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Research Article

Cold adaptation strategies in lab-reared European grapevine moth *Lobesia botrana*: Exploring diapause induction, supercooling point, and cold hardiness

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Abstract: The European grapevine moth, *Lobesia botrana* (Denis and Schiffermueller) (Lepidoptera: Tortricidae), is a significant pest causing economic damage to vineyards worldwide. In this research, the cold tolerance of the pupae and its relationship with diapause was investigated at 23 ± 0.5 °C, 70 ± 5% RH, and LD 12:12 h. One-day-old eggs were transferred to LD 12:12 h to induce diapause at the pupal stage. Diapausing pupae exhibited a mean supercooling point (SCP) of -24.35 °C, whereas in the non-diapausing pupae (23 ± 0.5 °C, 70 ± 5% RH, LD 16:8 h), it was -23.06 °C, with no significant difference between the two groups. Furthermore, diapausing pupae demonstrated significantly higher cold tolerance (LT_{50} of -14.43 °C) than non-diapausing pupae (LT_{50} of -3.33 °C). Diapausing pupae tolerated subzero temperatures without significant changes in the SCP, tolerating 11 °C lower than control pupae due to the short daylength alone. Our results suggest that the diapause state and cold hardiness of *L. botrana* are independent of changes after SCP, and the insect employs a freeze-intolerant strategy to overcome subzero temperatures. Cold acclimation at -5 and -10 °C for 72 h induced a significant decrease in the SCP of diapausing pupae, while a 72-h cold acclimation had no notable impact on the SCP of non-diapausing pupae. These findings provide valuable insights into the survival mechanisms of the European grapevine moth under cold conditions and diapause-related adaptations.

Keywords: European grapevine moth, diapause induction, cold hardiness, supercooling point, tolerance

Introduction

Diapause, a stage-specific developmental arrest, is widely exploited by insects to bridge unfavorable seasons (Denlinger, 2023). Insects employ diverse survival strategies to thrive in challenging environmental conditions, with diapause and prolonged developmental arrest as prominent adaptations for synchronized life cycle progression (Hodek, 2012). Among the pivotal cues for arranging diapause are photoperiod and temperature, regulating its induction and termination (Denlinger, 2002; Denlinger, 2008). This intricate interplay carries ecological and agricultural implications, particularly for insects like the European grapevine moth *Lobesia*

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botrana (Denis and Schiffermueller) (Lepidoptera: Tortricidae), a notorious vineyard pest (Ioriatti *et al*., 2011). The grapevine pest causes substantial damage to vineyards worldwide. It is a polyvoltine species that produces from two to four generations per year, depending on the geographical area and microclimate (Ioriatti *et al*., 2011; Pavan *et al*., 2013; Rank *et al*., 2020). The insect has a wide geographical distribution, including Europe, North Africa, the Middle East, and West Asia (Thiéry *et al*., 2014). Native to Europe, it has gathered attention due to its economic impact on grape production and the subsequent challenges in devising effective management strategies. The complex relationship between *L. botrana* and its host plants involves complex interactions directed by vine phenology, temperature, and photoperiod (Ioriatti *et al*., 2012; Pavan *et al*., 2014).

Photoperiod is a critical environmental cue influencing diapause induction in insects (Danks, 1987; Koštál, 2006; Lee, 2010; Andreadis and Athanassiou, 2017; Denlinger, 2023). For *L. botrana*, the interplay between photoperiod and temperature has been recognized as a determinant of its life cycle timing and diapause behavior (Bale, 1996; Ioriatti *et al*., 2012; Ioriatti *et al*., 2023). Shortening photoperiods are linked to the initiation of diapause in pupal stages, which is crucial for the moth's survival during unfavorable conditions (Denlinger, 2023). Understanding the complex photoperiodic response in *L. botrana* and its intersection with cold adaptation is crucial for the underlying mechanisms governing its seasonal dynamics. The supercooling point (SCP) acts as a critical physiological parameter reflecting an insect's ability to withstand cold temperatures by preventing ice formation within its body (Storey and Storey, 2013; Andreadis and Athanassiou, 2017; Lee *et al*., 2019; Li *et al*., 2020). Diapause has been associated with shifts in SCP in various insects (Sinclair *et al*., 2003; Hahn and Denlinger, 2007; Koštál *et al*., 2016). In the context of *L. botrana*, inquiring into the SCP alterations during diapause promises insights into its cold adaptation strategies and the potential role of SCP as a critical trait influencing its overwintering success. In cold regions, *L. botrana* has demonstrated the ability to supercool and withstand subzero temperatures (Andreadis *et al*., 2005; Masoudmagham *et al*., 2021). This ability has been linked to cryoprotectants and modifications in cellular structures that inhibit ice nucleation (Khani and Moharramipour, 2010; Andreadis and Athanassiou, 2017). Understanding the supercooling capacity of *L. botrana* is crucial as it influences the insect's ability to withstand temperature fluctuations and overwintering conditions. (Andreadis *et al*., 2005).

