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Research Article

Cryoprotectants in overwintering larvae of leopard moth, *Zeuzera pyrina* (Lepidoptera: Cossidae), collected from northwestern Iran

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Abstract: Leopard moth, *Zeuzera pyrina* L. (Lepidoptera: Cossidae), is one of the important woodboring pests whose larvae bore into twigs, branches, and trunks of various woody species, weakening and sometimes killing trees or shrubs. Recently it has caused severe losses of walnut trees in Iran. This pest overwinters as different larval instars inside trees. Overwintering larvae were collected monthly from October 2020 to March 2021 from Maragheh walnut orchards, northwestern Iran, to determine the presence of cryoprotectants and their changes during autumn and winter. Overwintering larvae accumulated sorbitol, trehalose, and myo-inositol during winter. During cold months there was approximately 11 fold and 7.5-fold increase in trehalose and sorbitol contents, respectively. Glycogen content was the highest in October and decreased significantly with decreasing ambient temperature. Our results suggest that the accumulation of sorbitol, trehalose, and myo-inositol plays an important role in the harsh-season survival of *Z. pyrina*.

Keywords: cold hardiness, polyols, glucose, glycogen

Introduction

Temperature is an important determining factor in the geographical distribution of insects (Chown and Nicolson, 2004). Low temperature is a major adverse environmental factor that insects must overcome in temperate and cold regions (Storey, 1997). Many insects in temperate habitats enter diapause or quiescence. These insects enhance their cold hardiness by accumulating energy reserves before the onset of cold weather and reducing their metabolic activity and energy consumption. During overwintering, these insects synthesize and accumulate low molecular weight sugars and polyols (Storey and Storey, 1991). Accumulated compounds function as colligative and/or non-colligative, enhancing the level of insects' cold-hardiness and thus increasing the chances of their winter survival (Lee, 1991; Kostal *et al.*, 2001, 2004).

The polyols are considered effective cryoprotectants with various roles in either supercooled or frozen organisms. They depress melting and supercooling points of body fluids (Zachariassen 1985), stabilize proteins in non-frozen systems (Carpenter and Crow, 1988), protect cell membranes against dehydration caused both by freezing and desiccation (Crowe *et al.*, 1990), and regulate

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minimal cell volume during extracellular freezing (Storey and Storey, 1988). Sugars such as trehalose have cryoprotecting properties by protecting enzymes and membrane structure under stress caused by low temperatures (Hodkova and Hodek, 2004). The role of glycogen is complex; it may act as a precursor for cryoprotectants such as glycerol or as a fuel for basal metabolism during the overwintering period (Somme, 1982; Kimura *et al.*, 1992).

The leopard moth, *Zeuzera pyrina* L. is a cossid moth whose larvae bore into twigs, branches, and trunks of various woody species, weakening and sometimes killing trees or shrubs. Recently it caused severe losses of walnut trees in Iran. The species has one generation per two years, but moths and damages appear yearly (Kutinkova *et al.*, 2006). Females lay eggs on the branches of trees, and newly hatched larvae bore into young shoots, killing them. Larvae of the subsequent instars move to the lower and thicker branches up to the tree trunk, causing the drying of parts or the death of the entire tree depending on the larval infestation density (Almanoufi *et al.*, 2012). Control of this pest appears to be complicated by chemicals because the flight period of moths lasts about three months and larvae bore into twigs, branches, and trunks (Kutinkova *et al.*, 2006).

