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# Physiological and Biochemical Differences in Diapausing and Nondiapausing Larvae of *Eurytoma plotnikovi* (Hymenoptera: Eurytomidae)

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## Abstract

The pistachio seed wasp, *Eurytoma plotnikovi* Nikol'skaya (Hymenoptera: Eurytomidae), is one of the main pests in various pistachio growing regions of Iran. This pest passes the winter as diapausing last instar larvae. In this study, the relationship between diapause and cold hardiness and also the physiological and biochemical characteristics the diapausing and nondiapausing larvae of *E. plotnikovi* were investigated. Digestive  $\alpha$ -amylase enzyme showed a high activity ( $70.41 \pm 2.36$   $\mu$ g maltose/min per mg protein) in nondiapausing larvae, but its activity vigorously decreased during the diapause period. Glycogen declined at the beginning of diapause until March. Decrease in glycogen content was proportional to increase in total simple body sugars, trehalose, myo-inositol, and sorbitol contents. Lipid accumulated from the onset of diapause in September until January reaching a high concentration of 28.74 mg/g fresh body weight, but then declined from March to end of diapause in April. The supercooling points were decreased from August ( $-17.68 \pm 0.14^\circ\text{C}$ ) to January and reached to its lowest point in January ( $-23.14 \pm 0.27^\circ\text{C}$ ), the coldest month of the year, then gradually increased through April ( $-21.38 \pm 0.32^\circ\text{C}$ ). The survival rates at low temperature indicate that last instar larvae of *E. plotnikovi* are most cold tolerant in December–February when total body sugars, trehalose, myo-inositol, and sorbitol concentration is high, suggesting an alternative cryoprotective role for these compounds. The experimental data show that *E. plotnikovi* is freeze avoidance insect.

**Key words:** Amylolytic activity, cold hardiness, metabolic rate, supercooling point, the pistachio seed wasp

The pistachio wasps, serious pests of pistachio, are widely distributed in various pistachio growing regions of Iran, Greece, and several areas of Middle East (Nikol'skaya 1934, Davatchi 1956, Mourikis et al. 1998). This pest is an univoltine and monophagous pest in pistachio planting areas of Iran. In Rafsanjan, the larvae of *Eurytoma plotnikovi* Nikol'skaya (Hymenoptera: Eurytomidae) which feed on the growing cotyledons complete their development in August. Initiation and termination of diapause, respectively, occurs in September and end of March followed by a period of post-diapause quiescence till May (Basirat and Sayed-Al-Eslami 2001).

Temperature varies on multiple temporal and spatial scales in the environment that affect the abundance and distribution of insects. The insects face the multiple stresses of winter, and adjust their life histories around this important season (Danks 2006, Hahn and Denlinger 2011). During cold seasons, low temperatures and freezing can induce physiological damage in all overwintering developmental stages, which can lead to mortality (Lee 2010, MacMillan and Sinclair 2011). The ability of insect to cope with low temperatures depends on the basal thermal tolerance and on their capacity to

respond to thermal variations via change in membrane composition, enzyme activity, and concentration of compatible solutes (Chown and Terblanche 2007, Clarke and Worland 2008, Nyamukondiwa et al. 2011).

Diapause has been defined as dormancy resulting from responding to adverse environmental conditions (Denlinger 1991). It is a dynamic process consisting of several distinct phases, each characterized by a particular set of biochemical and physiological changes (Košťál 2006, King and MacRae 2015). Many species of insects that establish in temperate or colder climate areas enhance their cold tolerance before the onset of winter and spend the winter in diapause in a characteristic life stages (Denlinger 1991, Lester and Irwin 2012).

Based on physiological investigations, it is often stated that there are three main strategies for survival as diapausing at low temperatures in winter, which are chill-susceptible, freeze-tolerance, and freeze-avoidant (Sinclair et al. 2015). According to this classification, the freeze-intolerant (or freeze avoiding) insects are capable of decreasing their supercooling points (SCPs) under cold temperature stress, but freeze-tolerant initiates ice formation in extracellular

spaces to survive below their SCPs. The cold hardness of an insect species can be measured by its SCP (Bale 1996, Renault et al. 2002). For the unfavorable thermal conditions, an insect synthesizes and accumulates various types of low-molecular-weight compounds, such as polyhydric alcohols (polyols), sugars, and some of the amino acids (Teets and Denlinger 2013). The synthesis of these compounds whose main task is to inhibit the growth of ice lattice has been reported in some insects for resistance to future hazardous changes of environmental conditions (Hodkova and Hodek 2004, Andreadis et al. 2011, Bemani et al. 2012, Wasielewski et al. 2013, Heydari and Izadi 2014).

Several works focused on seasonal cold-hardening, in which insects used environmental stimuli such as decreasing photoperiod, temperature, or both to trigger physiological changes enhancing cold tolerance (Li et al. 2003, Kurban et al. 2007, Everatt et al. 2012, Bemani et al. 2012, Kořtal et al. 2014, Khanmohamadi et al. 2016, Mollaei et al. 2016). However, the physiological and biochemical characteristics of overwintering pistachio wasps are not understood. Therefore, we hypothesized that diapause might contribute to the supercooling capacity, energy reserves, and accumulation of cryoprotectants of *E. plotnikovi* larvae.

