* larvae to prepare for winter, contributing to the dramatic reduction in population density in the following year. Changes to population density in response to fall conditions have largely been unexplored and warrant further study.

## Conclusion

The overall impact of changing fall climates on *C. fumiferana* and other temperate species is uncertain. Elevated temperatures and longer durations of the early diapause period may impact population survival and persistence in parts of its range. Here, we show that diapause initiation and winter survival in *C. fumiferana* are significantly influenced by fall conditions. Warmer, longer falls deplete energy reserves and reduce the ability for overwintering larvae to successfully emerge in the spring. On the other hand, short, cool falls do not provide enough time for the completion of critical developmental processes. Optimal conditions for *C. fumiferana* diapause initiation exist in a balance between these two extremes, thus perturbations of this season may have widespread impact on *C. fumiferana* populations. However, to understand the broader impacts of changing fall climates on insect survival, we need to identify which physiological and molecular processes are active during diapause initiation. The diapause initiation period has received much less attention than diapause maintenance and termination (Denlinger, 2022), thus we have an opportunity to identify key processes that underpin diapause initiation, explore how these contribute to survival during winter, and provide insight as to why so many insect species enter diapause early.

## Authors' Contributions

A.D.R. and A.A.W. conceived the ideas and designed the methodology; A.A.W. and S.B. collected the data; K.M. and A.D.R. analysed and interpreted the data; A.D.R. and K.M. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

## Statement on Inclusion

We bring together collaborators from different countries, including scientists from where the study was conducted. All authors were engaged early on with the research to ensure that diverse sets of perspectives were considered. We cite literature published by other local scientists from the region.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Amanda D. Roe and Katie E. Marshall are Editorial Board members of the journal.

## Data availability

Data available from the Open Science Framework ([https://osf.io/7ns4t/?view\\_only=da34563a92a64015b012603212b73bae](https://osf.io/7ns4t/?view_only=da34563a92a64015b012603212b73bae)).

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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.2024.100073](https://doi.org/10.1016/j.cris.2024.100073).

