

Research Article

## Cold hardiness process of beet armyworm larvae, *Spodoptera exigua* (Lepidoptera: Noctuidae)

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**Abstract:** Cold hardiness is one of the most common adaptations of insects at low temperatures. To understand the cold hardiness strategy of beet armyworm, *Spodoptera exigua* (Hübner), six temperature treatments were tested on the last instar larvae. Supercooling points of treatments were not significantly different. Two sugars, glucose and trehalose, as well as three polyols, *myo*-inositol, glycerol and sorbitol, were identified in these larvae. Trehalose was more affected in response to low temperatures in longer duration, and had a significant negative correlation with glycogen content. Despite the extensive sugar beet cultivation areas in Iran, beet armyworm population has been decreased in recent years. It seems that climate change regarding the global warming especially in autumn and winter could be a reason for decline in population outbreak of beet armyworm in the next generation. Based on findings of this study, it is suggested that overwintering larvae are freeze-intolerant insects but long term acclimation at sub-zero temperatures makes larvae tolerate presence of ice crystals in their body fluid.

**Keywords:** ambient temperature, beet armyworm, *Spodoptera exigua*, cold hardiness, trehalose

### Introduction

Insects have developed behavioral and physiological adaptations to survive low temperatures during winter months. Developing cold hardiness, the ability to endure temperatures below 0 °C, is one of the most common adaptations. Two well-known cold hardiness strategies are freeze tolerance and freeze intolerance. In freeze-intolerant insects ice formation is lethal, so they develop

adaptations to avoid freezing by reducing supercooling point (SCP) and increasing the supercooling capacity. Freeze-tolerant insects can survive ice formation in extra-cellular spaces so often increase SCP before cold months and then show no conspicuous seasonal changes (Block, 1991; Lee, 1991).

Cryoprotectants, such as low molecular polyhydroxy alcohols and sugars, are accumulated in many overwintering insects. Their mode of action depends on the cold hardiness strategy of insects. In freeze-tolerant insects, cryoprotectants regulate the amount of water available for freezing, protect frozen tissues by reducing the extent of cell dehydration and stabilize cell membrane structure and proteins (Crowe *et al.*, 1987; Storey and Storey, 1988, 1991). In freeze-intolerant insects they provide colligative

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depression of freezing point and stabilize the native state of proteins at low temperatures (Zachariassen, 1985; Storey and Storey, 1991; Kostal *et al.*, 2001).

The beet army worm, *Spodoptera exigua* (Hübner), is a very broad-host-range pest accruing throughout Iran, especially in the beet cultivation areas (about 160,000 ha in 21 out of the 30 provinces of Iran), which does serious damages especially to sugar beet, potato and cotton when there is an insect outbreak. In the southern provinces with warmer climate such as Khuzestan [31° N, 48° E; 23 m above sea level (a.s.l.)] all life stages can be found throughout the winter. In Qazvin [36° N, 50° E; 1330 m a.s.l.] and Khorasan [36° N, 59° E; 1000 m a.s.l.] with severe winters, this pest enters a dormant stage as fifth (last) instar larvae or pupae on the surface or inside the soil (Khanjani, 2005). Since annual damage depends on the overwintering population of *S. exigua* (Kheyri, 1985), the study of cold hardiness strategies is concerned with much importance. Kim and Kim (1997) investigated the cold hardiness strategy of the beet army worm in South Korea. They concluded that this pest employs freeze intolerant strategy because all laboratory-rearing stages did not survive at temperatures even above their SCPs. Our previous study showed that overwintering larvae collected from November 2008 to March 2009, increased their SCPs and could successfully survive outdoor temperatures below SCPs (similar to freeze-tolerant insects) (Atapour and Moharramipour, 2011b).

On the other hand, in recent years despite extensive areas of sugar beet cultivation (Abdollahian Noghahi, 2007), the economic damage and importance of the *S. exigua* is decreasing so that it has switched from key pest to an occasional or a moderate pest (Malekzade, 2009). Therefore, this study was conducted to determine if there are any physiological changes during chilling after various treatments. We also determined cold hardiness strategy of *S. exigua* larvae in such conditions.

