

## Overwintering inside the food in the diapausing larvae of pomegranate fruit moth

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### Abstract

Low temperatures are a major survival challenge for insects living in temperate zones. The most common features that guaranty survival in the most overwintering insects are diapause and cold hardiness. In spite of obvious advantages, damages due to diapause associated stresses such as cold shock and depletion of energy reserves make diapause a costly life history strategy. *Apomyelois ceratoniae* (Zeller), a major pest of pomegranate in Iran, overwinters as different larval instars. In comparison to larger counterparts, early instars of pomegranate fruit moth must have sequestered fewer energy reserves, thus they might be less tolerant to cold. By designing experiments similar to natural conditions in December, we demonstrated that overwintering inside pomegranate fruits provides opportunity for larvae to feed and compensates energy depletion. Our results also indicated that there is no significant difference between supercooling point and cold tolerance of the small and the large field collected larvae during autumn and winter, thus overwintering mortality might not be a key driver in population dynamics.

**Key words:** cold tolerance, supercooling point, *Apomyelois ceratoniae*, overwintering, diapauses

### چکیده

وضعیت زمستان‌گذرانی لاروهای دیابوزی کرم گلوگاه انار در داخل غذا

بنفشه احمدی و سعید محرمی‌پور

دماهای پایین فصل زمستان چالش‌های زیادی را برای حشراتی که در مناطق معتدل زندگی می‌کنند ایجاد می‌کند. دیابوز و سرماسختی دو استراتژی رایج برای تضمین بقا در حشرات است. با وجود این که دیابوز مزایای بسیاری دارد، مرحله‌ای پرهزینه است، زیرا حشره با شوک‌های ناشی از سرما و کاهش ذخایر مواجه است. کرم گلوگاه انار، *Apomyelois ceratoniae* (Zeller) آفت کلیدی باغ‌های انار است که به صورت لاروهای سنین مختلف زمستان‌گذرانی می‌کند. لاروهای سنین پایین ذخیره انرژی کم‌تری دارند، براین اساس تصور می‌شود که سرماسختی آن‌ها نسبت به لاروهای بزرگ کم‌تر باشد. با انجام آزمایش‌های الگوبرداری شده از شرایط طبیعی در آذر ماه، مشخص شد که زمستان‌گذرانی داخل انار به عنوان منبع تغذیه، شرایط را برای جبران کاهش ذخایر انرژی مهیا می‌سازد. هم‌چنین بررسی‌ها نشان داد، نقطه انجماد و سرماسختی در لاروهای کوچک و بزرگ یکسان است و دینامیسم جمعیت این آفت نمی‌تواند تحت تأثیر مرگ و میر در فصل زمستان باشد.

واژگان کلیدی: تحمل سرما، نقطه انجماد، *Apomyelois ceratoniae*، زمستان‌گذرانی، دیابوز

### Introduction

Winter is a key driver of individual performance, community composition and ecological interaction in temperate, alpine and polar environments as terrestrial organisms living there may spend more than half of their life overwintering (Williams *et al.*, 2015). During winter, insects must survive low temperature exposure, as well as desiccation and energy drain (Williams *et al.*, 2015) that may reduce overall physiological performance and population growth (Carcamo *et al.*, 2009). Many insects enter diapause (e.g. *Sarcophaga crassipalpis* Macquart; Lee & Denlinger, 1985; *Sesamia nonagrioides* (Lef), Lopez *et al.*, 1995) to pass the environmental rigors of winter periods, while others (e.g. *Thaumatotibia leucotreta* (Meyrick); Broadman *et al.*, 2012; *Eurosta solidaginis* (Fitch); Baust & Lee, 1981) must simply withstand these harsh conditions (Leather *et al.*, 1993). Diapausing insects characteristically

feed very little or not at all, thus they are largely or totally dependent on energy reserves sequestered prior to entry into diapause (Hahn & Denlinger, 2007). Costs of diapause are commonly reflected in the lower post-diapause survival and fecundity (Denlinger, 1981; Williams *et al.*, 2003; Munyiri & Ishikawa, 2004). The two most likely physiological mechanisms underlying these costs are damage due to diapause-associated stresses, such as desiccation or cold shock and depletion of metabolic reserves that could contribute to decrease in post diapause fitness (Hahn & Denlinger, 2007). The stage of developmental arrest in which diapause occurs may take many different forms: a) various immobile stages such as diapausing embryos, cocooned mature larvae and pre-pupae which do not accept food and display deep metabolic suppression; b) diapausing free-living larvae and adults with movement and low metabolic suppression; c) some extreme cases including

diapausing larvae which continue to feed and grow during winter (Kostal, 2006). Even though, diapause mostly occurs at a specific age, (e.g. *S. nonagrioides* (Lopez *et al.*, 1995), *Chilo suppressalis* Walker (Xu *et al.*, 2011)).