In this study, we monitored SCP, survival at subzero temperatures, and levels of amylolytic activity, total body sugars, glycogen, lipid, protein, trehalose, sorbitol, myo-inositol, and glucose at the nondiapausing and diapause stages of *E. plotnikovi* last instar larvae. This information is essential to the development of a theoretical foundation for a better understanding of overwintering strategy in this pest and for improving forecasts of the population dynamics of this pest according to thermal field condition in various years.

## Materials and Methods

### Weather Data

Environmental temperature data were obtained from the nearest Data Processing Center of the Iran Meteorological Organization (IMO; Fig. 1). The Rafsanjan township with an area of about 7,678 km<sup>2</sup> and an altitude of 1,469 m, located in the North West of Kerman province (31°13'N, 55°59'E). This county is the main pistachio producing area of the world. About 96% of the agricultural farms are dedicated to pistachio trees. Because of proximity

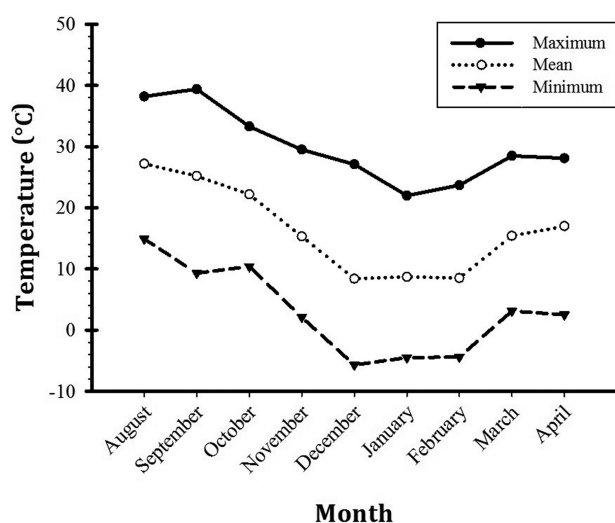


Fig. 1. Seasonal changes in maximum, mean, and minimum ambient temperature in Rafsanjan between August 2015 and April 2016.

to the desert, the county has a semiarid climate with relatively hot summers, and dry and cold winters (the average yearly temperature ranging from an average minimum of -18°C to a maximum of 43°C). High temperature and constant wind blowing increased the evaporation rate. The annual average rainfall in this area is less than 150 mm.

### Chemicals

Substrates, buffers, reagents, standards and dinitrosalicylic acid (DNS) were purchased from Sigma Chemical Co. (St. Louis, USA). Bovine serum albumin (BSA) was purchased from Roche Co. (Germany). Alcohols were purchased from Merck Co. (Germany).

### Experimental Insects

Experiments were conducted in 2015–2016 during the growth and overwintering periods of *E. plotnikovi* larvae. Nondiapausing (August 2015) and last instar diapausing (from September 2015 to April 2016) larvae of *E. plotnikovi* were collected from infected fruits that were remained upon the pistachio trees, in Rafsanjan, Iran (35°39'N, 52°05'E; alt. 1,800 m).

### Preparation of Whole-Body Homogenates and Enzyme Assays

The experiments started in August after larvae reached last instar. The experimental material was collected once in the beginning of each month. Nondiapausing (August) and diapausing (January) last instar larvae were dissected following the procedure described by Borzoui and Bandani (2013). In brief, larvae were dissected in pre-cooled distilled water under stereomicroscope (Stemi SV6 ZEISS, Germany). The midguts were homogenized in 0.15 M NaCl on ice using a pre-cooled homogenizer (Teflon pestle). The homogenates were centrifuged at 15,000 × g for 15 min at 4°C. The supernatant was recovered and frozen (-20°C) for enzymatic assays.

### Enzymes Activity Assay

Amylolytic activity of nondiapausing (August) and diapausing (January) last instar larvae was measured with the dinitrosalicylic acid (DNS) method using soluble starch (1%) as substrate, following the method adapted (Borzoui and Bandani 2013) from Bernfeld (1955). The activity assays were carried out incubating 20 µl of the enzyme extracted from 30 midguts + 500 µl of MES buffer (2-(N-morpholino)ethanesulfonic acid) (50 mM; pH 5.0) and 40 µl of starch solution at 37°C for 30 min. The reaction is interrupted by adding 100 µl of DNS and heating in boiling water for 10 min. The absorbance was measured at 540 nm. Activity values of amylase were expressed as micograms of maltose per minute per milligram protein, which refers to the amount of amylase that produce 1 mg maltose in 30 min at 37°C. A control containing no α-amylase extract with substrate was run simultaneously with the reaction mixture. This experiment was repeated five times.