There are examples of cryoprotective polyols in Lepidoptera, including glycerol, sorbitol, fructose, glucose, and trehalose (Pullin and Bale, 1989 a, b; Vrba *et al.*, 2014; Williams *et al.*, 2014). Atapour and Moharramipour (2009) studied changes in cryoprotectants in overwintering larvae of *Chilo suppressalis* Walker and found that glycerol and trehalose were the major cryoprotectants. Atapour and Moharramipour (2014) reported glycerol, myo-inositol, and trehalose as the major cryoprotectants of *Spodoptera exigua* (Hübner) larvae. Khani *et al.* (2007) and Vrba *et al.* (2017) showed that trehalose is the main cryoprotectant accumulated during winter in *Cydia pomonella* (L.) and *Erebia* larvae, respectively. Feng *et al.* (2018) reported trehalose as a major cryoprotectant in *Sphenoptera* sp. larvae, followed by inositol. Tian *et al.* (2018) studied the effect of fatty acids on the cold hardiness of *Eogystia hippophaecolus* (Hua, Chou, Fang & Chen, 1990) (Lep: Cossidae) and concluded that their larvae cold hardiness is enhanced by increasing fatty acid unsaturation. Pei *et al.* (2020), studying the factors influencing cold hardiness of *Streltzoviella insularis* (Staudinger) (Lep: Cossidae), believed that lipid metabolism and carbohydrate metabolism are key pathways involved in the cold tolerance of this cossid moth larvae.

Although a considerable amount of information has been gathered on changes in cryoprotectants in overwintering insects (Kostal and Simek, 1995; Goto and Honma, 2001; Khani *et al.*, 2007; Soudi and Moharramipour, 2011; Hamedi and Moharramipour, 2013; Hamedi *et al.*, 2013; Saeidi and Moharramipour, 2017), no attention has been paid to leopard moth, despite the high economic importance of this species. The main purpose of the present study is to identify the major cryoprotectants in overwintering larvae of the leopard moth and to determine which factors may be associated with cold-hardiness and the winter survival of this pest.

Materials and Methods

Insects

Overwintering larvae of *Z. pyrina* used in the tests were collected monthly from October 2020 to March 2021 from Maragheh walnut orchards, northwestern Iran. Collected larvae in each month were weighted on a semi-micro balance AND No. EPS-302 (sensitivity 0.1 mg) and stored at -25 ºC pending analysis.

Weather data

Available weather information was obtained from the agro-meteorological station, located 1 km from the sampling place.

Chemical analysis

The whole insect body was taken as one sample, and three or four larvae were used every month. Each larva was weighed and extracted in 1.5 ml of 80% ethanol. The extracts were dried at 35 ºC in an oven and resuspended in 200 ml of high-performance liquid chromatography (HPLC) grade water. The samples were filtered using syringe filter units (Millex, USA), and 30 ml of each sample was injected into HPLC (Waters, USA). The HPLC was fitted with a C18 carbohydrate column (Ca, 59305-U, 300 × 7.8 mm, Supelco, USA). The mobile phase was HPLC grade water at a flow rate of 0.5 ml/min, and the detector type was a refractive index [RI] detector. Separations were performed at room temperature; compounds were identified and quantified from the retention time of carbohydrate standards (Fluka, Buchs, Switzerland). Glycogen was determined by the method described by Hansen *et al*. (1952), using a UV-Visible spectrophotometer (Shimadzu Japan, UV-1800). The absorbance was read at 660 nm. The amount of glycogen in an unknown sample was determined by comparing it with the glycogen standard (BioBasic, Canada GB0301) curve.

Statistical analysis

Statistical analysis of the sugar content data was performed for three or four replicates by one-way analysis of variance (ANOVA) with a post-hoc Tukey test at $P < 0.05$ using SPSS version 16.00 for windows. The normality test was done using a Kolmogorov-Smirnov test before ANOVA. A Pearson correlation was used to investigate the relationship between variables. The results were expressed as mean ± SE.

Results

The average, maximum and minimum ambient temperature in Maragheh, Iran, during the sampling period is presented in Fig. 1. The minimum monthly temperature was below 0 ºC from December to the end of February, with the lowest in January (-5 ºC) (Fig. 1).