## Materials and Methods

### Insects

The last instar larvae of *S. exigua* were collected from the sugar beet fields in Qazvin and Karaj during July to October (35° N, 51° E; 1312 m a.s.l.) and colonized in 2009-2010. The laboratory-reared larvae used in experiment were obtained from this colony. Larvae were reared on fresh beet leaves which were changed daily and leaves' end was wrapped with wet cotton. Adults were supplied with 10% natural honey solution. All stages were kept in growth chamber set at 25 ± 2 °C, RH: 60% and a photoperiod of 16 L: 8 D (Mehrkhoh, 2011). These larvae were considered as control and 6 temperature treatments were applied as below:

- 1- Larvae reared at 25 °C (16 L: 8 D), then transferred to 15 °C (12 L: 12 D) for 72 h.
- 2- Larvae reared at 25 °C (16 L: 8 D), then transferred to 5 °C (12 L: 12 D) for 72 h.
- 3- Larvae reared at 25 °C (16 L: 8 D), then transferred to 0 °C (12 L: 12 D) for 72 h.
- 4- Larvae reared at 25 °C (16 L: 8 D), kept at 15 °C for 24 h, then 24 h at 5 °C and finally 24 h at 0 °C (12 L: 12 D).
- 5- Larvae reared at 15 °C from egg stage (12 L: 12 D).
- 6- Larvae reared at 15 °C from egg stage (12 L: 12 D), then transferred to 5 °C for 5 days (12 L: 12 D).

Temperature data were provided by the weather station of the Iran Meteorological Organization in Qazvin and Karaj.

### Measurement of supercooling points (SCPs)

The SCP of larvae (n = 6-7) was measured by using a thin thermocouple probe (NiCr-Ni probe) placed in close contact with the ventral side of individuals by means of adhesive tape. The temperature was recorded with a four channel data logger (model 177-T4; Testo, Lenzkirch, Germany) that transferred data at 10 second intervals to a computer. Data were read using Comsoft 4.0 software (Testo, Lenzkirch, Germany). Larvae with the thermocouple attached were cooled inside a Binder test chamber (model MK 53; Binder, Tuttlingen,

Germany). Temperature decreased from + 25 to -25 °C and the cooling rate was 0.5 °C /min. SCP of each individual was indicated by a sharp increase in temperature of 5-8 °C caused by the heat of fusion of ice crystallization (Neven, 1999; Woodman, 2010).

### Measurement of carbohydrates

For each treatment 3-5 replicates, which consisted of one larva, were used to measure concentrations of polyols and sugars. The extraction and other analytical procedures were similar as described by Atapour and Moharramipour (2009). Briefly, each larva was weighed and extracted in 1.5 ml of 80% ethanol. Solvent evaporated and then resuspended in 150 µL of HPLC grade water. After filtration, 30 µL of each sample was injected into HPLC with a refractive index (RI) detector controlled by Empower chromatography software (all from Waters, Milford, USA). The Ca carbohydrate column (Ca59305-U, 300 by 7.8 mm; Supelco) with a guard column (Ca59306-U; Supelco) was used for polyols and sugars separation. The mobile phase was HPLC grade water at a flow rate of 0.5 ml/min at 80 °C. Identification of the revealed components was established against authentic standards and by the LC-MS instrument using the same carbohydrate column coupled to an ion trap mass spectrometer instrument (Thermo Finnigan, USA), equipped with an ESI ion source, which was used in the positive-ion mode.

### Glycogen

Quantification of glycogen was made by a UV-Visible spectrophotometer (Sinco, model S-2100, South Korea) using the method described by Hansen *et al.*, (1951). The residue from sugars and sugar alcohols extraction was dissolved in 30% KOH. After 24 h, 0.6 ml of solution (mixture of 1 part of NaCl 2% and 7 parts of ethanol 95%) was added. Each sample was heated on a boiling water bath for 15-30 min and then cooled in the refrigerator for at least 3 h. After centrifugation at approximately 1200 g for 10 min, the supernatant was

discarded and 1 ml of ethanol 70% was added to the residue and centrifuged at 12000 g for 15 min. Current step was replicated and finally 5 ml alcohol reagent (210 ml ethanol 95%, 30 ml NaCl 2%, 30 ml SDS 1% and 30 ml distilled water) was added to the last residue. The samples were then mixed and kept at room temperature for 15 min before readings were made. Levels of absorbance were read at 660 nm in spectrophotometer. Glycogen levels were calculated with reference to a standard curve prepared using glycogen (Merck, Darmstadt, Germany).