### Biochemical Analysis

#### Total Body Sugars

Total body sugars (mono- and disaccharides) were extracted from individual larvae by the method of Warburg and Yuval (1997). The nondiapausing (August) and diapausing (September–April) last instar larvae of *E. plotnikovi* were weighed, and homogenized in 200 µl of 2% Na<sub>2</sub>SO<sub>4</sub>. For extraction of carbohydrates, 1,300 µl of chloroform-methanol (1:2) was added to the homogenate and centrifuged for 10 min at 7,150 × g at 4°C. Three hundred microliters of the supernatant was dissolved in 200 µl of distilled water,

and then 1 ml of anthrone was added to the solution. The mixture was stirred and heated for 15 min at 90°C and then cooled to room temperature. The absorbance was measured at 630 nm on a spectrophotometer (T60U, Harlow Scientific, Arlington, United States) using glucose (Sigma) as a standard. This experiment was repeated five times in each month with individual sample.

#### Glycogen Content

The pellet resulting from the centrifugation mentioned earlier was used for the determination of glycogen content. The pellet was washed by vigorous shaking in 400  $\mu$ l of 80% ethanol, thus removing possible remnants of sugars. To extract the glycogen, the washed pellet was dissolved in 250  $\mu$ l of distilled water and the final mixture was heated for 5 min at 70°C. Subsequently, 200  $\mu$ l of the solution was incubated with 1 ml of anthrone for 10 min at 90°C. After cooling at room temperature, the absorbance of the solution was measured at 630 nm. The glycogen content was determined by comparison to a standard curve that was prepared using glycogen (Sigma). This experiment was repeated five times in each month with individual sample.

#### Lipids Content

To measure lipid content of nondiapausing (August) and diapausing (September–April) last instar larvae of *E. plotnikovi*, 300  $\mu$ l of supernatant from the total body sugar determination experiment was evaporated at 35°C in an oven. Then, 300  $\mu$ l of H<sub>2</sub>SO<sub>4</sub> was added to the sample of each tube. The sample was mixed well, heated at 90°C for 15 min, cooled to room temperature, and stirred. Then 2,700  $\mu$ l of vanillin was added to the samples. Tubes were shaken for 30 min at room temperature. The total lipid content was determined by measuring the absorbance at 530 nm using on a spectrophotometer. Tripalmitin (Sigma) was used as a standard (Warburg and Yuval 1997). This experiment was repeated five times in each month with individual sample.

#### Protein Content

The residue from the polyol assay was resuspended in a solution of 1% SDS containing 0.4% sodium hydroxide, 2% sodium carbonate, and 0.18% sodium tartarate, and left overnight to solubilize the protein and centrifuged for 3 min at 8,000  $\times$  g. The concentration of protein in the samples was determined with the procedure of Lowry et al. (1951) adapted by Markwell et al. (1978). Bovine serum albumin (Sigma) was used as a standard. The absorption was measured at 595 nm. This experiment was repeated five times in each month with individual sample.

#### Low-Molecular-Weight Carbohydrates and Polyols

Trehalose, glucose, sorbitol, and myo-inositol were studied from all samples (nondiapausing and diapausing last instar larvae). The extraction, derivatization, and analytical procedures (high-performance liquid chromatography) were basically similar to those of Khani et al. (2007). The nondiapausing (August) and diapausing (September–April) last instar larvae of *E. plotnikovi* were carefully brushed to remove contaminating particles, weighed, and homogenized in 1.5–2 ml of 80% ethanol. The homogenate was centrifuged at 12,000  $\times$  g for 15 min at 4°C. Following this, the supernatant was taken and evaporated at 40°C in vacuum drying oven and then resuspended in 1 ml of HPLC grade water. Before the sample injection, the samples were further cleaned by passing through a 20  $\mu$ m syringe filter. Analyses of sugars and sugar alcohols were performed by high-performance liquid chromatography (Knauer, Berlin, Germany) using a carbohydrate column with 4  $\mu$ m particle size (250  $\times$  4.6 mm, I.D., Waters, Ireland). Glucose, myo-inositol, sorbitol, and trehalose were identified by comparison of retention time's authentic standards and their concentrations were determined

by the external standard method. This experiment was repeated five times in each month with individual sample.

#### Determination of SCP

The SCP of *E. plotnikovi* was determined for the nondiapausing (August) and diapausing (September–April) last instar larvae ( $n = 19$ –27). To determine SCPs, individual larvae were placed on a thermocouple (NiCr–Ni probe) connected to an automatic temperature recorder (Testo 177-T4, Testo, Germany) within a programmable refrigerated test chamber. The temperature of the refrigerated test chamber was reduced from 15 to –25°C, at a rate of 0.5°C/min. The lowest temperature reached before an exothermic event that occurred due to the release of latent heat was taken as the SCP of the individual (Mohammadzadeh and Izadi 2016).

#### Determination of Low-Temperature Survival

The nondiapausing (August) and diapausing (September–April) last instar larvae ( $n = 5$ –7) were kept in a programmable refrigerated test chamber, whose temperature was lowered slowly (0.5°C/min) from environmental temperature to the desired treatment temperature (–15, –20 and –25  $\pm$  0.5°C) and held at each temperature for 24 h. The mortality of larvae was recorded via direct observation. The larvae showing no movement in their appendages were judged to be dead (Mohammadzadeh and Izadi 2016).