The pomegranate fruit moth, *Apomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae), is a polyphagous fruit pest in many tropical and subtropical countries (Gothilf, 1984). In Iran, the larvae attack pomegranate, fig and pistachio and they emerged as the major pest of pomegranate orchards since 1980 (Mehrnejad, 1993; Shakeri, 1993). The second and third instar larvae enter the fruit from the calyx and consume the interior tissue and seeds. Fruit decay occurs as a result of saprophytic fungi entrance, leading to 20-80% reduction of yield (Shakeri, 2004). In Iran, larvae of different ages enter diapause in mid-autumn (November) (Shakeri, 2004; Karimi *et al.*, 2011) and overwinter inside fruits remaining on or under the trees (Shakeri, 2004). Although there are several studies on biology (e.g. Yousefi *et al.*, 2004) and control measures of pomegranate fruit moth (e.g. parasitoids Norouzi *et al.*, 2009), our information is limited on its overwintering biology and diapause.

Cox (1979) induced diapause in larvae of *A. ceratoniae* when reared them at 20 °C and 12: 12 (L: D) photoperiod. In addition Heydari & Izadi (2014) reported that the last instar larvae of pomegranate fruit moth are able to tolerate harsh conditions during winter. Our observation during collecting infested pomegranate indicated that only 20% of larvae overwinter as fully grown non feeding larvae. Objectives of the current study were; 1) to determine the difference in supercooling point (SCP) and cold tolerance of small (2<sup>nd</sup> and 3<sup>rd</sup>) and large (4<sup>th</sup> and 5<sup>th</sup>) overwintering larvae, 2) how do small larvae compensate energy drain during winter?

## Material and methods

### Insects

*A. ceratoniae* larvae were collected during autumn and winter 2012-2013 by gathering infested pomegranate fruit from orchard located in Chandab Village, Semnan, Iran (35° 25' N, 51° 56' E, 1130 m above sea level). We divided larvae into small (2<sup>nd</sup> and

3<sup>rd</sup>) and large (4<sup>th</sup> and 5<sup>th</sup>) instars and kept them separately on pomegranate seeds. The larvae were kept outdoor at ambient temperature in shade until use in the experiments.

### SCP of field collected larvae

To determine the SCP, field collected larvae (n = 24-50) were attached to chromel-alumel thermocouples (Type k, 1.5 mm diameter) using adhesive tape and placed inside a programmable refrigerating test chamber (Model MK 53, Binder GmbH Bergstr., Tuttlingen Germany). Temperature of test chamber was decreased from 15 °C to -30 °C at 0.5 °C/min. Temperature of the larval body was recorded every 30s with a four-channel data logger (Testo Model 177-T4) and monitored using Comsoft 4 software. The SCP was recorded at the temperature where an exothermic reaction occurred, indicating a latent heat release and initiation of freezing. SCP of small (2<sup>nd</sup> and 3<sup>rd</sup>) and large (4<sup>th</sup> and 5<sup>th</sup>) larvae were compared.

### SCP of larvae that have discharged their gut

To determine if discharging the gut content affects the SCP, larval mouth parts were stimulated with a fine brush prior to measuring SCP (n= 24). The SCP was compared to a group of non-disturbed larvae.

### SCP of fed and starved larvae

Field collected larvae were divided into two groups in early November: 1) larvae were placed individually in Petri dishes (3 cm diameter) (n=60); 2) larvae were placed individually in Petri dishes containing 5 g of pistachio powder (3 cm diameter) (n = 60). Both treatments were placed in growth chamber (Binder GmbH, model KBWF 240, Germany) simulating December conditions in pomegranate orchard (10L: 14D photoperiod, 60 ± 5% RH, 14 °C day time, 5 °C night time). SCP was measured after one, two and four weeks.

### Inoculative freezing

To determine SCP with external inoculation, 100 mg of a known ice nucleator, silver iodide (Sigma Aldrich), was suspended in 1 ml of distilled water.