We demonstrated the putative cryoprotectants in leopard moth larvae based on the HPLC analysis. The leopard moth larvae accumulated glucose, trehalose, sorbitol, and myo-inositol (Fig. 2). Among the accumulated polyols during cold months, sorbitol was detected in the highest mean concentrations, followed by trehalose. The concentration of sorbitol ($F = 215.4$, $df = 5,12$, $p < 0.0001$), trehalose ($F = 221.01$, $df = 5,12$, $P < 0.0001$), glucose ($F = 21.47$, $df = 5,12$, $P < 0.0001$) and myo-inositol ($F = 27.43$, $df = 5,12$, $p < 0.0001$) in overwintering larvae differed significantly among different months. In October, trehalose and sorbitol contents were low (1.15 and 2.07 mg/g, respectively). Most accumulation occurred during overwintering and reached maximum levels of 11.73 mg/g in January and 15.81 mg/g in February for trehalose and sorbitol, respectively. Trehalose and sorbitol decreased rapidly after that (Fig. 3), which coincided with the ambient temperature increase. There was a negative and significant correlation between trehalose and sorbitol concentrations and ambient temperature (Table 1). There was a positive and significant correlation between these two concentrations (Table 1). Glucose increased gradually from October to December, then decreased rapidly in January and increased after that (Fig. 3). Myo-inositol increased from October to January and then decreased after that and reached the lowest level in March as ambient temperature increased (Fig. 4). There was no significant correlation between glucose and myo-inositol concentrations and ambient temperature and also other cryoprotectants concentration (Table 1). Glycogen content was the highest in October and then decreased significantly with decreasing ambient temperature (Fig. 4). There was a positive and significant correlation between glycogen content and ambient temperature, but the correlation between glycogen concentration and other cryoprotectants concentration was not significant (Table 1).

[DOR: 20.1001.1.22519041.2022.11.3.1.6]

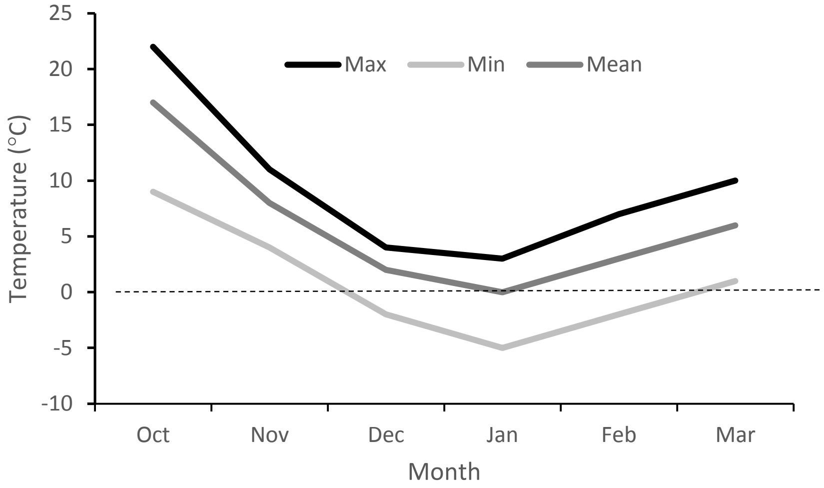

Figure 1 Seasonal changes in average, minimum, and maximum ambient temperature in sampling location of *Zeuzera pyrina* larvae at Maragheh, Iran.