#### Statistical Analysis

All the data were analyzed using SAS ver.9.2 program (PROC GLM; SAS Institute 2009). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test at  $\alpha = 0.05$ . Data were initially tested for normality (Kolmogorov–Smirnov test) and homoscedasticity (Levene's test) before subjecting them to ANOVA. Nonparametric test (Mann–Whitney *U* and Kruskal–Wallis test) was used to test for differences in the non-normal SCP and cold hardiness data. The Pearson correlation coefficient was used to investigate the relationships between SCP and physiological and biochemical characteristics of larvae, using SPSS 16.0.

## Results

#### Ambient Temperature

The mean ambient temperature in Rafsanjan, Iran, during 2015 to 2016 was 25.2°C in August, declining to 22.2°C in October, 8.4°C in December, 8.7°C in January and close to 8.5°C in February, before increasing to 15.4°C in March. The minimum monthly temperature was <0°C from December until the end of February, with the lowest in January and February (–4.5 and –4.4°C, respectively) (Fig. 1).

#### Seasonal Changes in Amylolytic Activity of *E. plotnikovi* Larvae

Diapause significantly affected digestive  $\alpha$ -amylase activity in the last instar larvae of *E. plotnikovi* ( $T = 86.47$ ,  $P < 0.001$ ). Amylolytic activity was vigorously higher in the nondiapausing larvae (70.41  $\pm$  2.36  $\mu$ g maltose/min per mg protein) than the diapausing larvae (2.23  $\pm$  0.17  $\mu$ g maltose/min per mg protein) (Fig. 2).

#### Seasonal Changes in Carbohydrates Content of *E. plotnikovi* Larvae

Data in Fig. 3 show changes in total body sugar levels in nondiapausing and different months of overwintering ( $F_{8,36} = 52.93$ ,

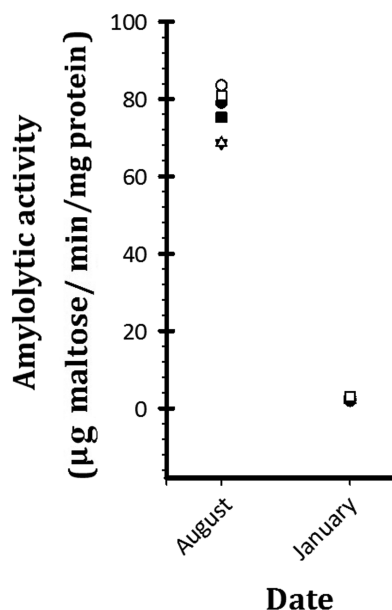


Fig. 2. Amylolytic activity in midgut of nondiapausing (August) and diapausing (January) last instar larvae of *E. plotnikovi* ( $n = 5$ ). Bars with different superscripts denote significant difference ( $P < 0.05$ ) using one-way ANOVA followed by Tukey's multiple comparison test. Data are expressed in micrograms of maltose per minutes.

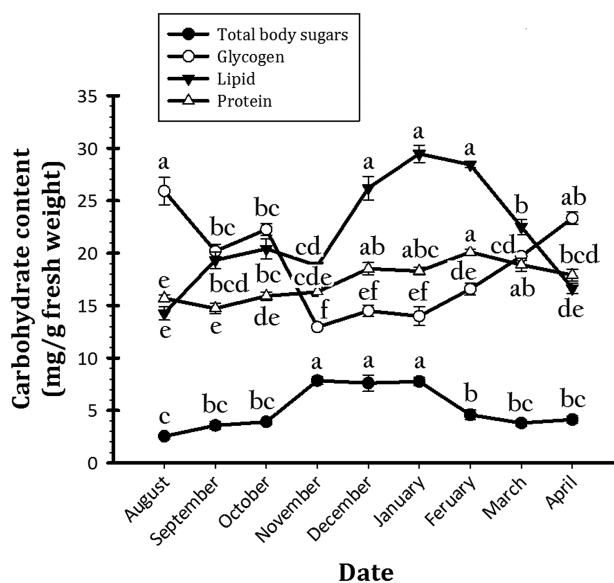


Fig. 3. Profile chemical contents of nondiapausing (August) and diapausing (September–April) last instar larvae of *E. plotnikovi* in 2015–2016. Data for each month are mean  $\pm$  SD of five individuals sampled. Bars with different superscripts denote significant difference ( $P < 0.05$ ) using one-way ANOVA followed by Tukey's multiple comparison test. Data are expressed in milligrams per gram fresh body weight.

$P < 0.001$ ). The total body sugars level remains stable in September and October, but it showed a sharp increase at the beginning of November and drop again in February (Fig. 3). Furthermore, the concentration of the total body sugars of diapausing (January) larvae was three times higher than that of nondiapausing (August) larvae (Fig. 3). Glycogen content was at relatively low level during the cold season ( $F_{8,36} = 42.15$ ,  $P < 0.001$ ). The lowest level of glycogen in different months of overwintering was observed in November

(Fig. 3). In addition, glycogen content of diapausing (January) larvae ( $14.19 \pm 1.16$  mg/g fresh body weight) was significantly lower than glycogen content of nondiapausing (August) larvae ( $25.87 \pm 1.02$  mg/g fresh body weight) (Fig. 3). Changes in glycogen content were reversely proportional to total simple sugar content.