Our study investigates the influence of cold acclimation on the SCP of induced diapause pupae. Cold acclimation, involving gradual temperature reductions, confers enhanced insect cold tolerance (Storey and Storey, 1988). The synergistic effects of photoperiodic diapause response and cold acclimation on SCP represent an avenue of inquiry with implications for understanding the adaptability of *L. botrana* to fluctuating environmental conditions. During cold acclimation, *L. botrana* pupae undergo physiological changes that involve adjustments in metabolism, accumulation of cryoprotectants, and modification of cell membrane fluidity (Rozsypal and Kostál., 2018; Masoudmagham *et al*., 2021). These adaptations enable the insect to better cope with extreme cold conditions and contribute to its capacity to endure sub-freezing temperatures (Hemmati *et al*., 2017). As part of the insect physiology and adaptation field, our study aims to elucidate the complicated link between photoperiodic cues, diapause induction, and cold acclimation on SCP in *L. botrana* pupae under controlled laboratory conditions. We address key questions: How does photoperiod influence diapause induction in *L. botrana* pupae under short daylight conditions at room temperature (23 ± 0.5 °C). To what extent does cold acclimation influence the SCP of *L. botrana* pupae in induced diapause? How does the interplay between photoperiodic diapause response and cold acclimation influence the overall cold hardiness of *L. botrana* pupae? Our research seeks to illuminate the dynamics leading to photoperiodic diapause response, cold acclimation, and SCP in *L. botrana* pupae. Furthermore, we investigated the role of cold acclimation in shaping the SCP

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and LT_{50} of diapausing and nondiapausing pupae. This study contributes to our understanding of the adaptability of insects to changing environments, enhancing insect-environment interactions.

Materials and Methods

Insect rearing

Mass rearing of *L. botrana* was conducted within a controlled climatic chamber, maintaining a temperature of 23 ± 0.5 °C and a relative humidity of 70 ± 5%. A balanced photoperiod of 16 hours of light to 8 hours of darkness (L: D) was sustained to mimic natural light cycles. The larvae were nourished through an artificial diet formulated during this process. This diet was developed based on the work of Rapagnani *et al*. (1990), although with particular modifications for optimization. To ensure a consistent supply of nourishment, a monthly production of approximately 500 g of the modified artificial diet was prepared. The exact formulation details can be found in Table 1. This diet served as the sole sustenance for the rearing insects throughout their developmental stages. The initial larvae were sourced from a vineyard in Iran's Qazvin Province, specifically, the region of Takestan at coordinates 36°01'37 "N, 49°42'18 "E. Fresh larvae were periodically collected from the same vineyard and carefully introduced into square plastic containers measuring 20 × 20 × 5 cm. A thorough disinfection process involving 70% ethanol was consistently applied to hands, containers, and tools before any interactions to ensure a clean and sterile rearing environment. This method provided the successful mass rearing of *L. botrana*, maintaining genetic diversity and a hygienic rearing environment.

The induction of diapause in pupae at room temperature by short daylength

To generate diapausing pupae in *L. botrana*, we employed a method involving exposure to reduced daylength at 23 ± 0.5 °C. The European grapevine moth exhibits a facultative pupal diapause in which short daylengths (< 13h) during egg and larval stages induce very high incidences of pupal diapause (Roditakis and Karandinos, 2001). Eggs of *L. botrana* were reared under controlled conditions at 23 °C.

Table 1 The artificial diet formula was prepared based on the formula of Rapignani *et al*. (1990), with modifications to prepare approximately 500 g of artificial diet for the mass rearing of *Lobesia botrana*.

Ingredients	Quantity	Unit	Remarks*
Acetic acid	1.20	ml	3
Agar	12.20	g	1
Alfalfa powder	12.20	g	2
Ascorbic acid	9.75	g	3
Casein	21.45	g	2
Cholesterol	0.60	g	3
Grape sap	10.00	ml	3
Linolenic acid	1.00	g	3
Multivitamin mix	3.00	ml	3
Propionic acid	1.25	ml	3
Sorbic acid	0.98	g	3
Sucrose	18.10	g	2
Tetracycline 250	2.00	pill	3
Vegetable oil	1.20	ml	3
Water	360.00	ml	1
Wesson salt	0.32	g	2
Wheat germ	45.40	g	2
Yeast extract	9.30	g	2

* The materials listed as number 1 were added to the mixture at the beginning and when the water was boiling, and materials numbered 2 and 3 were added to the mixture at 70 and 50 °C, respectively.

A short daylength cycle was established to initiate the diapause induction process, consisting of 12 h light followed by 12 h of darkness. This modified photoperiod was chosen based on prior research findings that indicated the first instar larvae as the critical developmental stage for effectively inducing diapause in *L. botrana* (Roditakis and Karandions, 2001). This approach was chosen to mimic the natural conditions that trigger diapause in response to changing daylengths. By changing the photoperiod during the early stages of development (LD 12: 12 h), we aimed to replicate the environmental cues that prompt the insect to enter a state of diapause. Using LD 12: 12 h and controlled temperature settings ensured a controlled environment conducive to our

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study's objectives. This method was designed to investigate how diapause induction occurs under specific conditions, providing valuable insights into the physiological and developmental mechanisms underlying diapause in *L. botrana*. Focusing on the first instar larvae as the critical stage for diapause induction, we aimed to alter daylength such that leads to subsequent formation of diapausing pupae.

Recognition of diapause-induced pupae

Diapausing pupae exhibit certain morphological traits that are distinct from nondiapausing pupae. These traits could involve changes in coloration, size, or other external features. For *L. botrana*, recognizing diapausing pupae could involve a combination of morphological and developmental factors. Diapausing pupae exhibit changes in eye coloration compared to nondiapausing pupae. According to the studies of Roditakis and Karandions (2001), in nondiapausing pupae, the color of the eyes turns dark red after a few hours, while in pupae that have gone to diapause, the color of the eyes does not change and remains light green. We used this criterion to distinguish diapausing from nondiapausing pupae. Nondiapausing pupae could have a slightly lighter appearance that sets them apart from diapausing pupae. Diapausing pupae might also display an extended development time that doubles that of nondiapausing pupae.