### Statistical Analysis

Statistical analysis was performed using the SPSS version 16.0. All data were expressed as mean ± SE. Differences between treatments were done by one-way analyses of variance (ANOVAs), followed by a Tukey's test for multiple comparisons at  $P < 0.05$ . Pearson correlation was used to investigate relationships between two variables.

### Results

#### Temperature changes and supercooling points

The average of minimum temperature in January and the number of days with below -4 °C in Karaj and Qazvin are shown in Fig. 1. The Karaj weather statistics report were available from 1985 to 2005 (20 years). During this period, the average of minimum temperature in January has increased especially in 1994 and 2004, and reached 0 °C. The lowest recorded value was in 1989 with -7.8 °C (Fig. 1A). In 1989, recorded days below -4 °C were 51 days, but it was reduced to 28 days in 1998 (Fig. 1B). In Qazvin, the average minimum temperature in January has been increasing since 1994 (Fig. 1C, similar to Karaj station). Also, the number of recorded days below -4 °C has been decreasing since 1994 (Fig. 1D). Before 1994, the lowest monthly recorded temperature was -13 °C in January in 1977; however it was -5.7 °C in 1996. Overall, these

data indicate that during recent 10 years, the air temperature has relatively increased so that overwintering insects encounter fewer days of sub zero temperatures during the cold seasons.

The lowest SCP of last instar larvae in *S. exigua* belonged to control larvae ( $-9.4 \pm 0.74$  °C) that was not significantly different from other treatments ( $F_{6, 38} = 1.21$ ;  $P = 0.319$ , Fig. 2).

### Sugars and polyols

Trehalose, glucose, *myo*-inositol, glycerol and sorbitol were identified in *S. exigua*. Trehalose was dominant sugar that significantly differed among treatments ( $F_{6, 17} = 7.89$ ;  $P < 0.001$ , Fig. 3). Trehalose content remained unchanged in control and other treatments except those reared at 15 °C followed by 5 °C for 5 days. In this case, trehalose increased up to maximum level of  $3.9 \pm 0.31$  (mg/g f.w., fresh weight) compared to  $0.52 \pm 0.06$  (mg/g f.w.) in control (Fig. 3).

Glucose, in contrast to trehalose, showed the highest level in control group ( $3.6 \pm 0.36$  mg/g f.w.) compared to treatments ( $F_{6, 17} = 3.22$ ;  $P < 0.05$ ). The lowest glucose content was observed in larvae reared at 25 °C followed by 15 °C for 72 h ( $1.68 \pm 0.06$  mg/g f.w.) (Fig. 4A).

As shown in figure 4B, *myo*-inositol changes were statistically significant ( $F_{6, 17} = 3.97$ ;  $P < 0.05$ ). It had a trace amount in control group ( $0.57 \pm 0.01$  mg/g f.w.) but gradually reached the highest level of  $1.7 \pm 0.27$  (mg/g f.w.) in the last treatment.

Glycerol content was significantly affected in response to low temperatures especially in long period ( $F_{6, 17} = 3.74$ ;  $P < 0.05$ ). The changes of glycerol were similar to trehalose but at a relatively reduced level (Figs. 3 and 4-C). In control group it was at minimum level of  $0.44 \pm 0.11$  (mg/g f.w.) but reached a peak of  $2.17 \pm 0.5$  (mg/g f.w.) in the last treatment (Fig. 4C).

Sorbitol was the only component changes of which at different rearing temperature