#### Seasonal Changes in Lipids Content of *E. plotnikovi* Larvae

Lipid contents of whole-body of *E. plotnikovi* last instar larvae showed a significant change in different months of overwintering ( $F_{8,36} = 24.78$ ,  $P < 0.001$ ). The amount of lipids of diapausing (January) larvae was two times more than that of nondiapausing (August) larvae. In overwintering months, lipids content was at the lowest level in August, increased in September, reached to the maximum levels during December till February and, finally, decreased during spring (Fig. 3).

#### Seasonal Changes in Protein Content of *E. plotnikovi* Larvae

The protein content of the diapausing larvae showed little changes during overwintering, ranging between 20.32 and 14.84 with a significant rise in February, March, and April ( $F_{8,36} = 14.61$ ,  $P < 0.001$ ) (Fig. 3). When the diapausing (January) and nondiapausing (August) larvae were compared, there was a significant difference in the protein content ( $20.32 \pm 0.14$  and  $16.28 \pm 0.14$  mg/g fresh body weight, respectively) (Fig. 3).

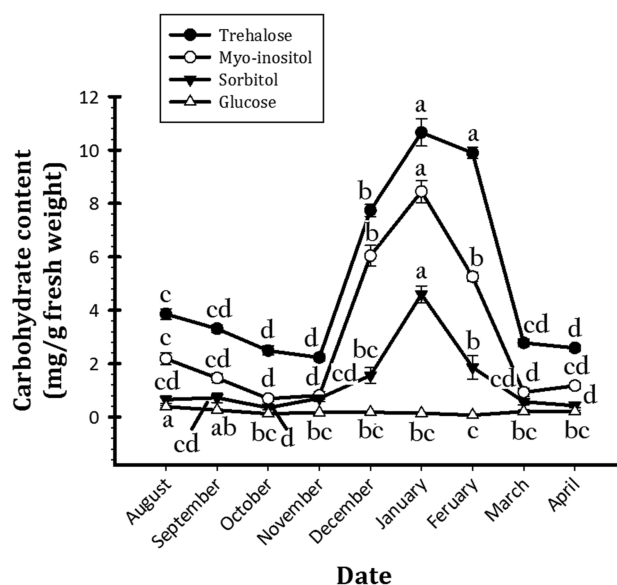
#### Seasonal Changes in Low-Molecular-Weight Carbohydrates and Polyols Content of *E. plotnikovi* Larvae

Four major carbohydrates in *E. plotnikovi* last instar larvae were found to be trehalose, myo-inositol sorbitol, and glucose. The carbohydrate levels of the larvae differed significantly during months of overwintering ( $F_{8,36} = 109.13$ ,  $P < 0.001$  for trehalose;  $F_{8,36} = 49.24$ ,  $P < 0.001$  for myo-inositol;  $F_{8,36} = 6.24$ ,  $P = 0.123$  for glucose;  $F_{8,36} = 34.53$ ,  $P < 0.001$  for sorbitol). The trehalose level in *E. plotnikovi* diapausing larvae remained stable in September to November but increased from 2.23 mg/g fresh body weight in November to 7.74 mg/g fresh body weight in December, reached to the highest level in January and February and drop again in March (Fig. 4). Myo-inositol and Sorbitol shows the same pattern of accumulation in diapausing larvae, reaching peak of concentration in January (Fig. 5). Glucose was found in high level in September, but decreased until February and again increased in March (Fig. 4). Also, a significant difference in trehalose, glucose, sorbitol, and myo-inositol levels was detected between diapausing (January) and nondiapausing (August) larvae. Diapausing larvae had higher levels of these carbohydrates compared with nondiapausing larvae (Fig. 4).

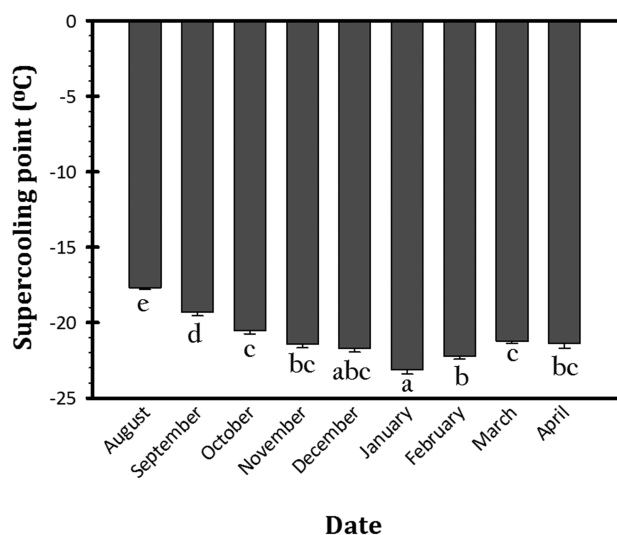
#### Seasonal Changes in SCPs and Low-Temperature Survival of *E. plotnikovi* Larvae