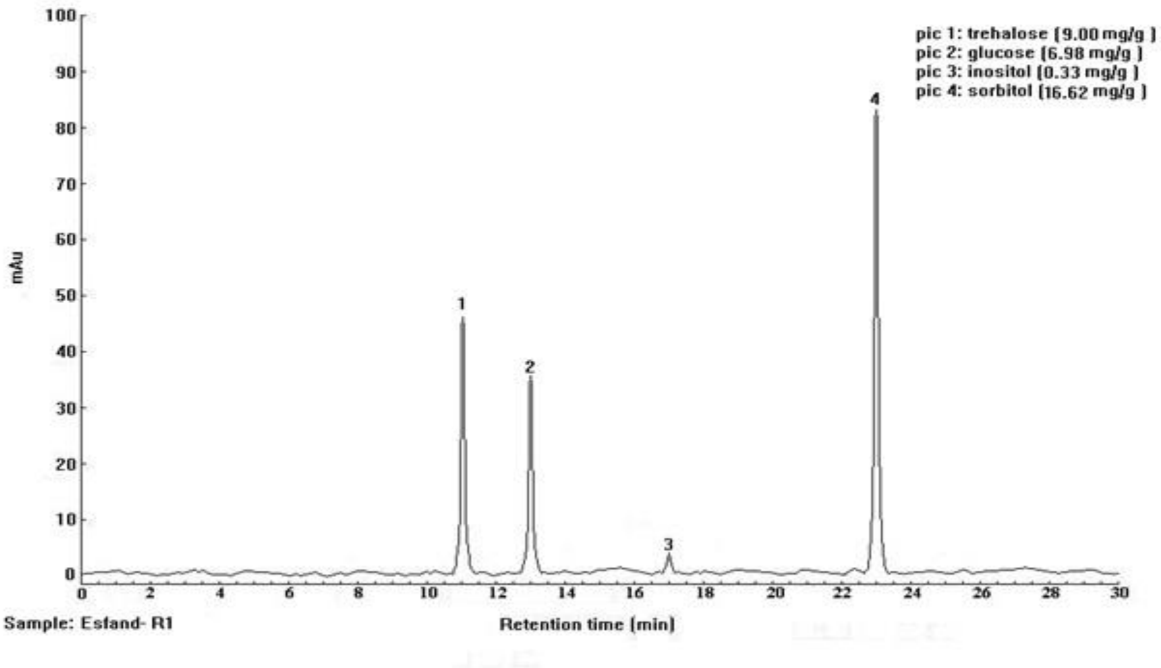

Figure 2 HPLC chromatogram of cryoprotectants separation in a sample of larvae of *Zeuzera pyrina* from overwintering regions in Maragheh.