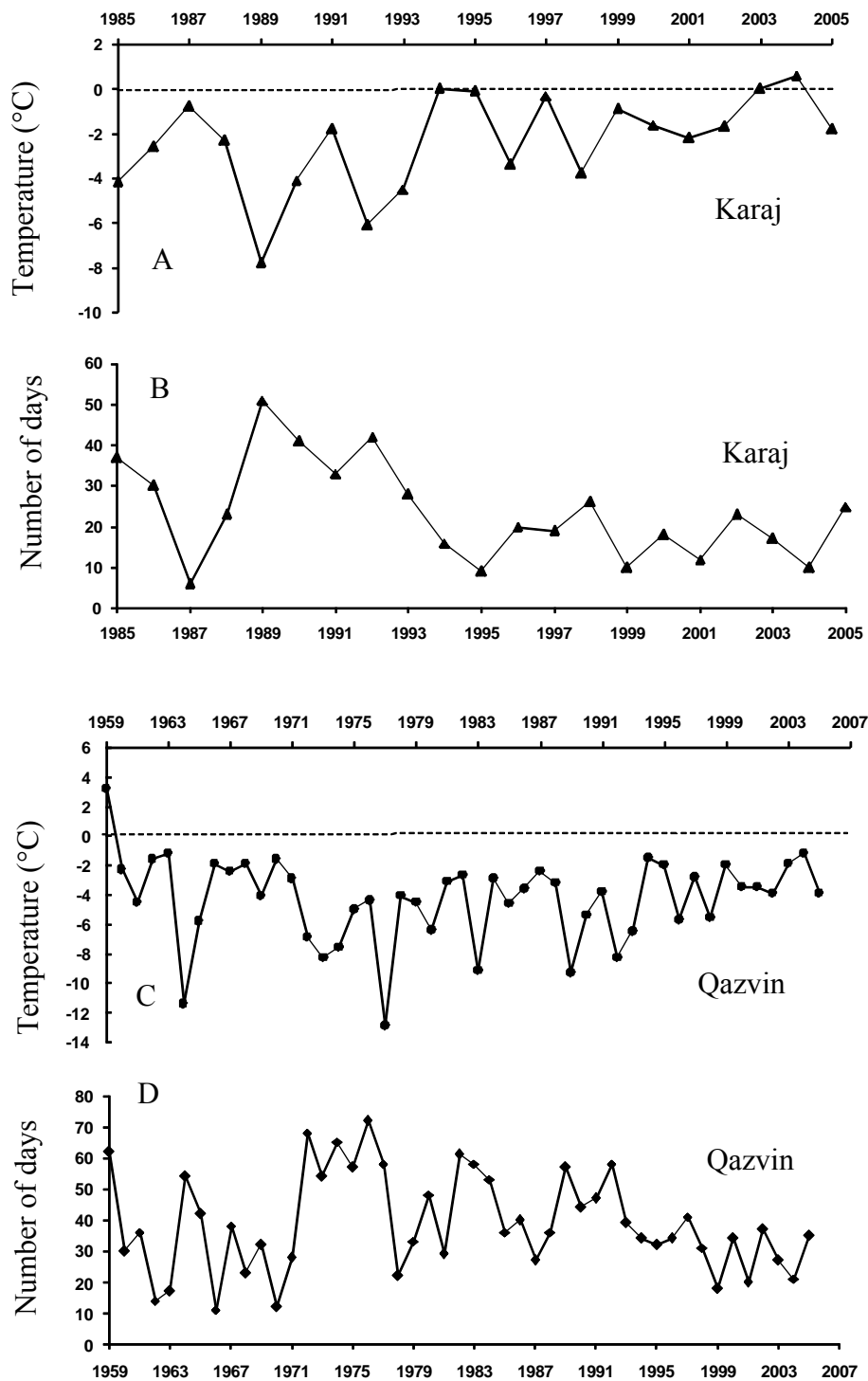
combinations were not significantly different however, it slightly increased with gradual increase of exposure times to lower temperatures from  $0.91 \pm 0.14$  (mg/g f.w.) in control larvae to  $1.98 \pm 0.15$  (mg/g f.w.) in the last treatment (Fig. 4D).

### Glycogen

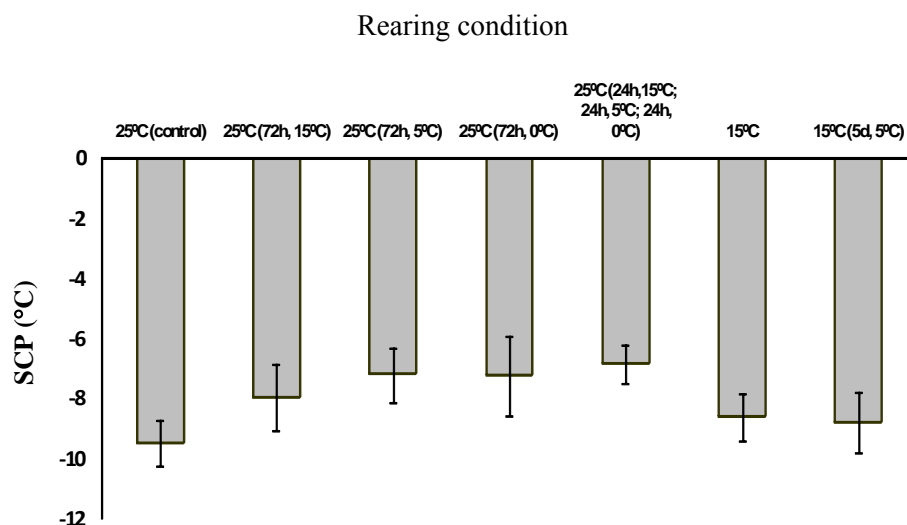
Glycogen content varied significantly among control and treatments ( $F_{6, 14} = 10.39$ ;  $P < 0.01$ ). Moreover, there was negative correlation between glycogen and trehalose ( $r = -0.558$ ,  $P = 0.009$ , Fig. 3). The highest and lowest amounts of glycogen were measured in control and last treatment (those reared at 15 °C followed by 5 °C for 5 days) with about 49% decrease.