SCPs of diapausing larvae changed significantly during different months of diapause ( $F_{8,199} = 52.73$ ,  $P < 0.001$ ). The diapausing larvae which were collected from overwintering sites in September had a mean SCP of  $-19.32 \pm 0.22^\circ\text{C}$ . The SCP drop to its lowest point in January ( $-23.14 \pm 0.27^\circ\text{C}$ ), the coldest month of the year, then gradually increased through April ( $-21.38 \pm 0.32^\circ\text{C}$ ) (Fig. 5). The diapausing larvae that were collected in January had significantly lower SCP than those of nondiapausing larvae ( $-17.68 \pm 0.14^\circ\text{C}$ ) collected in August (Fig. 5). The survival rates of larvae exposed to low temperatures for 24 h are shown in Table 1. Diapausing (January)





**Fig. 4.** Profile carbohydrates content of nondiapausing (August) and diapausing (September–April) last instar larvae of *E. plotnikovi* ( $n = 5$ ). Data for each month are mean  $\pm$  SD of five individuals sampled. Bars with different superscripts denote significant difference ( $P < 0.05$ ) using one-way ANOVA followed by Tukey's multiple comparison test. Data are expressed in milligrams per gram fresh body weight.



**Fig. 5.** SCPs of nondiapausing (August) and diapausing (September–April) last instar larvae of *E. plotnikovi* in 2015–2016. Data for each month are mean  $\pm$  SD of 19–27 individuals sampled. Bars with different superscripts denote significant difference ( $P < 0.05$ ) using one-way ANOVA followed by Tukey's multiple comparison test.

larvae of *E. plotnikovi* showed considerably higher survival rate at low temperatures compared with nondiapausing (August) larvae. The survival rate of the larvae following 24 h exposure at  $-15^{\circ}\text{C}$  increased from 18.89% in August to 92.00% in January (Fig. 6). The survival rate of the larvae after 24 h exposure at  $-20$  and  $-25^{\circ}\text{C}$  reached about 58 and 21%, respectively, in January. The cold hardiness of diapausing larvae increased gradually from September to January, and this increase was expressed as greater capacity to survive at  $-15^{\circ}\text{C}/24$  h ( $F_{8,46} = 32.17$ ,  $P < 0.001$ ),  $-20^{\circ}\text{C}/24$  h ( $F_{8,46} = 26.54$ ,  $P < 0.001$ ) and  $-25^{\circ}\text{C}/24$  h ( $F_{8,46} = 11.01$ ,  $P < 0.001$ ). The cold hardiness of diapausing larvae decreased again in February to April (Fig. 6).

### Correlation Analysis

The analysis of correlation coefficients of the examined SCP with biochemical content of *E. plotnikovi* last instar larvae is shown in Tables 1 and 2. The results of this study showed that significant correlations existed between the SCP and low temperatures survival rate on one side and lipid, glycogen, total body sugars, protein, trehalose, myo-inositol, and sorbitol levels on the other. A positive correlation was found between SCP and glycogen content ( $r = 0.678$ ) (Table 1). Moreover, high negative correlations were found between SCP and trehalose ( $r = -0.732$ ), myo-inositol ( $r = -0.747$ ), and sorbitol ( $r = -0.752$ ) levels (Table 2). Very high positive correlations were also found between survival rate at  $-15^{\circ}\text{C}$  ( $r = 0.886$ ),  $-20^{\circ}\text{C}$  ( $r = 0.882$ ), and  $-25^{\circ}\text{C}$  ( $r = 0.779$ ) and lipid content of larvae (Table 1). Also, cold tolerance exhibited a positive correlation with trehalose, myo-inositol, and sorbitol content and a negative correlation with glucose content (Table 2).

### Discussion

This study helps to broaden our insight into the physiology of cold tolerance of *E. plotnikovi* diapausing and nondiapausing larvae. Results obtained in this study showed the presence of digestive  $\alpha$ -amylase in the *E. plotnikovi* larvae. In compatibility with Abraham et al. (1992), we found that the activity of digestive  $\alpha$ -amylase assayed in the last instar larvae had a noticeable difference in diapausing (January) and nondiapausing (August) larvae of *E. plotnikovi*. In diapausing larvae, amylolytic activity showed a 30-fold decrease when compared with nondiapausing larvae. This decline may result from a greater degradation or a lower synthesis of digestive enzymes in the passive feeding period. This trade-off between enzyme activity and feeding is consistent with the endocrinological control model of insect development (Karasov et al. 2011). In *Osmia bicornis* Linnaeus (Hymenoptera: Megachilidae),  $\alpha$ -amylase and protease activity substantially changed over the course of the overwintering season (Wasielewski et al. 2013). Furthermore, it was shown that genes encoding trypsin and a chymotrypsin-like protease are down-regulated in diapause-destined *Culex pipiens*

**Table 1.** Correlation coefficients ( $r$ ) of SCPs and cold hardiness with chemical contents of diapausing and nondiapausing (July) larvae of *E. plotnikovi*

