Zoological Studies **59:**25 (2020)
doi:10.6620/ZS.2020.59-25

Zoological Studies

Open Access

Simultaneous Occurrence of Diapause and Cold Hardiness in Overwintering Eggs of the Apple Oystershell Scale, *Lepidosaphes Malicola* Borchsenius (Hem.: Diaspididae)

Parvaneh Nazari[1], Nafiseh Poorjavad[2], and Hamzeh Izadi[3,*]

[1]*Department of Plant Protection, College of Agriculture, Isfahan University of Technology, P.O. Box 8415683111, Isfahan, Iran. E-mail: parvanehnazari@ymail.com (Nazari)*
[2]*Department of Plant Protection, College of Agriculture, Isfahan University of Technology, P.O. Box 8415683111, Isfahan, Iran. E-mail: npoorjavad@cc.iut.ac.ir (Poorjavad)*
[3]*Department of Plant Protection, Faculty of Agriculture, Vali-e-Asr University of Rafsanjan, Rafsanjan, Iran. *Correspondence: Tel: +98 34 3131 2012. Fax: +98 34 3131 2042. E-mail: izadi@vru.ac.ir (Izadi)*

Received 27 March 2020 / Accepted 20 May 2020 / Published 6 July 2020
Communicated by Benny K.K. Chan

As the key pest of apple fruits, the oystershell scale, *Lepidosaphes malicola* Borchsenius (Hem.: Diaspididae), overwinters as diapausing eggs under the protective, waxy cover of females. In this research, the effects of diapause development, cold acclimation, and rapid cold hardening were studied on the cold hardiness of the eggs. The changes in some physiological components were also investigated. The results indicated cold exposure to be a prerequisite for the survival of the diapausing eggs of *L. malicola*. No eggs hatched without exposure to cold. In addition, a direct relationship was observed among cold hardiness, cold acclimation, and diapause of the eggs based on the results. The highest level of hatching (the highest cold hardiness) of the eggs (80%) occurred in the cold-acclimated eggs at the end of diapause (March). Rapid cold hardening also influenced the cold hardiness of the eggs with diapause development. At the end of diapause, the lowest (61%) and the highest (77%) rates of egg survival were observed when the eggs were exposed to 5 and -10°C for 24 h, respectively. Cold hardiness of the diapausing eggs of *L. malicola* was also accompanied by some physiological changes, *i.e.*, a decrease in glycogen content and an increase in simple sugar, lipid, and protein contents. The lowest glycogen content (about 50 μg/g) and the highest amounts of total simple sugars (454 μg/g) of lipids (542 μg/g) and proteins (84 μg/g) were observed in the cold-acclimated eggs at the end of diapause.

Key words: Cold hardiness, Cold acclimation, Diapause, Apple.

BACKGROUND

The oystershell scale, *Lepidosaphes malicola* (Hem.: Diaspididae), is a serious pest that injures fruits, shade trees, and shrubs, and is the most common pest of apple fruits. This scale insect, which is a bivoltine pest, overwinters as diapausing eggs beneath the protective, waxy cover of females. The overwintered eggs hatch from late May to early June. First-instar nymphs, which are known as crawlers, wander over the bark of the host plant for a short period and then settle down to feed. Nymphs reach maturity in late summer or early fall, and adults immerge. In contrast to males having three nymphal stages, females usually go through five distinct stages (Esmaili 1983).

The survival, development, and distribution of insects, as poikilothermic animals, are vigorously affected by the ambient temperature (Cira et al. 2016).

Citation: Nazari P, Poorjavad N, Izadi H. 2020. Simultaneous occurrence of diapause and cold hardiness in overwintering eggs of the apple oystershell scale, *Lepidosaphes Malicola* Borchsenius (Hem.: Diaspididae). Zool Stud **59:**25. doi:10.6620/ZS.2020.59-25.

In the temperate regions, insects employ different adaptation strategies to survive harsh conditions, of which diapause and cold hardiness are two essential components used by insects for surviving winters in temperate zones (Lee 1991). The relationship between these two strategies has remained unclear. Cold hardiness may be achieved independent of diapause (Khanmohamadi et al. 2016; Mollaei et al. 2016; Mohammadzadeh et al. 2017), or it can be a component of the diapause syndrome (Bemani et al. 2012; Heydari and Izadi 2014; Lee 1991; Milonas and Savopoulou-Soultani 1999).