### Discussion

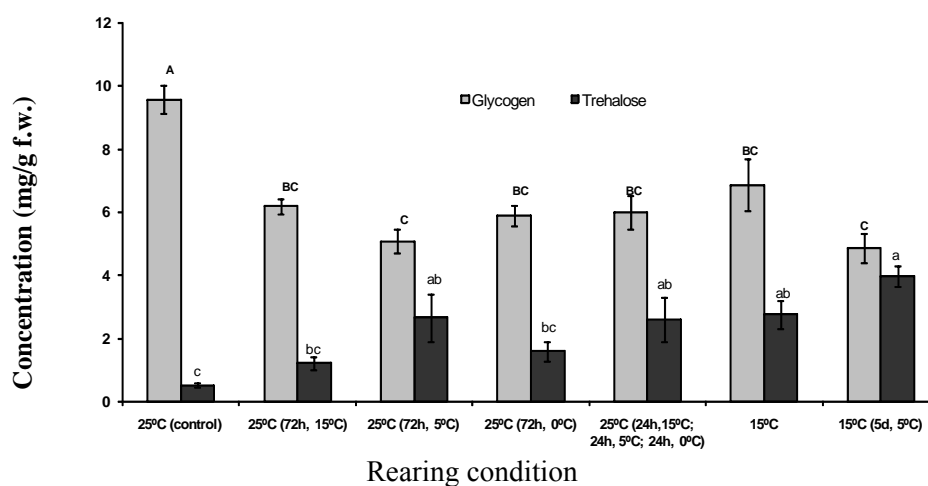
During our previous study on the overwintering larvae collected from November 2008 to March 2009, SCPs were significantly increased from -12 °C in November to -6 or -7 °C in February and March. All of these larvae could survive after measuring SCPs, while laboratory-reared larvae could not withstand (Atapour and Moharramipour, 2011b). In another study established by Kim and Kim (1997), all laboratory-reared stages of *S. exigua* could not survive at temperatures below SCPs reporting a freeze intolerant strategy in this insect. Also in their study, pretreatment of larvae at 0 and 5 °C for 2 h, increased the survival rate at -10 °C, suggesting the rapid cold hardiness ability for this pest. Nevertheless, in our preliminary tests, pretreatment of larvae in 0 and 5 °C for 2, 24 h and even 48 h could not increase survival rate at -15 °C and so there was not any rapid cold hardiness. However, all field-collected larvae were well able to tolerate low temperatures below their whole body SCPs. Therefore in the present study, we tried to understand how overwintering larvae were able to successfully overcome such low temperatures.



**Figure 1** The average of minimum temperature in January in Karaj (A) and Qazvin (C) and the number of days with minimum temperature below -4 °C in Karaj (B) and Qazvin (D).