## References

- Andrewartha, H.G., 1952. Diapause in relation to the ecology of insects. *Biological Reviews* 27 (1), 50–107. <https://doi.org/10.1111/j.1469-185X.1952.tb01363.x>.
- Blais, J.R., 1958. Affects of 1956 spring and summer temperatures on spruce budworm populations (*Choristoneura fumiferana* Clem.) in the Gaspé Peninsula. *The Canadian Entomologist* 90, 354–361.
- Bosch, J., Sgolastra, F., Kemp, W.P., 2010. Timing of eclosion affects diapause development, fat body consumption and longevity in *Osmia lignaria*, a univoltine, adult-wintering solitary bee. *Journal of Insect Physiology* 56 (12), 1949–1957. <https://doi.org/10.1016/j.jinsphys.2010.08.017>.
- Butterson, S., Roe, A.D., Marshall, K.E., 2021. Plasticity of cold hardiness in the eastern spruce budworm, *Choristoneura fumiferana*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 259, 110998. <https://doi.org/10.1016/j.cbpa.2021.110998>.
- Candau, J.-N., Dedes, J., MacQuarrie, C.J.K., Perrault, K., Roe, A.D., Wardlaw, A., 2018. Validation of a spruce budworm phenology model across environmental and genetic gradients: Applications for budworm control and climate change predictions. *Information Report GLC-X-20 (GLX-C-20)*. <https://cfs.nrcan.gc.ca/publications?id=39098>.
- Danks, H.V., 1987. *Insect dormancy: An ecological perspective*. *Biological Survey of Canada (Terrestrial Arthropods)*: Available from the Entomological Society of Canada.
- Denlinger, D.L., 2022. *Insect Diapause*. Cambridge University Press. <https://doi.org/10.1017/9781108609364>.
- Denlinger, D.L., Yocum, G.D., Rinehart, J.P., 2012. Hormonal control of diapause. In: Gilbert, L.I. (Ed.), *Insect Endocrinology*. Academic Press, San Diego, pp. 430–463.
- Ebling, P.M., Dedes, J., 2015. Rearing diapause *Choristoneura fumiferana*. Standard Operating Procedure IPS/003/003. <http://cfs.nrcan.gc.ca/publications?id=35979>.
- Eveleigh, E.S., Johns, R.C., 2014. Intratree variation in the seasonal distribution and mortality of spruce budworm (Lepidoptera: Tortricidae) from the peak to collapse of an outbreak. *Annals of the Entomological Society of America* 107 (2), 435–444. <https://doi.org/10.1603/AN13136>.
- Forister, M.L., Halsch, C.A., Nice, C.C., Fordyce, J.A., Dilts, T.E., Oliver, J.C., Prudic, K.L., Shapiro, A.M., Wilson, J.K., Glassberg, J., 2021. Fewer butterflies seen by community scientists across the warming and drying landscapes of the American West. *Science* 371 (6533), 1042–1045. <https://doi.org/10.1126/science.abe5585>.
- Gallinat, A.S., Primack, R.B., Wagner, D.L., 2015. Autumn, the neglected season in climate change research. *Trends in Ecology & Evolution* 30 (3), 169–176. <https://doi.org/10.1016/j.tree.2015.01.004>.
- Glazaczow, A., Orwin, D., Bogdziewicz, M., 2016. Increased temperature delays the late-season phenology of multivoltine insect. *Scientific Reports* 6 (1). <https://doi.org/10.1038/srep38022>. Article 1.
- Gray, D.R., 2013. The influence of forest composition and climate on outbreak characteristics of the spruce budworm in eastern Canada. *Canadian Journal of Forest Research* 43 (12), 1181–1195. <https://doi.org/10.1139/cjfr-2013-0240>.
- Grisdale, D., McMorran, A.R., 1963. Rearing insects on artificial diet. *Canadian Forest Service*. <http://cfs.nrcan.gc.ca/publications?id=37015>.
- Hahn, D.A., Denlinger, D.L., 2011. Energetics of insect diapause. *Annual Review of Entomology* 56 (1), 103–121. <https://doi.org/10.1146/annurev-ento-112408-085436>.
- Han, E.-N., Bauce, E., 1993. Physiological changes and cold hardiness of spruce budworm larvae, *Choristoneura fumiferana* (Clem.), during pre-diapause and diapause development under laboratory conditions. *The Canadian Entomologist* 125 (6), 1043–1053. <https://doi.org/10.4039/Ent1251043-6>.
- Han, E.-N., Bauce, E., 1995. Non-freeze survival of spruce budworm larvae, *Choristoneura fumiferana*, at sub-zero temperatures during diapause. *Entomologia Experimentalis et Applicata* 75 (1), 67–74. <https://doi.org/10.1111/j.1570-7458.1995.tb01911.x>.
- Han, E.-N., Bauce, E., 1997. Effects of early temperature exposure on diapause development of spruce budworm (Lepidoptera: Tortricidae). *Environmental Entomology* 26 (2), 307–310. <https://doi.org/10.1093/ee/26.2.307>.
- Han, E.-N., Bauce, E., 1998. Timing of diapause initiation, metabolic changes and overwintering survival of the spruce budworm, *Choristoneura fumiferana*. *Ecological Entomology* 23 (2), 160–167. <https://doi.org/10.1046/j.1365-2311.1998.00111.x>.
- Han, E.-N., Bauce, E., Trempe-Bertrand, F., 2000. Development of the first-instar spruce budworm (Lepidoptera: Tortricidae). *Annals of the Entomological Society of America* 93 (3), 536–540. [https://doi.org/10.1603/0013-8746\(2000\)093\[0536:DOTFIS\]2.0.CO;2](https://doi.org/10.1603/0013-8746(2000)093[0536:DOTFIS]2.0.CO;2).
- Harvey, G.T., 1957. The occurrence and nature of diapause-free development in the spruce budworm, *Choristoneura fumiferana* (Clem.) (Lepidoptera: Tortricidae). *Canadian Journal of Zoology* 35 (4), 549–572. <https://doi.org/10.1139/z57-047>.
- Hervet, V.A.D., Laird, R.A., Floate, K.D., 2016. A review of the McMorran diet for rearing Lepidoptera species with addition of a further 39 species. *Journal of Insect Science* 16 (1), 19. <https://doi.org/10.1093/jisesa/iev151>.