Diapause is a phenomenon that occurs when food resources are limited or unavailable. Therefore, to survive this period and enable post-diapause development, reproduction, or distribution, it seems crucial to accumulate energy resources and manage food reserves before and during diapause, respectively. Insects usually sequester sufficient energy reserves, such as carbohydrates and lipids, before entering diapause (Hahn and Denlinger 2010; De Barro et al. 2011). The accumulation of food resources has well been documented in many overwintering insects (Behroozi et al. 2012; Sadeghi et al. 2012; Bemani et al. 2012; Heydari and Izadi 2014).

Cold hardiness or tolerance has been defined as the ability of an insect to resist low temperatures and maintain a supercooled conditions to prevent injuries related to harsh conditions (Andreadis and Athanassiou 2017; Su et al. 2017). The capacity of an insect to survive cold exposure depends on the seasonal temperature variations, geographical environment, developmental stages, physiological status (*i.e.*, the synthesis and accumulation of cryoprotectants; changes in body moisture and fat, sugar, protein, and amino acid contents, and fatty acid content), and exposure time (Cira et al. 2016; Su et al. 2017; Feng et al. 2018). One of the crucial factors in the induction of cold hardiness is the exposure to low temperatures (Andreadis and Athanassiou 2017). However, cold hardiness is an essential strategy adopted by the overwintering insects in temperate zones because, in these regions, changes in the ambient temperature often impact insect abundance strongly (Wang et al. 2017).

The rapid cold hardening (RCH) is defined as the phenotypic capacity of an insect to instantly (within minutes to hours) and substantially enhance its cold resistance. Moreover, this capacity acts as an agent protecting the performance of the insect species in an environment where thermally variable conditions prevail (Shreve et al. 2004; Lee and Denlinger 2010; Kawarasaki et al. 2013; Teets et al. 2020)

In the current study, we examined the effects of cold acclimation and RCH on the survival and energy reserves of the diapausing eggs of *L. malicola.*

MATERIALS AND METHODS

Insect collection

On the fifth day of each month, from November to March (2017–2018), some infested branches of apple trees were collected from a garden located in the vicinity of Semirom (31.42°N and 51.57°E), Isfahan, Iran. A 5-cm part was cut from the middle of each branch, the armor of the scales was removed with fine forceps, and the eggs were collected.

Cold acclimation assay

To estimate the impact of cold acclimation on the hatching and survival of the overwintering eggs, four batches of eggs (five eggs per batch) were acclimated at 5°C, -10°C, -20°C, and -25°C, and were kept for 24, 48, and 72 h at each temperature. After the time lapses (24, 48, and 72 h), one batch was removed and transferred to room temperature (25°C). The emerged larvae were counted after three months.

Rapid cold hardening (RCH) assay

To determine the effect of RCH on the survival of the overwintering eggs, six batches, each containing five eggs, were directly transferred to 5°C, -10°C, -20°C, and -25°C, kept in each temperature for 24 h, and then transferred to 25°C. The emerged larvae were counted after three months.

Biochemical analysis

Total simple sugars

The total simple sugars (mono- and disaccharides) were measured using a modified method proposed by Warburg and Yuval (1997). The eggs (n = 3 batches, equal to 10 mg /month) were homogenized in 200 µl of 2% Na_2SO_4. To extract the simple sugars, the homogenate was mixed with 1300 µl of chloroform-methanol (1:2). The mixture was centrifuged at 7150 × g for 10 min. To determine the amount of simple sugars in the eggs, 600 µl of supernatant was mixed with 400 µl of distilled water, and 2 ml of anthrone reagent (500 mg of anthrone dissolved in 500 ml of concentrated H_2SO_4) was allowed to react at 90°C for 10 min. The absorbance was read at 630 nm using a spectrophotometer (T60U, Harlow Scientific, USA). The amount of total simple sugars was measured by the

standard curve of the standard glucose solution (Sigma). This experiment was performed monthly with six individual eggs.

Glycogen assay

The pellet resulting from the analysis of total simple sugars was used to determine the glycogen content of the overwintering eggs. To remove the feasible remnants of sugars, the pellet was washed using 400 µl of 80% methanol. To extract the glycogen, the washed pellet was mixed with 750 µl of distilled water, and the mixture was heated at 70°C for 5 min. Subsequently, 600 µl of the solution was allowed to react with 3 ml of anthrone reagent (600 mg of anthrone dissolved in 300 ml of concentrated H_2SO_4) at 90°C for 10 min. The optical density was measured at 630 nm using a spectrophotometer (T60U, Harlow Scientific, USA). The amount of glycogen was determined based on the standard curve using glycogen (Sigma). This experiment was repeated six times per month with an individual egg.