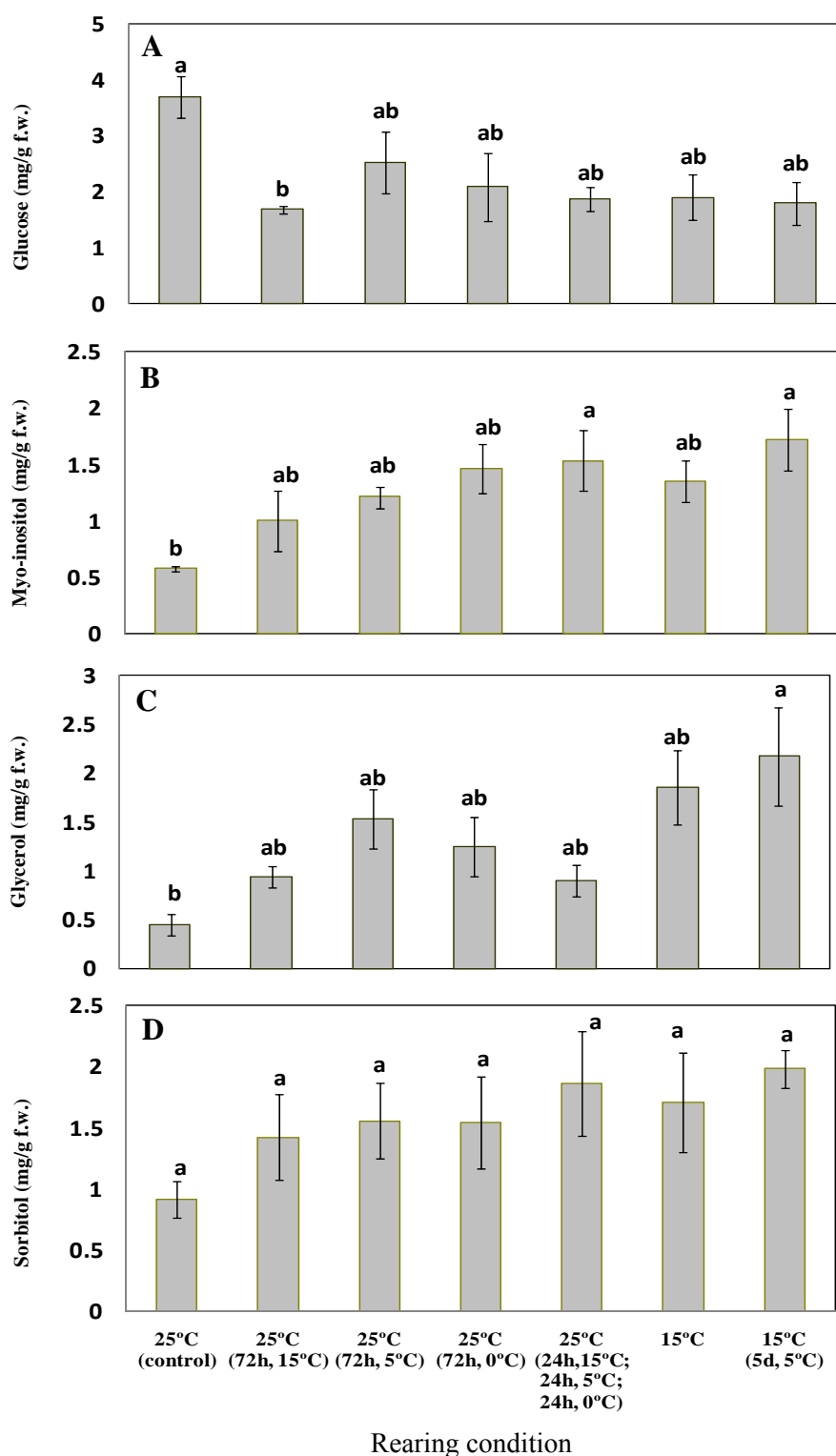
**Figure 2** Supercooling points (SCPs) of the overwintering larvae of the beet armyworm, *Spodoptera exigua* in control and different treatments. Error bars indicate standard error of mean.



**Figure 3** Influence of temperature treatments on changes of glycogen and trehalose content of beet armyworm larvae. Values labeled with the same letters are not significantly different at the 5% level by Tukey's test after ANOVA. Error bars indicate standard error of mean.

Despite no significant variation between SCPs of different treatments, all treatments had a relatively higher freezing point compared to control group. It seems that cooling rate is a very important factor in survival of these larvae. The larvae cooled from 0 °C to -15 °C at a rate of 1 °C/min could not tolerate -15 °C for 2 h, but all of those larvae cooled at a more similar to natural climatic cooling rate (1 °C / h), could withstand -15 °C for 2 h (Atapour and

Moharrampour, 2011a). On the other hand, Qazvin meteorological data showed that monthly minimum temperature reaches below -4 °C for about 30 days per year. Based on Sinclair (1999, 2001) classification, it seems that larvae of *S. exigua* are neither freeze-intolerant nor freeze-tolerant insects. If these larvae were gradually exposed to sub-zero temperatures, they would be able to increase their cold hardiness capacity to some extent.