Cold acclimation effects on diapausing and nondiapausing pupae

The impact of cold acclimation on the SCP and the cold hardiness of diapausing and nondiapausing pupae was investigated under laboratory conditions. This acclimation spanned 72 hours at +5, 0, -5, and -10 °C. Each temperature included 20 pupae. The experiments were performed with five replicates. This experiment assesses the shifts in SCP of diapause and nondiapausing pupae. Each pupa of diapausing and nondiapausing (5 days after pupation) was immobilized on a 2 × 3 cm plastic tape. Then, the ventral surface of the abdomen was attached to a thermocouple with a NiCr-Ni probe, connected to an automatic temperature recorder (Measurement Computing, model USB-5203, USA). The test chamber (Binder, model MK53; Tuttlingen, Germany) was programmed to gradually decrease the temperature at a controlled rate of 0.05 °C/min until reaching the target temperature. The cooling was recorded at 5 s intervals with an eight-channel data logger, and data were read in the computer (Khani and Moharramipour 2010) (Fig. 1).

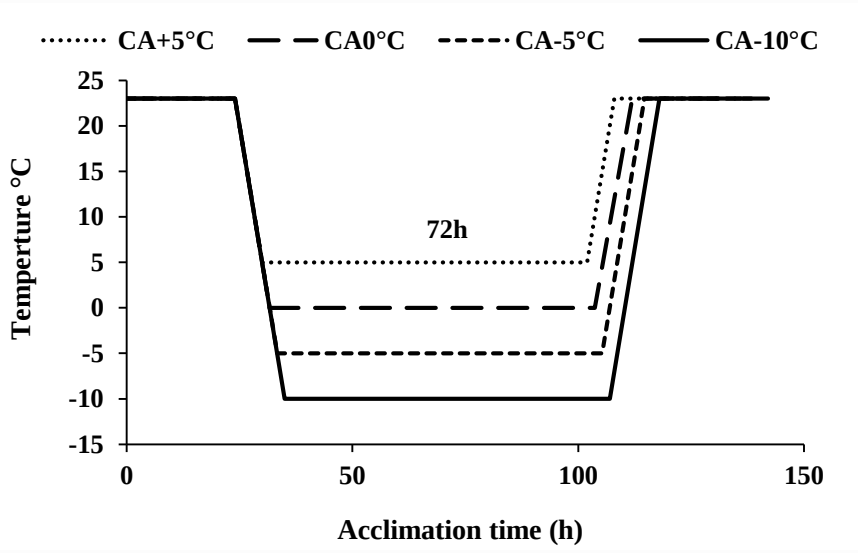

Figure 1 Experimental protocols for cold acclimation of pupae of *Lobesia botrana*. The pupae were acclimated for 72 h at 5, 0, -5, and -10 °C.

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Measurement of supercooling point

Individually, each pupa (five days after pupation), whether in the nondiapausing or diapausing stage, was secured onto a 2 x 3 cm plastic band post-pupation (5 days for diapausing pupae). This assembly was then equipped with a precision thermocouple. The thermocouple's NiCr-Ni probe was strategically attached to the ventral surface of the abdomen. This setup was connected to an advanced automatic temperature recorder (Measurement Computing, model USB-5203, USA) to ensure accurate data acquisition. Once the thermocouple arrays were configured, they were promptly transferred into a specialized test chamber (Binder, model MK53; Tuttlingen, Germany). A controlled cooling process was initiated inside this environment, gradually reducing the temperature at a controlled rate of 0.5 °C/min. This cooling procedure was tracked at 5-second intervals through an eight-channel data logger (Khani and Moharramipour, 2010). The primary objective was to determine the mean SCP across 50 pupae, encompassing both the nondiapausing and diapausing conditions. The SCP of each pupa was documented as the lowest temperature immediately before a distinct temperature surge – an event caused by the liberation of latent heat of crystallization (Lee, 1991).

Mortality at low temperatures

The cooling commenced at 23 °C, with exposure temperatures ranging from +5 to -22 °C. A range of +5, +3, 0, -3, -5, -7, -10, -12, -15, -17, -20 and -22 °C were used to find mortality between 0 and 100%. Executing this experiment involved five separate replicates, with each replicate containing a group of 20 pupae. These pupal groups were placed within Eppendorf tubes and a programmable test chamber (Binder, model MK53; Tuttlingen, Germany). The test chamber was programmed to gradually decrease the temperature at a controlled rate of 0.5 °C/min until reaching the target temperature. Diapausing and nondiapausing pupae were subjected to each temperature condition for 24 hours. Subsequently, a gradual rewarming at a rate of 0.5 °C/min brought the pupae back to the initial temperature of 23 °C, where they were maintained for an additional 24 hours. After these controlled treatments, the pupae in the Eppendorf tubes were carefully reintroduced to their original conditions. Live and dead pupae were accurately counted. Pupae were classified as dead if they failed to undergo adult emergence even after untreated pupae had completed their emergence. This criterion was complemented by visual cues such as the darker and dehydrated appearance of dead pupae. Pupae that did not give rise to adults, even upon the passage of time, were also classified as dead following the criteria established by Hemmati *et al*. (2017). In addition, a 2-hour survival/mortality test was conducted in the laboratory and natural conditions (insects collected from nature in January) to compare the results with those of previous studies conducted by Andreadis *et al*. (2005). The median lethal temperature (LT_{50}) was determined following the abovementioned mortality assay.