Larvae were individually dipped in silver iodide solution. SCP was compared with larvae which were not suspended in silver iodide.

#### **The cold hardiness**

Mortalities of small and large field collected larvae were determined after 3 h of exposure to -7, -10 and -12 °C. These temperatures were chosen based on SCP of the larvae to obtain 10 to 90% mortality. The larvae were placed in glass Petri dishes (100 mm × 15 mm) (n = 7; r = 3), the bottom of which were covered by dry tissue paper. The Petri dishes were then placed in the programmable refrigerator and cooled to the test temperature (-7, -10 and -12 °C) at the rate of 0.5 °C/min. The larvae were rewarmed after 3 h of exposure to test temperatures at the same rate to the room temperature. Larvae walking in a coordinated fashion after 24 h was recorded as alive.

#### **Cold tolerance inside dry and wet foods**

To determine cold tolerance inside dry and wet foods, survival at -10 °C, the discriminating temperature after reduced survival were compared in three groups: 1) larvae placed in glass Petri dishes (100 mm × 15 mm) (n = 7; r = 3) filled with dry wheat bran; 2) larvae placed in glass Petri dishes (100 mm × 15 mm) (n = 7; r = 3) filled with wheat bran suspended in water; 3) larvae placed in glass Petri dishes (100 mm × 15 mm) (n = 7; r = 3) the bottom of which was covered with dry tissue paper.

#### **Cold tolerance of fed and starved larvae**

Cold tolerance (exposure to -10 °C for 3 h) of fed and starved larvae was compared when those were kept in December conditions for four weeks in growth chamber (n = 7; r = 3). (See previous parts for detail).

#### **Determining the interior temperature of pomegranate**

In order to determine the interior temperature of pomegranate, thermocouples were placed inside the pomegranates and exposed to -7, -10 and -12 temperatures as described before. The changes in test chamber temperature were also recorded.

#### **Gut condition**

Guts of starved and fed larvae that were kept in December condition, as well as field collected larvae in January were dissected out with fine forceps.

#### **Statistical analysis**

A t-test was performed to compare between SCP of small (2<sup>nd</sup> and 3<sup>rd</sup>) and large (4<sup>th</sup> and 5<sup>th</sup>) larvae, the ones that had discharged their gut content vs. not stimulated ones as well as fed vs. starved larvae. Analyses were done using statistical software package SPSS for windows, version 20.0 (IBM. Corp, 2011 Armonk, NY, USA).

#### **Results**

SCP and cold tolerance of small (2<sup>nd</sup> and 3<sup>rd</sup>) and large (4<sup>th</sup> and 5<sup>th</sup>) larvae were compared to determine whether they are both able to overcome the winter in the same pattern or the age and body size affects the overwintering survival. Our results indicated that there is no statistically difference ( $t_{83} = -1.07$ ,  $P = 0.282$ ) between SCP of small ( $-10.6 \pm 0.6$  °C) and large ( $-9.7 \pm 0.6$  °C) larvae. In spite of significant differences ( $t_{190} = 20.1$ ,  $P = 0.001$ ) being between the weight of small ( $18.5 \pm 0.6$  mg/larva) and large ( $43.0 \pm 1.1$  mg/larva) larvae, cold tolerance of field collected larvae during autumn and winter followed the same pattern in both groups (12-25% mortality at -7 °C; 50-80% mortality at -10 °C and 90-100% at -12 °C).

Discharging the gut content neither affected the SCP value ( $t_{45} = 0.84$ ,  $P = 0.403$ ), nor the unimodal distribution (fig. 1)

Determining the SCP and cold tolerance of fed vs. starved larvae revealed that even though SCP of the starved larvae were lower than that of the fed ones, it showed no significant difference after one ( $t_{11} = 0.5$ ,  $P = 0.623$ ), two ( $t_{13} = 0.5$ ,  $P = 0.601$ ) and four weeks ( $t_{18} = 1.7$ ,  $P = 0.104$ ); however a shift from SCPs higher than the median to SCPs lower than that occurred over time (fig. 2). SCPs in upper median group decreased in both fed and starved larvae after one, two and four weeks, (86, 71 and 31% for fed larvae and 60, 44 and

33% for starved larvae respectively). In addition, exposure to -10 °C for 3 h resulted in 42 and 44 percent survival in starved and fed larvae, respectively.