**Figure 4** Changes of (A) glucose, (B) *myo*-inositol, (C) glycerol and (D) sorbitol content of beet armyworm larvae treated at different temperatures. Values labeled with the same letters are not significantly different at the 5% level by Tukey's test after ANOVA. Error bars indicate standard error of mean.

Kim and Song (2000) reported that in *S. exigua* lowering rearing temperature led to increase of hemolymph osmolality, glycerol content and cold hardiness but they supposed that "this pest may use other major cryoprotectants rather than glycerol". The present study, as the first report, shows that trehalose was the dominant cryoprotectant. Trehalose content was negatively correlated with glycogen. In many studies, glycogen has been reported as the main source of anti-freeze compounds. In overwintering larvae of *Enosima leucotaeniella* (Ragonot) (Goto *et al.*, 1998), pupae of *Hyphantria cunea* Drury (Li *et al.*, 2001) and pupae of *Mamestra brassicae* L. (Ding *et al.*, 2003) there was an interconversion between glycogen and trehalose content suggesting the glycogen as an important carbon source for synthesis of trehalose (Rojas *et al.*, 1983; Storey and Storey, 1988; Storey *et al.*, 1991).

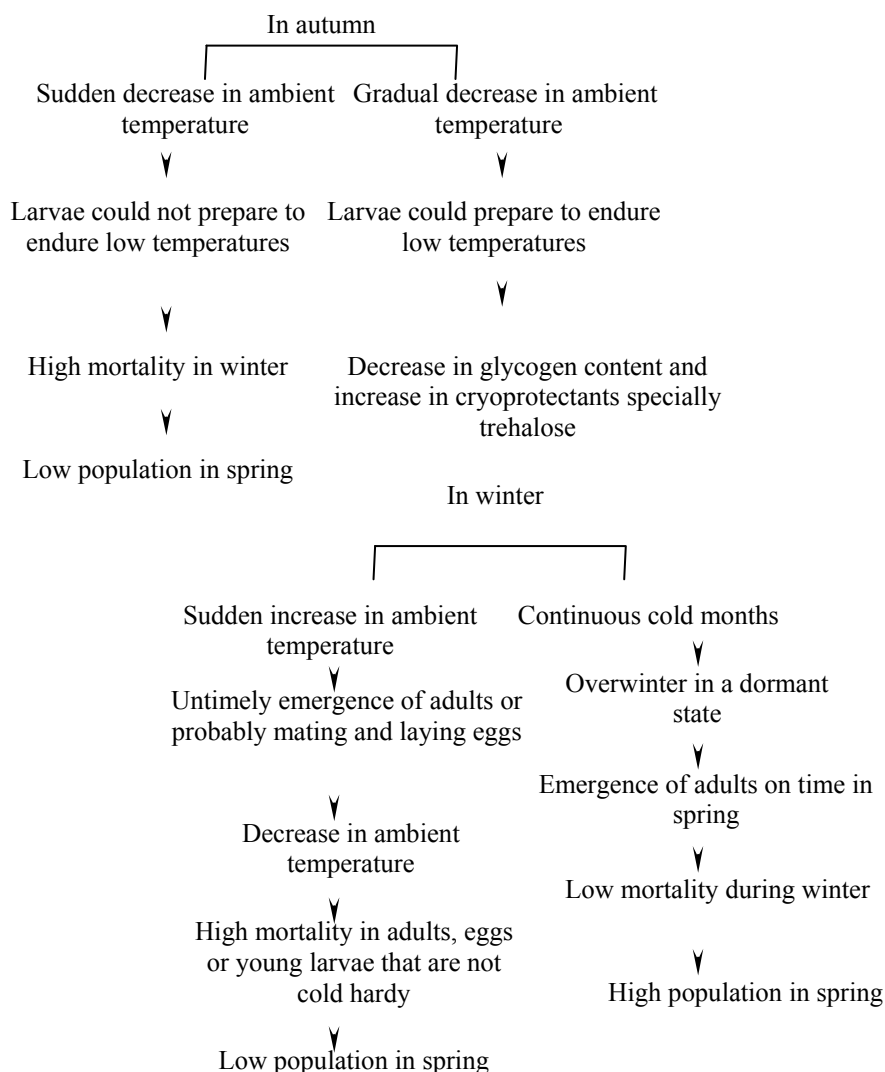
Other compounds especially glycerol and *myo*-inositol accumulated in larvae exposed to sub-zero temperature for longer duration (especially in last treatment) might be considered as cryoprotectants but their amount was less than trehalose. All of these data together show that production of cryoprotectants and increasing the cold hardiness in this pest is a long-term and gradual process.