Statistical analysis

The distinct variations in SCP between diapausing and nondiapausing pupae were compared using the independent t-student test. This analysis was conducted to identify any statistically significant disparities between these two states. A binary logistic regression model was used to explore how pupal death rates vary with different temperatures and to find the temperature that causes half of the pupae to die, called the LT_{50} (Saeidi *et al*., 2012). A non−significant chi-square outcome signified the unity of the collected data with the established model. Furthermore, to investigate potential associations between the LT_{50} values of diapausing and nondiapausing pupae, a Mantel Haenszel test was executed. This test aimed to ascertain the existence of any discernible relationships within these mortality indicators. A nonzero correlation was chosen within this analytical framework as a default parameter within the SPSS program. Crucially, the analysis treated individual pupae as distinct experimental units within the Mantel Haenszel test. This approach ensured the robustness of the statistical inferences drawn from the model. All data analyses were conducted using SPSS 26

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(IBM), establishing a solid foundation for the comprehensive interpretation of the results and contributing to the scientific rigor of the study's findings.

Results

Diapause induction at room temperature

A photoperiod of 12:12; L:D h induced diapause in *L. botrana* pupae, even at 23 °C. When the first instar larvae were exposed to this altered photoperiod, over 96% of the population entered diapause during the pupal stage. Subsequently, we examined these diapause-induced pupae, assessing their SCP and cold hardiness across both non-acclimated and subzero acclimated conditions.

Supercooling points of diapausing and nondiapausing pupae

Our research investigated the effects of photoperiod-induced diapause on the SCPs of diapausing pupae in *L. botrana*. We observed the relationship between diapause induction, cold acclimation, and SCP values. Our results illuminated that diapause induction through daylength alterations did not substantially impact the SCPs of diapausing pupae when they were not subjected to cold exposure. The mean SCP was -24.35 °C for diapausing pupae and -23.06 °C for nondiapausing pupae. The SCP values of diapausing pupae were not significantly different from those of nondiapausing pupae ($t = -1.997$, $P = 0.056$). Under these conditions, we noted a marginal disparity between these two groups.

Supercooling point variation with cold acclimation

In nondiapausing pupae, the SCP slightly increased from -22.96 °C to -21.90 °C as temperatures descended from 5 °C to -5 °C. However, this shift did not achieve statistical significance. In contrast, diapausing pupae exhibited a more pronounced decline in SCP, reducing from -24.66 °C to -25.89 °C, and this alteration proved to be statistically significant. Moreover, the results highlighted a difference in SCP between diapausing and nondiapausing pupae across all temperatures. Diapausing pupae consistently showed a lower SCP compared to their nondiapause counterparts, with this distinction achieving statistical significance at every temperature point examined (Fig. 2). At -10 °C, all nondiapausing pupae died and are not shown in Fig. 2. According to results, the temperature of -25.89 °C was the lowest SCP in diapausing pupae.

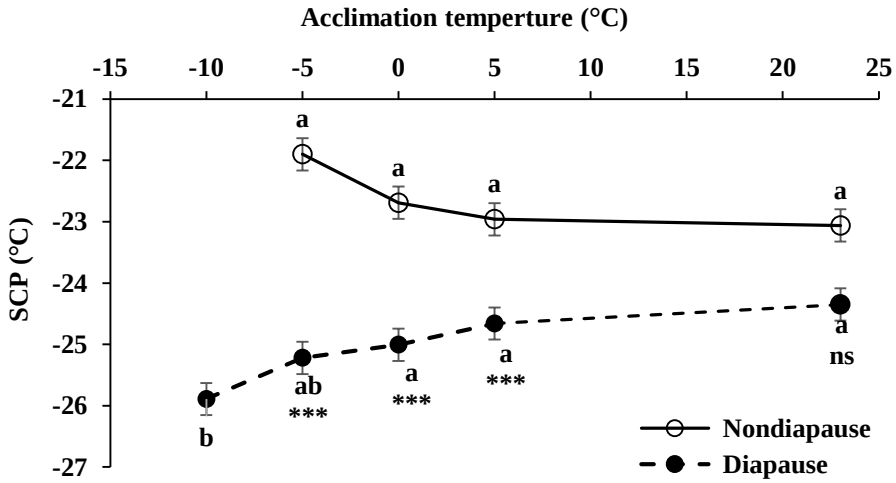

Figure 2 Effects of the cold acclimation of diapausing and non-diapausing *Lobesia botrana* pupae for 72 h on supercooling point (SCP). The non-diapausing pupae could not tolerate -10 °C and died before the SCP determination. Means with the same letters in each line indicate no significant differences among treatments at $P \leq 0.05$ by Tukey's test after ANOVA. ns and *** indicate non-significant and significant differences between SCP of diapausing and non-diapausing pupae at $P \leq 0.001$ using independent sample t-student test.

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These findings highlight the significant influence of cold acclimation in modulating SCP within diapausing pupae.

Cold hardiness in diapausing pupae

The LT_{50} values revealed a distinct pattern between diapausing and nondiapausing pupae, indicating their differential susceptibility to low temperatures. The LT_{50} for diapausing pupae was markedly lower than that of their nondiapausing counterparts, with values of -14.43 and -3.33 °C for 24-hour cold exposure. This difference was highly significant, as affirmed by the Mantel-Haenszel Chi-Square analysis ($\chi^2 = 32.992$; df = 1; $p < 0.0001$). The LT_{90} for diapausing and nondiapausing pupae were -18.04 and -10.31 °C respectively.