Lipid assay

To determine the lipid content of the eggs, 300 µl of the supernatant of simple sugars, empirically determined, was evaporated at 35°C in an oven. Then, 300 µl of H_2SO_4 was added to the supernatant and heated at 90°C for 10 min. After cooling, the sample was stirred, and 2700 µl of the vanillin reagent (600 mg of vanillin + 100 ml of distilled water + 400 ml of 85% H3Po4) was added to the sample. The tubes were vortexed and retained at room temperature for 30 min. The optical density was read at 530 nm using a spectrophotometer. The amount of lipids was determined by the standard curve using triolein solution (Sigma) as the standard (Warburg and Yuval 1997). This experiment was performed each month with six individual eggs.

Protein assay

To determine the protein content, the method proposed by Bradford (1976) was used. Thus, 25 µl of the supernatant from the glycogen assay was mixed with 475 µl of the Bradford reagent, which consisted of 50 mg of Coomassie Brilliant Blue dissolved in 100 ml of 85% (w/v) phosphoric acid and of 50 ml of methanol. A spectrophotometer was used to measure the optical density at 590 nm. Bovine serum albumin (Sigma) was used as the standard. This experiment was performed each month with six individual eggs.

Statistical analysis

All data were initially examined for normality (Kolmogorov-Smirnov tests) (PROC GLM; SAS Institute, 2009). The statistical analyses were then performed using a one-way analysis of variance (ANOVA) followed by Tukey's test ($P = 0.05$). The results were expressed as mean ± SE and considered significantly different at $P < 0.05$.

RESULTS

The results of the current study indicated that cold acclimation significantly increased egg survival, while cold exposure time had no significant impact on survival (Table 1). On the other hand, no differences were observed in the survival rates of the eggs at different exposure times.

The minimum number of emerged larvae was observed at the onset of overwintering (November). The rate of egg hatching increased as diapause lasted longer and reached the highest level, *i.e.*, 80%, in the fully-developed eggs in March. Without cold exposure, the hatching of the eggs was almost negligible.

The effect of rapid cold hardening on the rates of egg hatching of *L. malicola* is presented in table 2. As the results indicate, when the eggs were rapidly exposed to different temperatures, the survival rates increased with the prolongation of diapause and peaked in

Table 1. Effect of cold acclimation on hatching rate (%) of overwintering eggs of *Lepidosaphes malicola*

Cold exposure time (h)	Egg hatching rate (%)				
	November	December	January	February	March
24	10.67 ± 0.42e	20.67 ± 0.66d	57.33 ± 1.33c	61.33 ± 3.95b	80.33 ± 3.95a
48	8.50 ± 0.50d	18.50 ± 0.74c	56.00 ± 1.00b	57.33 ± 2.45b	74.83 ± 1.37a
72	6.67 ± 0.42d	14.67 ± 0.66ic	53.33 ± 1.33a	57.33 ± 1.97b	76.33 ± 1.97a

Values labeled with the same letters on each row are not significantly different ($P < 0.05$).

March ($F_{14,89}$ = 48.18, P < 0.0001). In addition, at each temperature, the lowest and the highest rates of egg hatching were observed at the onset and termination of diapause, respectively. In most cases, the survival rates at different temperatures and exposure times were not significantly different across the months. For example, at the onset of diapause, when the eggs were exposed to 5°C for 24 h, the survival rate was 13.48%, while the survival rate was 14% when the eggs were exposed to -25°C for 48 h. At the end of diapause, these rates reached 61.00 and 74.33%, respectively. However, the lowest and the highest survival rates were observed at the onset and end of diapause, respectively.