The association of cryoprotectants with cold hardiness but subsequent decrease in glucose content has been reported in some previous studies such as in pupae of cabbage root fly, *Delia radicum* L. (Kostal and Simek, 1995) or overwintering larvae of rice stem borer, *Chilo suppressalis* Walker (Atapour and Moharramipour, 2009). The reason for this coincidence is not clear, although the glucose in addition to glycogen may be converted to cryoprotectants (Kostal and Simek, 1995; Storey and Storey, 1991).

Additional studies are needed to realize these changes.

In a study on the biology and population fluctuation of *S. exigua* (Kheyri, 1985), it was reported that relatively severe and long winter, may cause the increase of the pest population in the next year. In decades of 1970 and 1980 (especially 1970-1972) the beet armyworm was the key pest of sugar beet in Iran (Kheyri, 1976). Reviewing weather data (Fig. 1) shows the fact that the coldest winter has occurred in these two years and thereafter the ambient temperature has relatively increased. After 1990, despite increasing cultivation of sugar beet in Iran, the importance of this pest decreased from key pest to a moderate pest (Khanjani, 2005). Thus it could be concluded that ambient temperature, especially during fall and winter, plays an important role in pest population in such cold areas. It should be noted that other factors such as rainfall, host species and quality of the food (Kheyri, 1985, 1989) or pest habitat and its shelter (Danks, 2006) affect cold hardiness, survival rate and the density of pest population in next generation, yet ambient temperature would be the most determining factor. As it is summarized in Fig. 5 a lower climate change damage, could be expected due to global warming. Because of long-term process of cold hardiness in *S. exigua* overwintering larvae, sudden decrease of temperature early in the autumn may cause high mortality in population. Also, in years with mild winter the untimely emergence of adults may be expected which followed by sub-zero temperatures, drastic mortality would be expected in adults or other susceptible stages. In contrast, in years with severe long winter an outbreak would be anticipated due to low mortality during winter as a state of cold tolerant larvae and pupae.





**Figure 5** A schematic charts representing the effect of ambient temperature changes on *S. exigua* population outbreak.

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## فرایند سرماسختی کرم برگ‌خوار چغندر قند (*Spodoptera exigua* (Lepidoptera: Noctuidae))

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**چکیده:** یکی از متداول‌ترین سازش‌های حشرات با دماهای پایین سرماسختی می‌باشد. به‌منظور درک استراتژی سرماسختی و تأثیر دما در این فرایند، لاروهای شاهد و شش تیمار دمایی مختلف از کرم برگ‌خوار چغندر مورد آزمایش قرار گرفتند. نقطه انجماد بدن لاروها در تیمارهای مختلف اختلاف معنی‌داری از خود نشان نداد. دو قند ترهالوز و گلوکز و سه پلی‌ال میواینوزیتول، گلیسرول و سوربیتول در این لاروها شناسایی شدند. ترهالوز در پاسخ به دمای پایین در درازمدت بیشتر تحت تأثیر قرار گرفت و رابطه همبستگی منفی و معنی‌داری را با میزان گلیکوژن از خود نشان داد. آمار هواشناسی نشان داد که در سال‌های اخیر دمای هوا به‌طور نسبی افزایش داشته است. علی‌رغم افزایش سطح زیرکشت چغندر، جمعیت این آفت در این سال‌ها کاهش یافته است. به‌نظر می‌رسد تغییرات آب و هوایی در ارتباط با گرم شدن کره زمین به‌ویژه در طول پاییز و زمستان نقش مهمی را در کاهش جمعیت آفت در نسل جدید ایفا نماید. براساس یافته‌های این مطالعه به‌نظر می‌رسد که لاروهای این آفت غیرمتمحمل به یخ‌زدگی هستند اما در سازش‌های طولانی به دماهای زیر صفر قادر به تحمل کریستال‌های یخ در بدن خود می‌باشند.

**واژگان کلیدی:** دمای هوا، کرم برگ‌خوار چغندر، *Spodoptera exigua*، سرماسختی، ترهالوز