Parameters		Total body sugars		Glycogen		Lipid		Protein	
		$r$	$P$ value	$r$	$P$ value	$r$	$P$ value	$r$	$P$ value
SCP		-0.707	0.025	0.678	0.047	-0.621	0.050	-0.766	0.013
Cold hardiness	$-15^{\circ}\text{C}$	0.680	0.044	-0.729	0.026	0.886	0.001	0.794	0.011
	$-20^{\circ}\text{C}$	0.636	0.066	-0.704	0.034	0.882	0.002	0.767	0.016
	$-25^{\circ}\text{C}$	0.564	0.114	-0.556	0.120	0.779	0.013	0.550	0.125

Linnaeus (Diptera: Culicidae) females (Robich and Denlinger 2005). The difference in enzyme activity between diapausing and nondiapausing specimens of some other species has also been reported. Košťál et al. (2004a) reported significant differences in the activities of the enzymes involved in glycogen hydrolysis and reduction of sugars to polyols between the nondiapausing and diapausing specimens of *Pyrrhocoris apterus* (L.) (Hemiptera: Pyrrhocoridae). In nondiapausing and diapausing adults of the bug, nearly 100 and 40% of glycogen phosphorylase was in its active form, respectively. Košťál et al. (2004b) found a relatively rapid increase in the activities of enzymes involved in low-molecular-weight carbohydrates and polyols metabolism during the early diapause of *P. apterus*.

Lipids, glycogen, and protein are major forms of energy reserves, and patterns of utilization of these energy substrates can differ in nondiapausing stage and during diapause (Hahn and Denlinger 2007). According to our findings, lipid content was higher in diapausing larvae than that in nondiapausing larvae, which means that diapausing larvae have the ability to reserve energy in the form of lipids and utilize it during repressed metabolism of overwintering period. Same results have been reported by Behroozi et al. (2012), Sadeghi et al. (2012), Bemani et al. (2012), and Heydari and Izadi (2014). Conversely, some other diapausing insects do not rely on lipids as the source of energy during the overwintering period (Goto et al. 1998, Košťál et al. 1998, Khanmohamadi et al. 2016, Mollaei et al. 2016). However, this was not the case for glycogen that was lower in diapausing larvae. In diapausing larvae, glycogen content

decreased and reached to the lowest levels in November till February. Low-molecular-weight carbohydrates and total simple sugar contents increased with decrease in the glycogen level. This suggests that glycogen in diapausing larvae is mobilized for the production of cryoprotectant molecules to decrease the SCP and increase cold hardiness (Heydari and Izadi 2014, Khanmohamadi et al. 2016). It might be attributed to correlation of glycogen content with supercooling ( $r = 0.678$ ) and cold hardiness of the larvae ( $r = -0.729$  for  $-15^{\circ}\text{C}$ ;  $r = 0.704$  for  $-20^{\circ}\text{C}$ ). The same results were reported by Hayakawa and Chino (1981) in diapausing pupae of the silkworm, *Philosamia cynthia pryeri* Butler (Lepidoptera: Saturniidae), Bemani et al. (2012) in diapausing larvae of *Arimania comaroffi* (Ragonot) (Lepidoptera: Pyralidae) and Khanmohamadi et al. (2016) in diapausing larvae of *Eurytoma amygdali* Enderlein (Hymenoptera: Eurytomidae). Accumulation of low-molecular-weight carbohydrates (e.g., trehalose and glucose), polyols (e.g., glycerol, sorbitol, and myo-inositol), or both in response to harsh winter conditions has been well studied in some diapausing insects (Goto et al. 2001, Košťál et al. 2007, Behroozi et al. 2012, Sadeghi et al. 2012, Bemani et al. 2012, Heydari and Izadi 2014, Khanmohamadi et al. 2016). In diapausing larvae of *E. plotnikovi*, trehalose, myo-inositol, and glycerol were found to be three major cryoprotectants. Trehalose, myo-inositol, and sorbitol levels increased as ambient temperature decreased and reached to the highest levels in January, the coldest month of the year. Accumulation of these cryoprotectants may contribute to enhancement of cold hardiness, stabilization of membranes and proteins, enhancing SCP and preventing damage to cells (Yancey 2005, Rozsypal et al. 2013, Teets and Denlinger 2013). According to correlation coefficients analysis, increase in the amounts of low-molecular-weight carbohydrates was proportional to decrease in SCP and increase in cold hardiness (Table 2). The same results have been reported by Goto et al. (2001), Khani et al. (2007), Behroozi et al. (2012), Sadeghi et al. (2012), and Bemani et al. (2012).

In the current study, protein content was greater in diapausing larvae than in nondiapausing larvae, which means that diapausing larvae reserve nutrition to maintain the development of diapause (Denlinger 1991). These have also been suggested to be the case in other diapausing insects (Adedokun and Denlinger 1985, Ding et al. 2003, Hahn and Denlinger 2007, Behroozi et al. 2012, Heydari and Izadi 2014, Khanmohamadi et al. 2016). The increased protein content during diapause may also be used to protect the insect against injury from ice crystal formation. In some insects, heat-shock proteins increased during diapause to enhance stress tolerance and maintain the integrity of cellular proteins in the harsh winters. An increase in the level of heat-shock proteins may also be used as a mechanism to enhance cold tolerance of insects during stressful periods of overwintering (Yocum 2001, Duman et al. 2004, Michaud and Denlinger 2004, Rinehart et al. 2007, Heydari and Izadi 2014).