The results of the biochemical analysis are presented in figure 1. The highest amount of glycogen was observed at the beginning of overwintering (November = 46.16 µg/ g body weight). The glycogen content substantially decreased ($F_{4,14}$ = 137.56, P < 0.0001) from November onward and reached the lowest level in February and March, *i.e.*, 22.88 µg/g body weight (Fig. 1). The changes in the total simple sugar contents showed the opposite trend to the glycogen changes, namely $F_{4,14}$ = 12610.3, P < 0.0001 (Fig. 1). The sugar content was lowest (97.27 µg/g body weight) in November and peaked, *i.e.*, 454.41 µg/g body weight, in March. The protein content of the overwintering eggs of *L. malicola* significantly increased as diapause lasted longer ($F_{4,14}$ = 1723.64, P < 0.0001). The changes in the protein content also showed a reverse trend compared to those of glycogen content. The lowest and the highest amounts of protein were observed in November (8.64 µg/g body weight) and March (84.35 µg/g body weight), respectively. The lipid content of the overwintering eggs of *L. malicola* also significantly increased in the prolonged diapause and peaked (542.61 µg/g body weight) in March ($F_{4,14}$ = 2242.14, P < 0.0001).

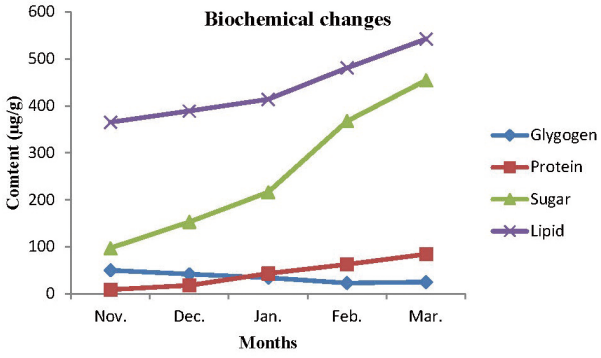

Fig. 1. Biochemical changes during overwintering of *Lepidosaphes malicola* eggs.

DISCUSSION

The results of the study strongly suggest that cold exposure is a prerequisite for diapause development and complementation in the eggs of *Lepidosaphes malicola*. Moreover, the results of this study indicate that cold acclimation significantly affect the survival and hatching rates of the diapausing eggs of the pest. The hatching rate of the acclimated eggs increased as diapause lasted longer. When the eggs were acclimated from 5°C to -25°C for 24 h, the survival rates increased from about 11% at the beginning of diapause (November) to about 80% at the end (March). Based on the result of the current study, both the temperature and duration

Table 2. Effect of rapid cold hardening on the hatching rate (%) of overwintering eggs of *Lepidosaphes malicola*

Temp (°C)	Time (h)	Survival rate (%)				
		November	December	January	February	March
5	24	13.84 ± 0.74dA	23.83 ± 0.99cA	49.33 ± 1.08bA	42.00 ± 1.71bA	61.00 ± 1.71aA
	48	12.67 ± 0.21eA	12.67 ± 0.45dB	36.00 ± 0.81cB	58.66 ± 1.97bB	77.66 ± 1.97aB
	72	14.00 ± 0.93dA	24.00 ± 1.17cA	59.00 ± 1.00bC	52.66 ± 0.66bB	73.00 ± 2.00aB
-10	24	10.00 ± 0.57dA	15.50 ± 0.82dA	23.67 ± 1.17cA	56.00 ± 1.78bA	77.00 ± 0.89aA
	48	12.33 ± 0.88dA	27.33 ± 1.12cB	47.00 ± 1.23bB	53.33 ± 1.68bA	74.33 ± 1.90aA
	72	12.17 ± 0.40cA	17.17 ± 0.64cA	25.33 ± 1.61bA	59.33 ± 1.22aA	54.33 ± 9.30aB
-20	24	5.33 ± 0.49cA	7.33 ± 0.73cA	10.67 ± 0.66cA	62.00 ± 0.89bA	73.50 ± 7.94aA
	48	16.00 ± 0.44cB	17.33 ± 0.69cB	18.67 ± 0.98cB	62.00 ± 1.71bA	82.33 ± 1.22aB
	72	10.67 ± 0.42cC	12.17 ± 0.66cC	13.33 ± 0.98cA	60.00 ± 1.03bA	79.66 ± 0.66aB
-25	24	9.17 ± 0.93dA	14.67 ± 0.89cdA	17.33 ± 0.88cA	55.33 ± 1.90bA	76.33 ± 1.97aA
	48	14.00 ± 0.73cB	15.50 ± 0.97cA	17.33 ± 0.21cA	57.33 ± 2.45bA	74.33 ± 2.17aA
	72	7.33 ± 0.71cA	8.83 ± 0.95cB	10.67 ± 0.61cB	52.66 ± 2.81bA	72.33 ± 2.45aA