The LT_{50} for 2-hour exposure to cold stress for lab-reared nondiapausing and diapausing insects were determined to be -5.14 and -16.78 °C, and the LT_{90} for diapausing and nondiapausing pupae were -20.9, and -12.07 °C respectively (Figs. 3, 4). The 2-hour LT_{50} data indicate that when the insect prepares to face the cold, the insect is likely to encounter subzero temperatures. In *L. botrana*, the tolerance is -16.78 °C, and this insect can adapt as much as possible, and these colds cannot be fatal for the insect. The results showed that the tolerance level for the field-collected insects in late January was -18.15 (-18.02, -18.34 °C), which shows that the insects gradually adapted to the cold and decreased their LT_{50} when the temperature changed and became colder. The tolerance of the insects from the field was lower than that of the laboratory test.

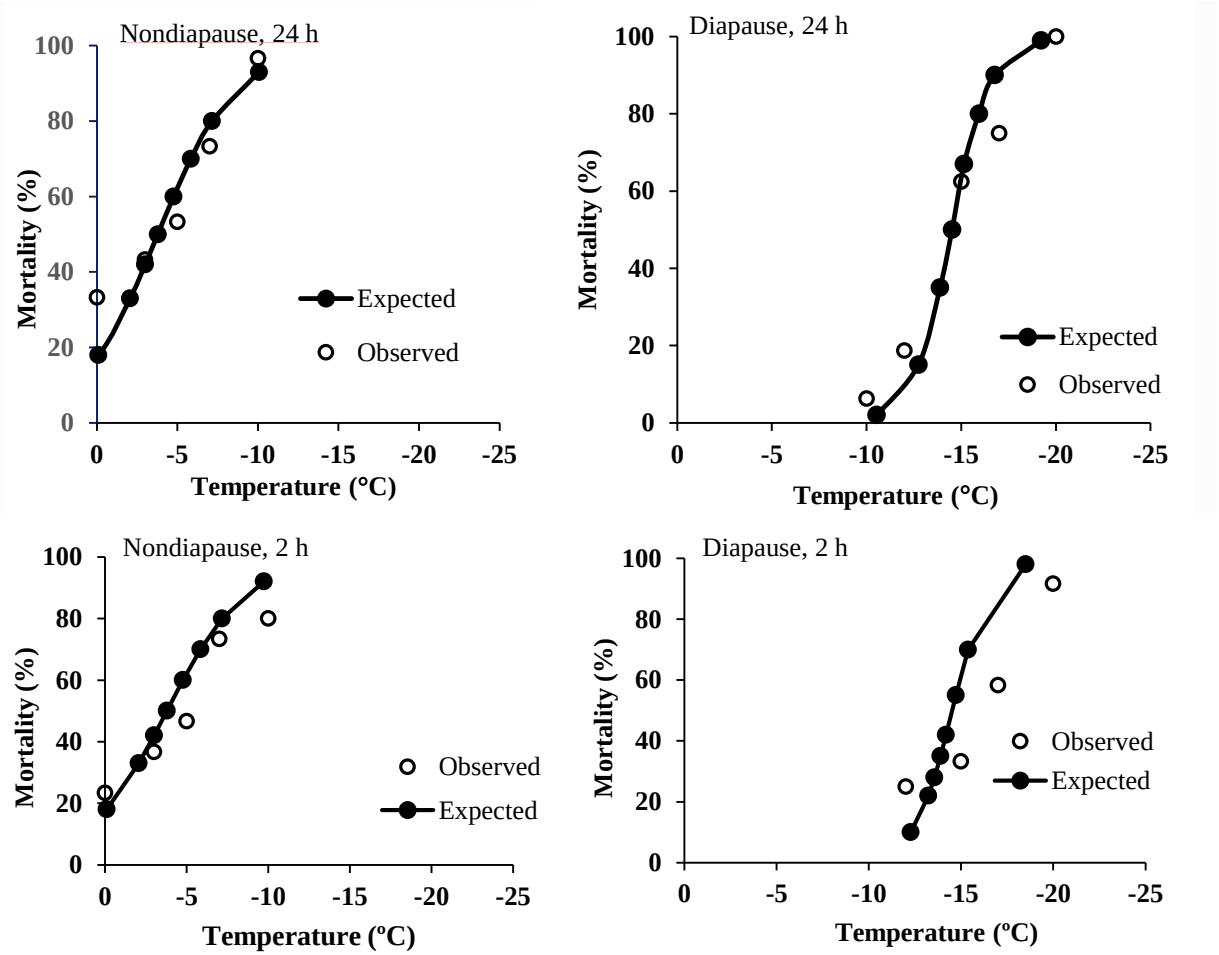

Figure 3 The observed and expected mortality rates at low temperatures under laboratory conditions after exposure to subzero temperatures for 2 and 24 h.