Silver iodide increased SCP significantly ( $t_{21} = 4.0$ ,  $P = 0.001$ ) and resulted in a shift in SCPs from values lower than median to higher degrees (100% in upper median; fig. 3). Also wet food inoculated freezing and decreased larval survival (32%) at -10 °C, where survival in dry food and Petri dishes covered with tissue paper were 53% and 60%, respectively.

The interior temperature of pomegranate decreased with a lower slope in comparison to test chamber. The recorded temperatures inside pomegranates were  $-4.1 \pm 0.5$ ,  $-7.4 \pm 0.6$  and  $-9.8 \pm 1$  °C after 3 h of exposure to -7, -10 and -12 °C respectively.

Dissecting out the larval guts kept in December conditions revealed that diapausing larvae of *A. Ceratoniae* had the same survival rate (90%) in warm hours of day in both fed and starved larvae even after a month.

## Discussion

SCP and cold tolerance of both small and large diapausing larvae had the same pattern indicating that body size and age do not modify the overwintering survival in *A. ceratoniae*. Even though, changes in body size and accumulation of energy reserves are among the most conspicuous alterations that occur during prediapauses period (Danks, 1987, Denlinger *et al.*, 2005); this is not always the case (Hahn & Denlinger, 2007). For example, neither diapausing post-feeding larvae of fly *Calliphora vicina* (Robineau-Desvoidy) (Saunders, 1997) nor diapausing pupae of tobacco hornworm *Manduca sexta* (Linnaeus) are heavier or store significantly greater quantities of lipids than their non-diapausing counterparts. It is important to understand the relative costs and benefits of the accumulation of additional reserve or size prior to diapause (Hahn & Denlinger, 2007). Some species that

accumulate greater reserves are known to feed more or longer during preparation for diapause, perhaps increasing the risk of attracting natural enemies or not completing development before the onset of inclement conditions (Hahn & Denlinger, 2007). In addition, whether a species increases lipid reserves prior to diapause or not may be related to environment experienced during diapause preparatory period (Hahn & Denlinger, 2007). Insects that have not sequestered sufficient reserves to survive a lengthy diapause, have four options: to die during the diapause or post diapause when all reserves have been depleted; to opt to avert diapause, producing more generations is better than dying; to terminate the diapause prematurely when energy reserves become dangerously low; or to compensate deficiency by feeding during diapause (Hahn & Denlinger, 2011). The last option is only available to insects that retain ability to feed during larval or adult diapause (Hahn & Denlinger, 2011). Pomegranate fruit moth consumes pomegranate seeds, a source rich in triacylglycerides (TAG) -the primary form of storage lipids in insects- and unsaturated fatty acid - responsible for cold tolerance- (Melo *et al.*, 2014)) and overwinters inside the food source, so this is possible that larvae feed to compensate energy drain. Examination of the guts of the field collected diapausing larvae of *A. ceratoniae* kept in December condition, suggested that they consume food to overcome energy depletion. De Block *et al.* (2007) also showed that overwintering larvae of damselfly, *Lesteseurinus* (Say) that enters diapause with poor energy reserves compensate by feeding more than their well-fed cohorts. On the other hand, the food in the gut would increase the risk of ice inoculative freezing so that many temperate insects lower their SCP through evacuation of gut to remove food particles that might initiate the freezing process (Zachariassen, 1985; Duman, 2001). Our observations while measuring SCP and cold tolerance indicated that diapausing fed larvae empty their guts contents when they are at risk of being frozen.