The temperature values labeled with the same lowercase letters on each row are not significantly different. The temperature values labeled with the same uppercase letters on each column are not significantly different (P < 0.05).

of cold acclimation modulated the cold hardiness of the diapausing eggs of *L. malicola*. The same results were reported by Hanson and Craig (1995) concerning the eggs of *Aedes albopictus* (Diptera: Culicidae). The 24-hr cold acclimation of the diapausing eggs substantially enhanced their cold hardiness. This enhancement was directly proportional to diapause development. On the other hand, the greatest effect (hatching of about 80% of the eggs) of cold acclimation was achieved in the well-developed diapausing eggs. Hanson and Craig Jnr (1995) found that the cold hardiness of the temperate *Aedes albopictus* could increase if both cold acclimation and diapause length increased; however, the influence of cold acclimation was greater than that of diapause length. An increase in the thickness of the middle serosa and in the separation of serosa from the endochorion of the cold-acclimated, diapause-induced eggs of the Asian tiger mosquito, *A. albopictus*, was reported by Kreß et al. (2016). This change was an adaptation strategy to prevent ice formation in the inter-membranous space of the cells. In this species, the eggs were the most cold-tolerant developmental stage (Kreß et al. 2016).

Cold acclimation usually takes place over days to weeks, whereas RCH has been defined as the capacity of an insect to rapidly (within minutes to hours) enhance its cold tolerance and protect itself against the deleterious effects of the cold (Shreve et al. 2004; Lee and Denlinger 2010; Kawarasaki et al. 2013). On the other hand, RCH is a diurnal adaptive response to short-term stimuli, while cold acclimation is a seasonal adaptive response to long-term stimuli (Lee 1989; Shintani and Ishikawa 2007), although they are both adaptation strategies for survival under low temperatures. In the current study, the rapid exposure of the eggs to 5°C enhanced the cold tolerance of the eggs as diapause developed; when the eggs were rapidly exposed to -10°C, from early to mid-diapause, the egg survival decreased. RCH, however, enhanced the cold tolerance of the eggs at the end of diapause. Shintani and Ishikawa (2007) studied the effects of the rapid cold hardening and the cold acclimation on the eggs of the yellow-spotted longicorn beetle, *Psacothea hilaris* (Pascoe) (Coleoptera: Cerambycidae). They found that RCH had a transient effect, while cold acclimation enhanced cold tolerance. Rapid cold hardening may increase chilling and cold-shock tolerance of some of insect the species. Lee et al. (2006) showed that RCH enhanced freezing tolerance of the Antarctic midge, *Belgica antarctica* (Diptera, Chironomidae). Li et al. (2001) reported that RCH increased the survival of the pine needle gall midge of the overwintering larvae of *Codiplosis japonensis* more effectively than the cold acclimation did. In this larva, the effect of cold acclimation was found to be transient.

Our study also indicates that cold acclimation and diapause are accompanied by physiological changes, *i.e.*, reduction of glycogen and elevation in simple sugars (mono- and disaccharides), protein, and lipid contents. In the cold-acclimated eggs of the *L. malicola* with early diapausing, the glycogen content was about twice that of the eggs with fully-developed diapause. The changes in the glycogen content were reversely proportional to the changes in the total simple sugar content. This finding suggests that inter-conversion occurs from glycogen to simple sugars under cold acclimation and diapause development. The same results were reported regarding the pistachio white leaf borer, *Ocneria terebinthina* Strg. (Lepidoptera: Lymantriidae) (Behroozi et al. 2012), the pistachio fruit hull borer, *Arimania comaroffi* (Ragonot) (Lep.: Pyralidae) (Bemani et al. 2012), the common pistachio psylla, *Agonoscena pistaciae* Burckhardt and Lauterer (Hemiptera: Psyllidae) (Sadeghi et al. 2012), the carob moth, *Ectomyelois ceratoniae* Zeller (Lep.: Pyralidae) (Heydari and Izadi 2014), the almond wasp, *Eurytoma amygdali* (Hymenoptera: Eurytomidae) (Khanmohamadi et al. 2016), and the pistachio twig borer, *Kermania pistaciella* (Lepidoptera: Tineidae) (Mollaei et al. 2016). Based on the results, it seems that the diapausing eggs of the *L. malicola* used glycogen as the source of the energy reserve and the main source of total simple sugars and polyols. Total simple sugar levels increased with the development of diapause. Therefore, these low molecular weight carbohydrates, as cryoprotectants, might help increase egg survival. Our results showed that the changes observed in the biochemical contents were directly proportional to diapause development and cold tolerance enhancement. The highest amounts of total sugars and polyols were reported for the cold-acclimated fully-developed diapausing eggs. The total amino acids and lipid contents also reached their highest levels in the cold-acclimated, fully-developed diapausing eggs. Šlachta et al. (2002) reported some physiological changes in the cold-acclimated non-diapausing adults of *Pyrrhocoris apterus* (Heteroptera). Ding et al. (2003) also reported several physiological modifications together with the accumulation of metabolic reserves in the cold-acclimated diapausing pupae of the cabbage armyworm, *M. brassicae*. Overwintering in most insects usually lasts for numerous months. The accumulation of the energy reserves is a prerequisite for survival during this stressful, prolonged period (Hahn and Denlinger 2010; Hodek 2012). Lipid and glycogen are the principal energy reserves for most overwintering insects (Goto et al. 2001; Koštál et al. 2007; Behroozi et al. 2012; Bemani et al. 2012; Sadeghi et al. 2012; Heydari and Izadi 2014; Mollaei et al. 2016). In some diapausing insects, lipids have been reported to be an important