As our results indicate, larvae of *E. plotnikovi* maintain a supercooled state during diapause to avoid freezing of their body fluid. The mean SCP of diapausing larvae (January) was about  $6^{\circ}\text{C}$  lower

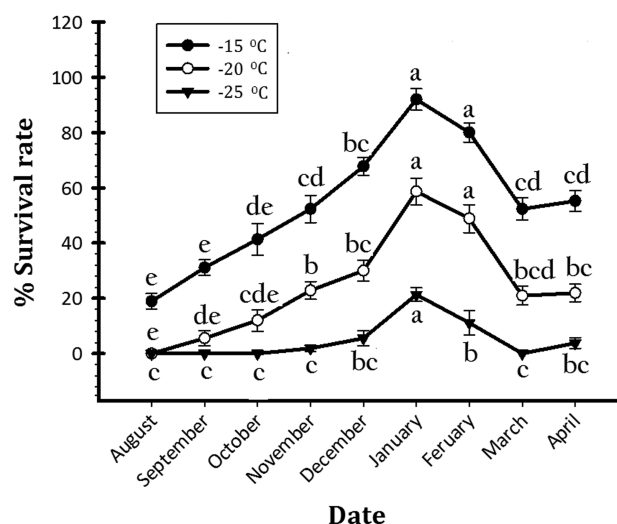


Fig. 6. Low temperatures survival rate of nondiapausing (August) and diapausing (September–April) last instar larvae of *E. plotnikovi* in 2015–2016. Data for each month are mean  $\pm$  SD of five to seven replicates. Bars with different superscripts denote significant difference ( $P < 0.05$ ) using one-way ANOVA followed by Tukey's multiple comparison test. Data are expressed in percent.

Table 2. Correlation coefficients ( $r$ ) of SCP and cold hardiness with carbohydrate contents of diapausing and nondiapausing (July) larvae of *E. plotnikovi*

Parameters	Trehalose		Myo-inositol		Sorbitol		Glucose	
	$r$	$P$ value	$r$	$P$ value	$r$	$P$ value	$r$	$P$ value
SCP	-0.732	0.020	-0.747	0.017	-0.752	0.016	0.610	0.054
Cold hardiness	-15°C	0.793	0.011	0.774	0.014	0.776	-0.765	0.016
	-20°C	0.842	0.004	0.807	0.009	0.834	0.005	0.028
	-25°C	0.884	0.002	0.897	0.001	0.962	0.000	0.183

than that of nondiapausing larvae. Continuing reduction of the SCP was proportional to enhancement of cold hardiness and accumulation of the cryoprotectants. According to the correlation analysis, there is a negative correlation between the SCP of *E. plotnikovi* last instar larvae and total body sugars, lipid, protein, trehalose, myo-inositol, and sorbitol levels. The highest levels of survival rate following 24 h exposure at  $-15$ ,  $-20$ , and  $-25$  °C were recorded in January with the lowest SCP. The increased survival rates of this pest found in the coldest month (January) might be attributed to their positive correlation ( $r = -0.900$ ) with trehalose, myo-inositol, and sorbitol levels (Table 2). In one of the earliest studies describing diapause in insects, Pullin et al. (1991) found that increased cold hardiness was associated with increased sorbitol and trehalose in *Pieris brassicae* L. (Lepidoptera: Pieridae). A recent metabolomic study of diapause in *Ectomyelois ceratoniae* Zeller (Lepidoptera: Pyralidae) also found increased levels of total body sugars, trehalose, and myo-inositol as well as reduction of glycogen (Heydari and Izadi 2014). By comparing the diapausing and nondiapausing larvae, a nearly 21 times increase in survival rate of the diapausing larvae were observed following 24 h exposure of the larvae at  $-25$ °C. High levels of survival rate following exposure of the diapausing larvae at different temperatures compared with very low levels of the nondiapausing larvae is an indication of enhanced cold hardiness. The considerable lower SCP of the diapausing larvae in comparison with nondiapausing larvae strongly supports this conclusion. Since most of the mortality diapausing larvae occurred at temperature near the lowest SCP and the larvae could not tolerate temperatures lower than the SCP, the diapausing larvae are considered to be freeze-avoidant.

In conclusion, our study shows a dynamic profile of turnover and use of nutrient reserves as well as a metabolic role for midgut for physiologically preparing *E. plotnikovi* last instar larvae for a successful overwintering. There was a sharp decline in amylolytic activity of diapausing larvae. Lipids were found to be the main source of energy during the diapausing period. Diapausing larvae of *E. plotnikovi* supercooled and enhanced their cold hardiness via accumulation of low-molecular-weight carbohydrates and specially trehalose. Diapausing last instar larvae of *E. plotnikovi* were found to be freeze-avoidant since the overwintering larvae could not tolerate temperatures lower than the SCP.

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