- Irwin, J.T., Lee Jr., R.E., 2003. Cold winter microenvironments conserve energy and improve overwintering survival and potential fecundity of the goldenrod gall fly. *Eurosta solidaginis*. *Oikos* 100 (1), 71–78. <https://doi.org/10.1034/j.1600-0706.2003.11738.x>.
- Jenni, L., Kéry, M., 2003. Timing of autumn bird migration under climate change: Advances in long-distance migrants, delays in short-distance migrants. Proceedings of the Royal Society of London. Series B: Biological Sciences 270 (1523), 1467–1471. <https://doi.org/10.1098/rspb.2003.2394>.
- Johnsen, S., Gutierrez, A.P., Jorgensen, J., 1997. Overwintering in the cabbage root fly *Delia radicum*: a dynamic model of temperature-dependent dormancy and post-dormancy development. *Journal of Applied Ecology* 34, 21–28.
- Joschinski, J., Bonte, D., 2021. Diapause and bet-hedging strategies in insects: A meta-analysis of reaction norm shapes. *Oikos* 130 (8), 1240–1250. <https://doi.org/10.1111/oik.08116>.
- Košťál, V., 2006. Eco-physiological phases of insect diapause. *Journal of Insect Physiology* 52 (2), 113–127. <https://doi.org/10.1016/j.jinsphys.2005.09.008>.
- Košťál, V., Štětina, T., Poupardin, R., Korbelová, J., Bruce, A.W., 2017. Conceptual framework of the eco-physiological phases of insect diapause development justified by transcriptomic profiling. Proceedings of the National Academy of Sciences 114. <https://doi.org/10.1073/pnas.1707281114>, 201707281–201707281.
- Liu, E.Y., Lantz, V.A., MacLean, D.A., Hennigar, C., 2019. Economics of early intervention to suppress a potential spruce budworm outbreak on crown land in New Brunswick, Canada. *Forests* 10 (6). <https://doi.org/10.3390/f10060481>. Article 6.
- Marshall, K.E., Gotthard, K., Williams, C.M., 2020. Evolutionary impacts of winter climate change on insects. *Current Opinion in Insect Science* 41, 54–62. <https://doi.org/10.1016/j.cois.2020.06.003>.
- Marshall, K.E., Roe, A.D., 2021. Surviving in a frozen forest: The physiology of eastern spruce budworm overwintering. *Physiology* 36 (3), 174–182. <https://doi.org/10.1152/physiol.00037.2020>.
- Marshall, K.E., Sinclair, B.J., 2015. The relative importance of number, duration and intensity of cold stress events in determining survival and energetics of an overwintering insect. *Functional Ecology* 29 (3), 357–366. <https://doi.org/10.1111/1365-2435.12328>.
- Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.-I., Lee, Y.C., 2005. Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. *Analytical Biochemistry* 339 (1), 69–72. <https://doi.org/10.1016/j.ab.2004.12.001>.
- McMorran, A., 1965. A synthetic diet for the spruce budworm, *Choristoneura fumiferana* (Clem.) (Lepidoptera: Tortricidae). *The Canadian Entomologist* 97 (1), 58–62. <https://doi.org/10.4039/Ent9758-1>.
- McMorran, A., 1973. Effects of pre-storage treatment on survival of diapausing larvae of the spruce budworm. *The Canadian Entomologist* 105 (7), 1005–1009. <https://doi.org/10.4039/Ent1051005-7>.
- Menzel, A., Sparks, T.H., Estrella, N., Koch, E., Aasa, A., Ahas, R., Alm-Kubler, K., Bissolli, P., Braslavská, O., Briede, A., Chmielewski, F.M., Crepinsek, Z., Curnel, Y., Dahl, Å., Defila, C., Donnelly, A., Filella, Y., Jatzcak, K., Måge, F., Zust, A., 2006. European phenological response to climate change matches the warming pattern. *Global Change Biology* 12 (10), 1969–1976. <https://doi.org/10.1111/j.1365-2486.2006.01193.x>.
- Meyers, P.J., Doellman, M.M., Ragland, G.J., Hood, G.R., Egan, S.P., Powell, T.H.Q., Nosil, P., Feder, J.L., 2020. Can the genomics of ecological speciation be predicted across the divergence continuum from host races to species? A case study in *Rhagoletis*. *Philosophical Transactions of the Royal Society B: Biological Sciences* 375 (1806), 20190534. <https://doi.org/10.1098/rstb.2019.0534>.