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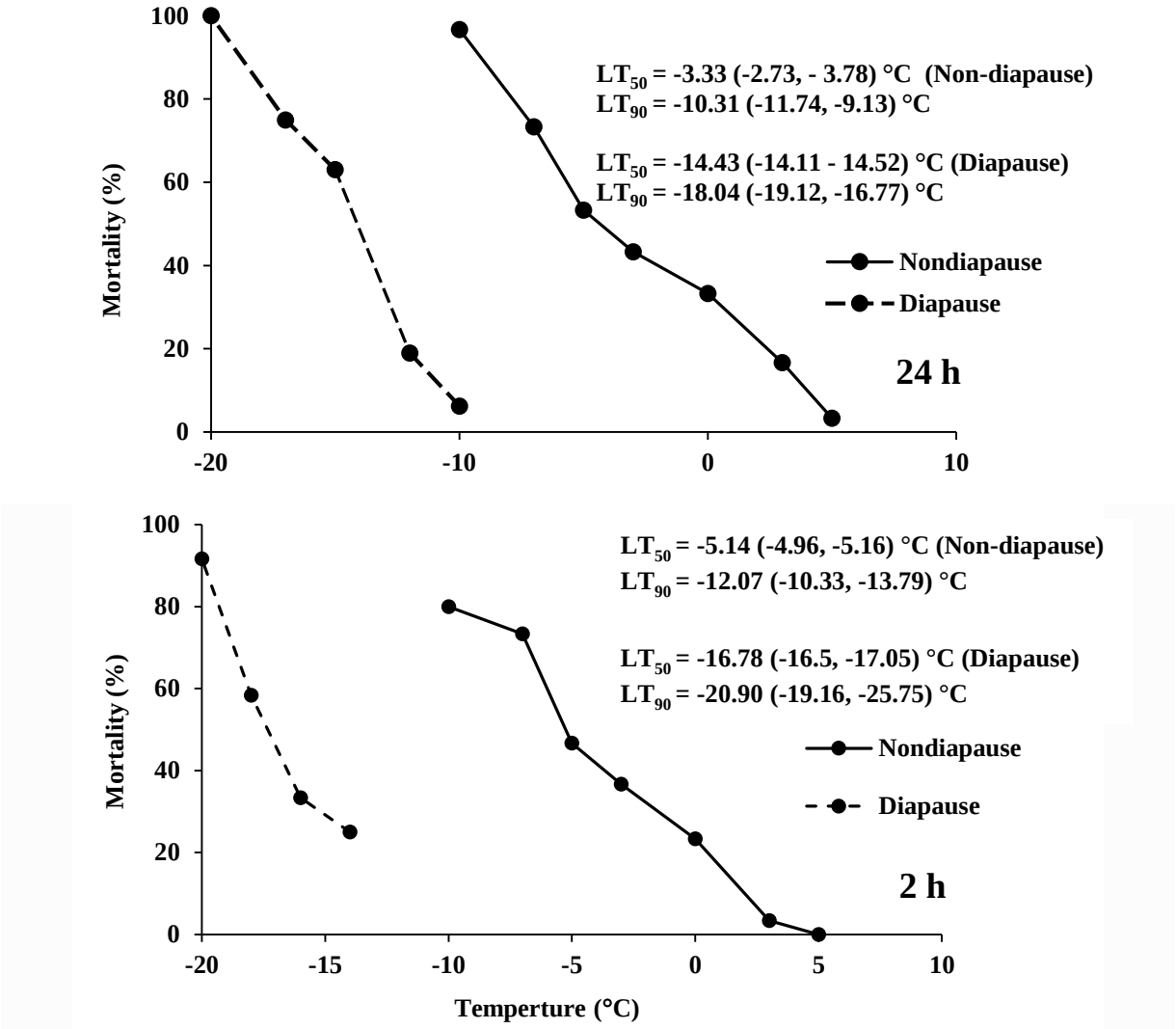

Figure 4 LT_{50} and LT_{90} values of diapausing and non-diapausing pupae of *Lobesia botrana* after exposure to subzero temperatures for 2 and 24 h. A range of temperatures was used to find LT_{50} and LT_{90} values.

Impact of cold acclimation on cold hardiness

The findings highlight distinct mortality trends based on the developmental stage. Mortality patterns in response to cold temperatures show that the mortality rate in nondiapausing pupae from zero to -10 °C reached 33 to 100%. In diapausing pupae, the mortality rate in the temperature range of -12 °C to -20 °C was from 20 to 100%. Also, the expected mortality can be used to estimate the predicted temperature for the mortality of 50% of the population. This is although the diapausing pupae experienced the temperature of 24 °C like the control without experiencing cold and were exposed to short daylength. This observation could indicate their sensitivity to photoperiod changes rather than thermal fluctuations.

Discussion

Diapause induction at room temperature

We found that a decrease in daylenght induces diapause pupae of *L. botrana* even at room temperature. Also, Roditakis and Karandinos (2001) found that *L. botrana* pupae reared under short daylengths (less than 13 h) at 20 °C or 25

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°C entered diapause. Moreover, Andreadis *et al*. (2005) found that diapause induction in *L. botrana* pupae was influenced by photoperiod and temperature, with a critical daylength of 12.43 h at 20 °C and lower temperatures. These studies suggest that photoperiod is more important than temperature for diapause induction in *L. botrana* pupae. Several other insect species show photoperiodic responses. In the case of the Monarch Butterfly *Danaus plexippus* (L.), a photoperiodic reaction to short daylight can induce diapause, even at room temperature. Monarch butterflies use changes in daylength as a cue to trigger their reproductive diapause. When exposed to decreasing daylengths, especially shorter daylight periods, Monarch butterflies initiate physiological changes that lead to diapause. Adult Monarchs become reproductively inactive during diapause, their metabolic rate drops significantly, and they can conserve energy and withstand unfavorable conditions. This mechanism is crucial for their long-distance migrations and survival through adverse seasons (Goehring and Oberhauser, 2002; Herman, 2002). Silkworm moths *Bombyx mori* L. have a well-defined photoperiodic response related to their reproductive diapause. They enter diapause during the pupal stage when exposed to short daylengths. This adaptation ensures that the adult moths emerge when environmental conditions favor mating and oviposition (Tobita and Kiuchi, 2022; Shimizu, 1982). Cabbage White butterfly *Pieris rapae* (L.) and Corn earworm *Helicoverpa zea* (Boddie) undergo diapause in response to photoperiodic cues. As days shorten, the moths enter diapause, enabling them to survive winter (Kono, Y. 1970; Roach *et al*., 1970; Denlinger, 2009). These examples highlight the diversity of insect photoperiodic responses and how they have evolved to synchronize their life cycles with changing environmental conditions.