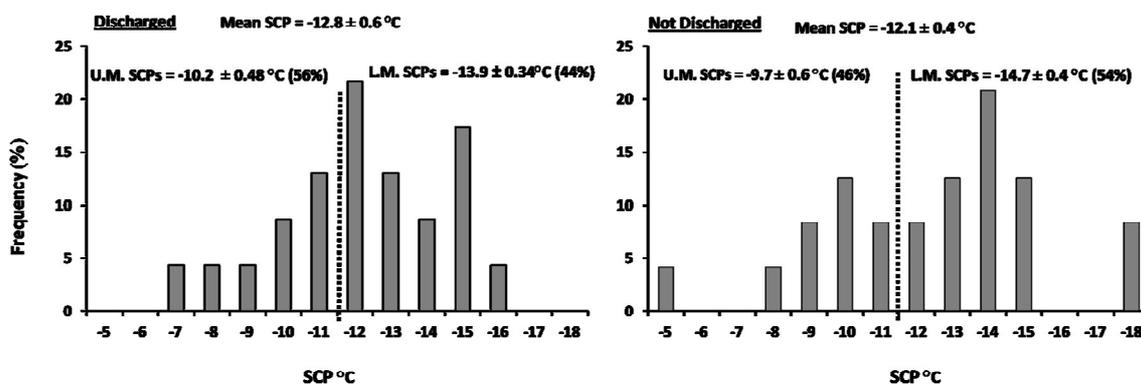


Fig. 1. Frequency (%) and distribution of supercooling points (SCPs) in the larvae with undischarged or discharged gut. A dotted line is drawn between upper median (U. M.) and lower median (L. M) SCPs.

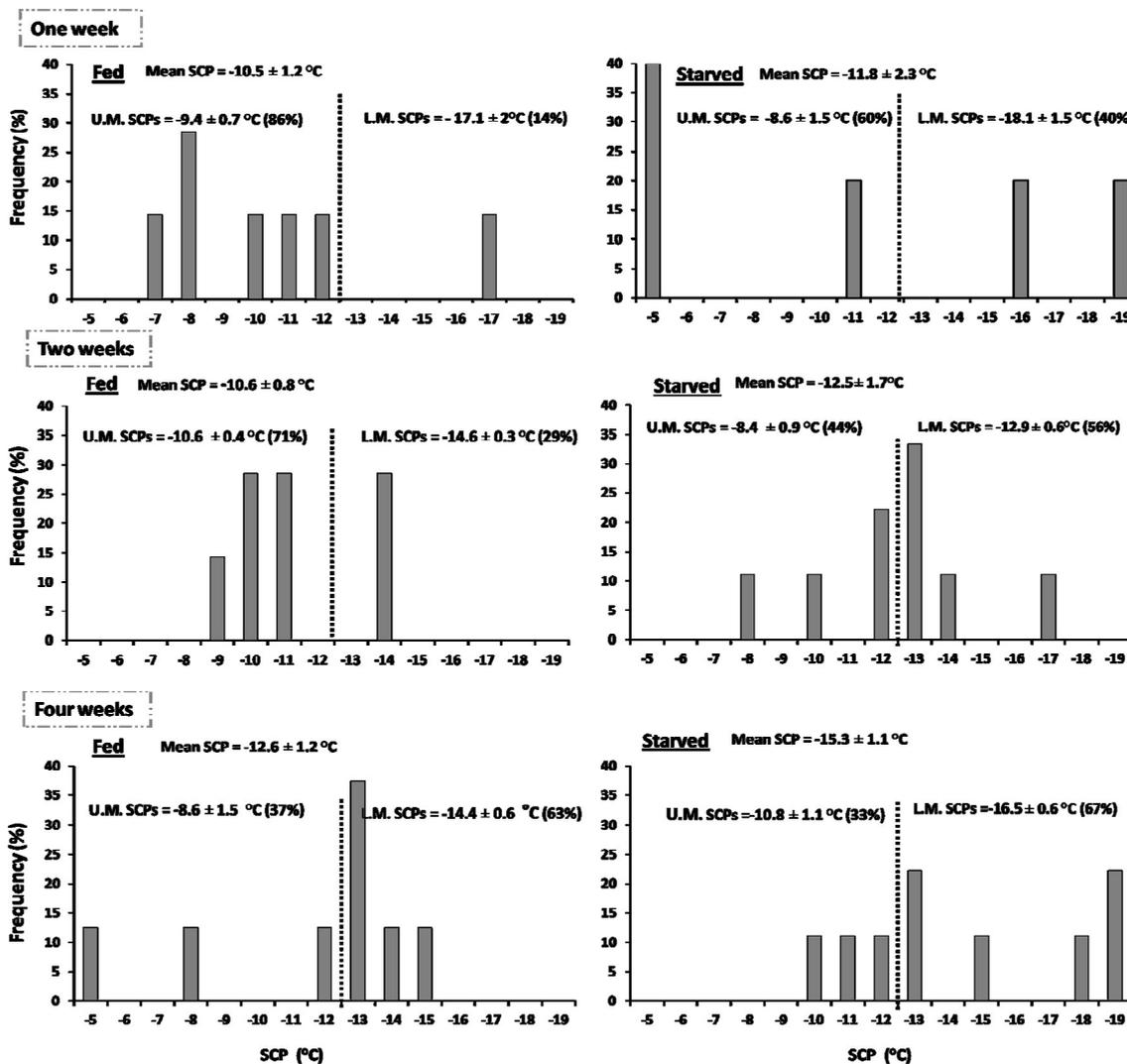
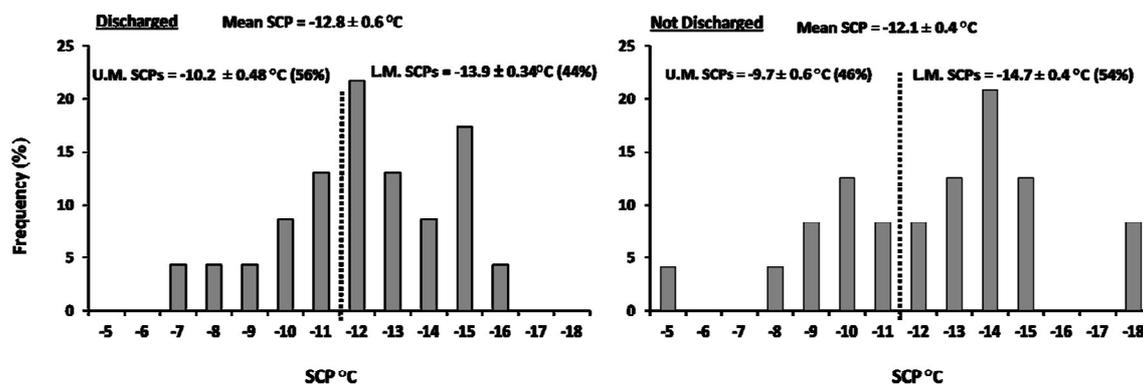