form of energy reserves (Han and Bauce 1998; Behroozi et al. 2012; Bemani et al. 2012; Sadeghi et al. 2012; Heydari and Izadi 2014; Yang et al. 2018). However, in the current study, lipid and protein contents significantly increased during diapause and peaked at the end of diapause (March). Therefore, in the diapausing eggs of *L. malicola*, lipids could not be considered as a source of energy. The same results were reported by Goto et al. (1997) and Koštál et al. (1998). Lehmann et al. (2016) found that lipid reserves did not decrease in the diapausing pupae of *Pieris napi*. Thus, based on our results, the lipid reserve was utilized by the eggs only at the onset of growth and metamorphosis. Indeed, it can be concluded that the diapausing eggs of the *L. malicola* mostly rely on the accumulation of simple sugars, polyols, proteins, and lipids to enhance cold tolerance during diapause development.

CONCLUSIONS

Cold exposure is essential for the survival of the diapausing eggs of the *L. malicola.* Moreover, cold acclimation and diapause are two interdependent phenomena, enhancing the survival of these eggs. A direct relationship was found between the survival rate of the eggs and the accumulation of some biochemical components.

Acknowledgment: We thank the Isfahan University of Technology, Deputy of Research for the grant to Dr. Poorjavad.

Authors' contributions: NP and PN conceived and designed the research and conducted the experiments. HI contributed to the analytical tools and the analysis of the data. HI wrote the manuscript.

Competing interests: The authors declare that they have no conflict of interests.

Availability of data and materials: Data are available upon reasonable request.

Consent for publications: All authors consent to the publication of this manuscript.

Ethics approval consent to participate: Not applicable.

REFERENCES

Andreadis SS, Athanassiou CG. 2017. A review of insect cold hardiness and its potential in stored product insect control. Crop Prot **91:**93–99. doi:10.1016/j.cropro.2016.08.013.

Behroozi E, Izadi H, Samih MA, Moharamipour S. 2012. Physiological strategy in overwintering larvae of pistachio white leaf borer, *Ocneria terebinthina* Strg. (Lepidoptera: Lymantriidae) in Rafsanjan, Iran. Ital J Zool **79:**44–49. doi:10.1080/11250003.2011.592152.

Bemani M, Izadi H, Mahdian K, Khani A, Samih MA. 2012. Study on the physiology of diapause, cold hardiness and supercooling point of overwintering pupae of the pistachio fruit hull borer, *Arimania comaroffi*. J Insect Physiol **58:**897–902. doi:10.1016/j.jinsphys.2012.04.003.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:**248–254. doi:10.1016/0003-2697(76)90527-3.

Cira TM, Venette RC, Aigner J, Kuhar T, Mullins D, Gabbert SE, Hutchison WD. 2016. Cold tolerance of *Halyomorpha halys* (Hemiptera: Pentatomidae) across geographic and temporal scales. Environ Entomol **45:**484–491. doi:10.1093/ee/nvv220.

De Barro PJ, Liu S-S, Boykin LM, Dinsdale AB. 2011. *Bemisia tabaci* : A statement of species status . Annu Rev Entomol **56:**1–19. doi:10.1146/annurev-ento-112408-085504.