Supercooling points of diapausing pupae

We found that daylenght inducing diapause did not affect SCP of diapausing pupae when they did not experience cold or were not cold-acclimated so that in this condition, SCP of diapausing pupae was not significantly different with nondiapausing pupae and ranged between -24.35 and -23.06 °C. Also, Masoudmagham *et al*. (2021) noted that the SCP of the grapevine moth, *L. botrana*, pupae was not affected by diapause status but by generation and season. However, cold acclimation caused a decrease in the SCP, but this change was not significant in nondiapausing pupae. Also, we found that SCP in cold-acclimated pupae was significantly lower than that of nondiapausing pupae. There are several studies inducing diapause through changes in daylength that do not substantially influence the SCP of diapausing pupae. In a study focused on the Eastern tent caterpillar *Malacosoma americanum* (F.) and Spruce Budworm *Choristoneura fumiferana* (Clemens), SCP of diapausing pupae did not differ with nondiapausing pupae when exposed to room temperature conditions (Mansingh, 1974; Delisle *et al.*, 2022). Also, the Fall armyworm *Spodoptera frugiperda* (J. E. Smith) and Gypsy moth *Lymantria dispar* (L.) reveal that diapause induced by daylength modifications does not significantly alter the SCP values (Tauber *et al*., 1990; Gray *et al*., 2001; Keosentse *et al*., 2021; Vatanparast and Park., 2022). However, cold-acclimated pupae, which had undergone diapause induction, exhibited SCP values that were markedly lower than those of their nondiapause counterparts within the *L. botrana* species. This finding suggests a complex interplay between diapause induction, cold acclimation, and the inherent SCP values of these pupae.

Impact of cold acclimation on supercooling point

In the cold-acclimated pupae, the SCP of nondiapausing pupae did not change significantly, but the SCP of diapause insects changed from -24.66 to -25.89 °C. Our finding suggests that diapausing pupae can lower their SCP, allowing them to withstand even colder temperatures. This adaptation is likely crucial for their survival in cold environments. Masoudmagham *et al*. (2021) found that the SCP of *L. botrana* pupae varied by generation

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and season, with the lowest SCP observed in the third generation that overwinters in diapause. This data suggests that cold acclimation may affect the SCP of *L. botrana* pupae. In another study, Andreadis *et al*. (2005) found that diapausing pupae of *L. botrana* had a higher survival rate than nondiapausing pupae after exposure to subzero temperatures and that it was related to the expansion of the supercooling capacity.

Cold hardiness of diapausing pupae

Our study delved into an intriguing aspect of *L. botrana* pupae's physiological responses by exploring the potential increase in cold tolerance among induced pupae without any prior cold exposure. This phenomenon could hold significant implications for understanding how diapause induction affects the insect's ability to withstand colder temperatures. Cold tolerance enhancement in induced pupae, even without cold exposure, reveals an opportunity for investigation. It is crucial to consider that such adaptations might involve intricate molecular and biochemical mechanisms that allow the insect to enhance its cold tolerance to better prepare for adverse conditions. Parallels with other examples of insects that exhibit enhanced cold tolerance due to various physiological adjustments, Woolly Bear Caterpillar *Pyrrharctia isabella* (J. E. Smith) is known for predicting winter weather conditions. They achieve enhanced cold tolerance by synthesizing antifreeze compounds and adjusting their metabolic rates, ensuring survival even without prior exposure to extreme cold (Marshall and Sinclair, 2011). Fruit Flies *Drosophila melanogaster* shows increased cold tolerance when subjected to specific genetic mutations. This change in cold resistance could be attributed to altered gene expression, which affects various physiological processes (Štětina *et al*., 2015). Cold-Hardy Aphids, e.g., *Aphis fabae* Scopoli can enhance their cold tolerance by accumulating cryoprotectants, safeguarding cells from freezing damage. These adaptations occur even without immediate cold exposure (Leather, 1993).

In the case of *L. botrana* induced pupae, increased cold tolerance without prior cold exposure adds depth to our understanding of how diapause influences physiological adjustments. This could encompass molecular, metabolic, and gene expression analyses to uncover the intricacies of enhanced cold tolerance and how they correlate with diapause induction.

Impact of cold acclimation on cold hardiness

Cold acclimation enhancing cold hardiness near the SCP is a well-established phenomenon in various insect species, and it is reasonable that *L. botrana* may exhibit similar responses. Cold acclimation typically involves physiological and biochemical changes that enhance an insect's ability to survive sub-freezing temperatures. These changes could include the accumulation of cryoprotectants, adjustments in metabolic processes, alterations in membrane fluidity, and the potential production of antifreeze proteins (Sinclair *et al*., 2003; Hahn and Denlinger, 2007). Cold hardiness refers to an organism's ability to withstand and survive freezing temperatures, and cold acclimation is a process through which an organism gradually becomes more resistant to cold stress by adjusting its physiological and biochemical mechanisms. During cold acclimation, insects undergo a series of changes that collectively improve their cold tolerance and increase their chances of survival when facing sub-freezing conditions (Denlinger, 2002; Sinclair *et al*., 2015). Examples of insects that exhibit enhanced cold hardiness near the SCP due to cold acclimation include the spruce budworm *C. fumiferana* and the woolly bear caterpillar *P. isabella*. In these examples, cold acclimation enables these insects to survive in frigid environments and endure near-freezing conditions without freezing or sustaining significant damage. This adaptation showcases the remarkable capacity of certain insects to adjust their physiology in response to seasonal changes and environmental challenges (Marshall and Sinclair, 2011; Butterson *et al*., 2021; Delisle *et al*., 2022).