Fig. 2. Frequency (%) and distribution of supercooling points (SCPs) in the fed and the starved larvae kept in December conditions for one, two and four weeks periods. A dotted line is drawn between upper median (U. M.) and lower median (L.M) SCPs.



**Fig. 3.** Frequency (%) and distribution of supercooling points (SCPs) in the larvae suspended in ice inoculative solution and the normal individuals. A dotted line is drawn between upper median (U. M.) and lower median (L. M) SCPs.



**Fig. 4.** a) starved and b) fed larvae of *Apomyelois ceratoniae* and their dissected gut, the larvae were kept in December condition (10L: 14D photoperiod,  $60\% \pm 5\%$  RH, 14 °C day time, 5 °C night time).

SCP of diapausing larvae kept in December conditions altered to lower degrees over time, indicating that cold acclimation changed the SCP patterns of both fed and starved larvae. This phenomenon was also observed in December, January and February during which the larvae acclimatize to the cold months of autumn and winter (Ahmdai *et al.*, 2016.). *A.ceratoniae* is a chill susceptible species, liable to die as a result of chill injury before freezing. Thus, altering the SCP pattern could be an adaptive physiological change to increase the probability of survival. Pomegranate fruit moth larvae showed no resistance to inoculative freezing as survival decreased in the larvae kept on wet food. Thus temperature drop which normally occurs following a rainy day would

increase the risk of mortality. Even though, *A. ceratoniae* is a major pest in tropical and subtropical countries, so below zero temperatures during winter in pomegranate orchards in Iran, helps overwintering larvae to manage their energy reserves. On the other hand, we have seen small larvae in infested pomegranate on March and even April, when the temperature exceeds the minimum growth temperature (Yosoufi *et al.*, 2004). Despite the fact that larvae overwinter inside the pomegranate, where the food source is available, existence of small larvae in pomegranate would occur only if larvae are in diapause. However further investigations on diapause of pomegranate fruit moth larvae such as estimation of metabolic rate would clarify this subject.

Pomegranate fruit moth is a polyphagous pest in tropical and subtropical countries. So the overwintering larvae are exposed to mild temperatures in autumns. Exposing to warm temperatures in autumn indicates that the bulk of energy is consumed during high temperature during autumn (Sinclair, 2015). Overwintering inside pomegranate provides the microclimate in which thermal variability is lower as

well as the opportunity to compensate the energy deficit. However, this microclimate can increase the probability of ice inoculative freezing. How does this opportunist species changes the conditions to win?

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