- Nealis, V.G., Régnière, J., 2016. Why western spruce budworms travel so far for the winter. *Ecological Entomology* 41 (5), 633–641. <https://doi.org/10.1111/een.12336>.
- Nielsen, M.E., Lehmann, P., Gotthard, K., 2022. Longer and warmer prewinter periods reduce post-winter fitness in a diapausing insect. *Functional Ecology* 36 (5), 1151–1162. <https://doi.org/10.1111/1365-2435.14037>.
- Perrault, K., Wardlaw, A.A., Candau, J.N., Irwin, C.L., Demidovich, M., MacQuarrie, C.J. K., Roe, A.D., 2021. From branch to bench: Establishing wild spruce budworm populations into laboratory colonies for the exploration of local adaptation and plasticity. *The Canadian Entomologist* 153 (3), 374–390. <https://doi.org/10.4039/tce.2021.1>.
- Ragland, G.J., Keep, E., 2017. Comparative transcriptomics support evolutionary convergence of diapause responses across Insecta. *Physiological Entomology* 42 (3), 246–256. <https://doi.org/10.1111/phen.12193>.
- Schebeck, M., Hansen, E.M., Schopf, A., Ragland, G.J., Stauffer, C., Bentz, B.J., 2017. Diapause and overwintering of two spruce bark beetle species. *Physiological Entomology* 42 (3), 200–210. <https://doi.org/10.1111/phen.12200>.
- Shingleton, A.W., Sisk, G.C., Stern, D.L., 2003. Diapause in the pea aphid (*Acyrtosiphon pisum*) is a slowing but not a cessation of development. *BMC Developmental Biology* 3, 7. <https://doi.org/10.1186/1471-213X-3-7>.
- Stehr, G., 1954. A laboratory method for rearing the spruce budworm, *Choristoneura fumiferana* (Clem.), (Lepidoptera: Tortricidae). *The Canadian Entomologist* 86 (9), 423–428. <https://doi.org/10.4039/Ent86423-9>.
- Storey, K.B., Storey, J.M., 2012. Insect cold hardiness: Metabolic, gene, and protein adaptation. *Canadian Journal of Zoology* 90 (4), 456–475. <https://doi.org/10.1139/z2012-011>.
- Süess, P., Roberts, K.T., Lehmann, P., 2023. Temperature dependence of gas exchange patterns shift as diapause progresses in the butterfly *Pieris napi*. *Journal of Insect Physiology* 151, 104585. <https://doi.org/10.1016/j.jinsphys.2023.104585>.
- Tauber, M.J., Tauber, C.A., Masaki, S., 1986. *Seasonal Adaptations of Insects*. Oxford University Press.
- Taylor, F., Spalding, J.B., 1989. Timing of diapause in relation to temporally variable catastrophes. *Journal of Evolutionary Biology* 2 (4), 285–297. <https://doi.org/10.1046/j.1420-9101.1989.2040285.x>.
- Teets, N.M., Marshall, K.E., Reynolds, J.A., 2023. Molecular mechanisms of winter survival. *Annual Review of Entomology* 68 (1), 319–339. <https://doi.org/10.1146/annurev-ento-120120-095233>.
- Toxopeus, J., Dowle, E.J., Andaloori, L., Ragland, G.J., 2023. Variation in thermal sensitivity of diapause development among individuals and over time drives life history timing patterns in an insect pest (p. 2023.05.31.543112). *bioRxiv*. <https://doi.org/10.1101/2023.05.31.543112>.
- Van Buskirk, J., Mulvihill, R.S., Leberman, R.C., 2009. Variable shifts in spring and autumn migration phenology in North American songbirds associated with climate change. *Global Change Biology* 15 (3), 760–771. <https://doi.org/10.1111/j.1365-2486.2008.01751.x>.
- Van Dyck, H., Bonte, D., Puls, R., Gotthard, K., Maes, D., 2015. The lost generation hypothesis: Could climate change drive ectotherms into a developmental trap? *Oikos* 124 (1), 54–61. <https://doi.org/10.1111/oik.02066>.
- Venables, W.N., Ripley, B.D., 2002. *Modern Applied Statistics with S*. Springer. <https://doi.org/10.1007/978-0-387-21706-2>.
- Williams, C.M., Marshall, K.E., MacMillan, H.A., Dzurisin, J.D.K., Hellmann, J.J., Sinclair, B.J., 2012. Thermal variability increases the impact of autumnal warming and drives metabolic depression in an overwintering butterfly. *PLOS ONE* 7 (3), e34470. <https://doi.org/10.1371/journal.pone.0034470>.
- Zhao, L., Wang, X., Liu, Z., Torson, A.S., 2022. Energy consumption and cold hardiness of diapausing fall webworm pupae. *Insects* 13 (9), 853. <https://doi.org/10.3390/insects13090853>.