Ding L, Li Y, Goto M. 2003. Physiological and biochemical changes in summer and winter diapause and non-diapause pupae of the cabbage armyworm, *Mamestra brassicae* L. during long-term cold acclimation. J Insect Physiol **49:**1153–1159. doi:10.1016/j.jinsphys.2003.08.012.

Esmaili M. 1983. Important pests of fruit trees. Sepehr Publication, Tehran, Iran.

Feng Y, Zhang L, Li W, Yang X, Zong S. 2018. Cold hardiness of overwintering larvae of *Sphenoptera* sp. (Coleoptera: Buprestidae) in Western China. J Econ Entomol **111:**247–251. doi:10.1093/jee/tox304.

Goto M, Fujii M, Suzuki K, Sakai M. 1997. Factors affecting carbohydrate and free amino acid content in overwintering larvae of *Enosima leucotaeniella*. J Insect Physiol **44:**87–94. doi:10.1016/S0022-1910(97)00098-X.

Goto M, Li YP, Kayaba S, Outani S, Suzuki K. 2001. Cold hardiness in summer and winter diapause and post-diapause pupae of the cabbage armyworm, *Mamestra brassicae* L. under temperature acclimation. J Insect Physiol **47:**709–714. doi:10.1016/S0022-1910(00)00164-5.

Hahn DA, Denlinger DL. 2010. Energetics of insect diapause. Annu Rev Entomol **56:**103–121. doi:10.1146/annurev-ento-112408-085436.

Han ERN, Bauce E. 1998. Timing of diapause initiation, metabolic changes and overwintering survival of the spruce budworm, *Choristoneura fumiferana*. Ecol Entomol **23:**160–167. doi:10.1046/j.1365-2311.1998.00111.x.

Hanson SM, Craig Jnr GB. 1995. Cold acclimation, diapause, and geographic origin affect cold hardiness in eggs of *Aedes albopictus* (Diptera: Culicidae). J Med Entomol **31:**192–201. doi:10.1093/jmedent/31.2.192.

Heydari M, Izadi H. 2014. Effects of seasonal acclimation on cold tolerance and biochemical status of the carob moth, *Ectomyelois ceratoniae* Zeller, last instar larvae. Bull Entomol Res **104:**592–600. doi:10.1017/S0007485314000364.

Hodek I. 2012. Adult diapause in Coleoptera. Psyche (Stuttg). **2012:**1–10. doi:10.1155/2012/249081.

Kawarasaki Y, Teets NM, Denlinger DL, Lee RE. 2013. The protective effect of rapid cold-hardening develops more quickly in frozen versus supercooled larvae of the Antarctic midge, *Belgica antarctica*. J Exp Biol **216:**3937–3945. doi:10.1242/jeb.088278.

Khanmohamadi F, Khajehali J, Izadi H. 2016. Diapause and cold hardiness of the almond wasp, *Eurytoma amygdali* (Hymenoptera: Eurytomidae), Two independent phenomena. J Econ Entomol **109:**1646–1650. doi:10.1093/jee/tow150.

Koštál V, Sula J, Simek P. 1998. Physiology of drought tolerance and cold hardiness of the mediterranean tiger moth *Cymbalophora pudica* during summer diapause. J Insect Physiol **44:**165–173. doi:10.1016/S0022-1910(97)00047-4.

Koštál V, Zahradníčková H, Šimek P, Zelený J. 2007. Multiple component system of sugars and polyols in the overwintering spruce bark beetle, *Ips typographus*. J Insect Physiol **53:**580–586. doi:10.1016/j.jinsphys.2007.02.009.

Kreß A, Kuch U, Oehlmann J, Müller R. 2016. Effects of diapause and cold acclimation on egg ultrastructure: New insights into the cold hardiness mechanisms of the Asian tiger mosquito *Aedes* (*Stegomyia*) *albopictus*. J Vector Ecol **41:**142–150. doi:10.1111/jvec.12206.

Lee RE. 1989. Insect cold-hardiness: to freeze or not to freeze. Bioscience **39:**308–313. doi:10.2307/1311113.

Lee RE. 1991. Principles of insect low temperature tolerance. *In*: Lee RE, Denlinger DL (eds) Insect at low temperature. Springer, Boston, MA, USA. doi:10.1007/978-1-4757-0190-6_2.