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Conclusion

The most significant inference drawn from the study is the evidence of a photoperiodic response in *L. botrana*. By investigating the complicated relationship between daylength and diapause, the study offers significant insights into the photoperiodic responses of this insect species. The study's findings showcase a critical role played by daylength in inducing diapausing pupae in *L. botrana*, even when subjected to room temperature conditions.

This observation indicates the insect's ability to perceive and respond to changes in daylength, revealing its finely tuned photoperiodic mechanism. The research aligns with previous studies that have demonstrated how various environmental cues, including daylength, temperature, and food availability, synchronize the life cycle of insects with seasonal changes. The observed diapause induction under altered daylength conditions may emphasize the interplay between the insect's internal clock and external environmental cues.

The implications of this study contribute to our broader understanding of how insects perceive and respond to changes in daylength, providing a platform for further investigations into the molecular and genetic underpinnings of photoperiodic responses. A broader range of temperature conditions could be considered to assess the observed diapause induction. Results of the study on the impact of daylength on diapause induction in *L. botrana* pupae provide a valuable addition to our understanding of insect photoperiodic responses.

In conclusion, our study provides evidence for increased cold tolerance in induced pupae of *L. botrana* without cold exposure. By synthesizing insights from related insect examples, we highlight the potential significance of understanding these adaptations in the context of diapause-induced changes in cold tolerance. Daylength constantly changes in nature, and outside temperatures can vary drastically during diapause. For additional studies, it is suggested to study the expression of genes related to diapause and examine the composition of the cryoprotectant and phospholipid compositions.

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استراتژی‌های سازگاری با سرما در کرم خوشه‌خوار انگور *Lobesia botrana* پرورش‌یافته در آزمایشگاه: بررسی القای دیاپوز: نقطه انجماد و سرماتابی

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چکیده: کرم خوشه‌خوار انگور *Lobesia botrana* (Denis and Schiffermueller) (Lepidoptera: Tortricidae)، آفت مهم تاکستان‌های سراسر دنیا می‌باشد که باعث ایجاد خسارت اقتصادی می‌شود. در این پژوهش، میزان تحمل حشره به سرما و رابطه آن با دیاپوز در شرایط دمایی ۰/۵ ± ۲۳ درجه سلسیوس و رطوبت نسبی ۵ ± ۷۰ درصد و دوره نوری روز کوتاه ۱۲ ساعت تاریکی و ۱۲ ساعت روشنایی مورد بررسی قرار گرفت. تخم‌های یک‌روزه در شرایط روز کوتاه قرار گرفته و در مرحله شفیره به دیاپوز رفتند. میزان نقطه انجماد (SCP) در شفیره‌های دیاپوزی ۲۴/۳۵- درجه سلسیوس بود، درحالی‌که در شفیره‌های غیردیاپوزی که در شرایط دمایی ۰/۵ ± ۲۳ درجه سلسیوس و رطوبت نسبی ۵ ± ۷۰ درصد و دوره نوری ۱۶ ساعت روشنایی و ۸ ساعت تاریکی پرورش یافته بودند، ۲۳/۰۶- درجه سلسیوس بود. بنابراین تفاوت معنی‌داری در میزان SCP دو گروه فوق مشاهده نشد. هم‌چنین حشرات دیاپوزی تحمل بالاتری نسبت به حشرات غیردیاپوزی داشتند. به‌طوری‌که میزان LT_{50} در شفیره‌های دیاپوزی و شفیره‌های غیردیاپوزی به‌ترتیب ۱۴/۴۳- و ۳/۳۳- درجه سلسیوس بود. جالب توجه است که شفیره‌های دیاپوزی بدون تغییر در میزان SCP، صرفاً با درک طول روز کوتاه، به میزان ۱۱ درجه سلسیوس بیش از شفیره‌های شاهد دماهای زیر صفر را تحمل کردند. نتایج پژوهش نشان می‌دهد که دیاپوز و سرماتابی کرم خوشه‌خوار انگور مستقل از SCP است و حشره از استراتژی حساس به یخزدگی استفاده می‌کند. سازگاری با سرما در دمای ۵- و ۱۰- درجه سلسیوس باعث کاهش قابل‌توجهی در SCP شفیره‌های دیاپوزی شد، درحالی‌که سازگاری ۷۲ ساعته به سرما تأثیر قابل‌توجهی بر SCP شفیره‌های بدون دیاپوز نداشت. این یافته‌ها اطلاعات ارزشمندی در مورد مکانیسم‌های بقای کرم خوشه‌خوار انگور در شرایط سرما و سازگاری‌های مربوط به دیاپوز ارائه می‌دهند.

واژگان کلیدی: کرم خوشه‌خوار انگور، القای دیاپوز، سرماتابی، جیره مصنوعی، نقطه انجماد، تحمل

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