Lee RE, Denlinger DL. 2010. Rapid cold-hardening: Ecological significance and underpinning mechanisms. *In*: Denlinger DL, Lee RE (eds) Low temperature biology of insects. Cambridge University Press, UK.

Lee RE, Elnitsky MA, Rinehart JP, Hayward SAL, Sandro LH, Denlinger DL. 2006. Rapid cold-hardening increases the freezing tolerance of the Antarctic midge *Belgica antarctica*. J Exp Biol **209:**399–406. doi:10.1242/jeb.02001.

Lehmann P, Pruisscher P, Posledovich D, Carlsson M, Kakela R, Tang P, Nylin S, Wheat CW, Wiklund C, Gotthard K. 2016. Energy and lipid metabolism during direct and diapause development in a pierid butterfly. J Exp Biol **219:**3049–3060. doi:10.1242/jeb.142687.

Li YP, Gong H, Park HY, Goto M. 2001. Rapid cold hardening providing higher cold tolerance than cold acclimation in the pine needle gall midge *Thecodiplosis japonensis* larvae. Insect Sci **8:**81–88. doi:10.1111/j.1744-7917.2001.tb00438.x.

Milonas PG, Savopoulou-Soultani M. 1999. Cold hardiness in diapause and non-diapause larvae of the summer fruit tortrix, *Adoxophyes orana* (Lepidoptera: Tortricidae). Eur J Entomol **96:**183–187.

Mohammadzadeh M, Borzoui E, Izadi H. 2017. Physiological and biochemical differences in diapausing and nondiapausing larvae of *Eurytoma plotnikovi* (Hymenoptera: Eurytomidae). Environ Entomol **46:**1424–1431. doi:10.1093/ee/nvx128.

Mollaei M, Izadi H, Šimek P, Koštál V. 2016. Overwintering biology and limits of cold tolerance in larvae of pistachio twig borer, *Kermania pistaciella*. Bull Entomol Res **106:**538–545. doi:10.1017/S0007485316000237.

Sadeghi R, Izadi H, Mahdian K. 2012. Energy allocation changes in overwintering adults of the common pistachio psylla, *Agonoscena pistaciae* Burckhardt & Lauterer (Hemiptera: Psyllidae). Neotrop Entomol **41:**493–498. doi:10.1007/s13744-012-0077-1.

Shintani Y, Ishikawa Y. 2007. Relationship between rapid cold-hardening and cold acclimation in the eggs of the yellow-spotted longicorn beetle, *Psacothea hilaris*. J Insect Physiol **53:**1055–1062. doi:10.1016/j.jinsphys.2007.05.012.

Shreve SM, Kelty JD, Lee RE. 2004. Preservation of reproductive behaviors during modest cooling: Rapid cold-hardening fine-tunes organismal response. J Exp Biol **207:**1797–1802. doi:10.1242/jeb.00951.

Šlachta M, Vambera J, Zahradníčková H, Koštál V. 2002. Entering diapause is a prerequisite for successful cold-acclimation in adult *Graphosoma lineatum* (Heteroptera: Pentatomidae). J Insect Physiol **48:**1031–1039. doi:10.1016/S0022-1910(02)00191-9.

Su H, Zou J, Zhou Q, Yu Q, Yong Y, Yang Y. 2017. Better cold tolerance of Bt-resistant *Spodoptera exigua* strain and the corresponding cold-tolerant mechanism. Pestic Biochem Physiol **140:**51–57. doi:10.1016/j.pestbp.2017.06.003.

Teets NM, Gantz JD, Kawarasaki Y. 2020. Rapid cold hardening: ecological relevance, physiological mechanisms and new perspectives. J Exp Biol **223:**jeb203448. doi:10.1242/jeb.203448.

Wang J, Gao G, Zhang R, Dai L, Chen H. 2017. Metabolism and cold tolerance of Chinese white pine beetle *Dendroctonus armandi* (Coleoptera: Curculionidae: Scolytinae) during the overwintering period. Agric For Entomol **19:**10–22. doi:10.1111/afe.12176.

Warburg MS, Yuval B. 1997. Effects of energetic reserves on behavioral patterns of Mediterranean fruit flies (Diptera: Tephritidae). Oecologia **112:**314–319. doi:10.1007/s004420050314.

Yang G, Wen J, Han Y, Hou M. 2018. Rapid cold hardening confers a transient increase in low temperature survival in diapausing *Chilo suppressalis* larvae. Insects **9(2):**53. doi:10.3390/insects9020053.