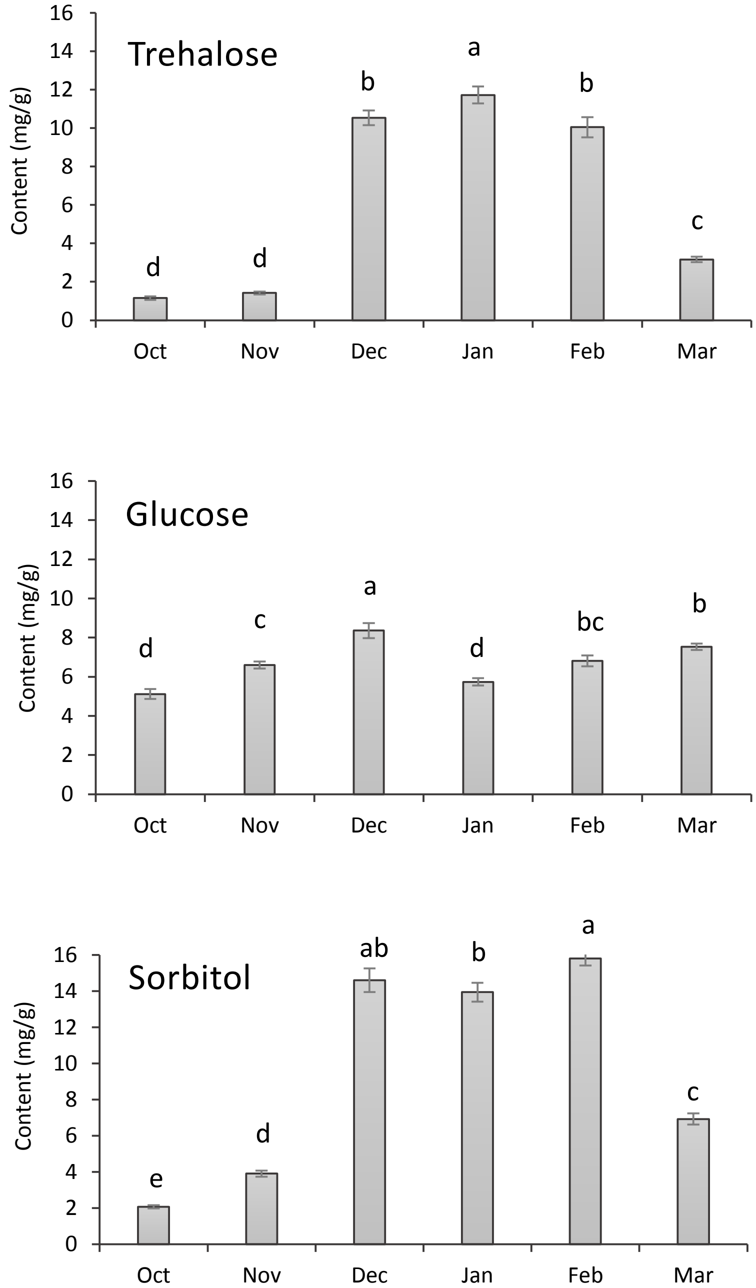

Figure 3 Changes of trehalose, sorbitol and glucose content in overwintering larvae of *Zeuzera pyrina.* The same letters indicate no significant difference at the 5% level by Tukey's test after ANOVA.

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Table 1 Pearson correlation (r) between cryoprotectants concentration of *Zeuzera pyrina* and mean ambient temperature.

Cryoprotectants	Mean ambient temperature	Trehalose	Glucose	Myo-inositol	Sorbitol	Glycogen
Trehalose	-0.845* (0.034)	1				
Glucose	-0.525 (0.285)	0.285 (0.584)	1			
Myo-inositol	-0.571 (0.236)	0.536 (0.273)	-0.293 (0.573)	1		
Sorbitol	-0.872* (0.024)	0.970** (0.001)	0.429 (0.395)	0.417 (0.410)	1	
Glycogen	0.849* (0.032)	-0.624 (0.185)	-0.654 (0.159)	-0.478 (0.337)	-0.706 (0.117)	1

P-value is written in parenthesis.

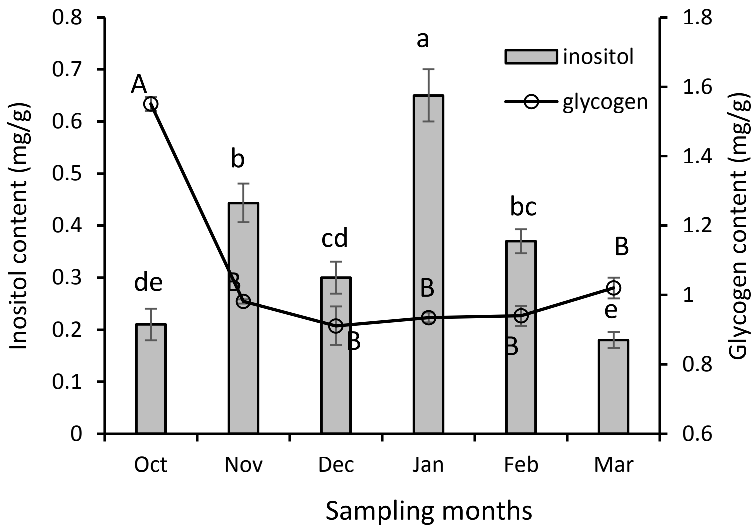

Figure 4 Changes of myo-inositol and glycogen content in overwintering larvae of *Zeuzera pyrina*. The same letters indicate no significant difference at the 5% level by Tukey's test after ANOVA.

Discussion

Insects prepare to survive the winter (Durak *et al*., 2021). Many insects have strategies to pass through or avoid the adverse effects of cold weather in winter. These can be either behavioral strategies such as migration or physiological strategies such as freeze avoidance and freeze tolerance (Storey and Storey, 2012). The accumulation of low molecular weight sugars and polyols is one of the major physiological mechanisms for increasing the cold tolerance of insects, but little is known about this process in the leopard moth. We found changes in the content of five compounds: glucose, trehalose, sorbitol, myo-inositol, and glycogen. Four low molecular weight sugars and polyols, including glucose, trehalose, sorbitol, and myo-inositol, are possible cryoprotectants in larvae of leopard moth.

Sorbitol and myo-inositol exhibited maximal and minimal concentrations (15.81 and 0.37 mg/g, respectively, in February) among the sugars and polyols we detected. Atapour (2017) and Atapour and Moharramipour (2014) reported 8 and 1.98 mg/g of sorbitol in *Pieris brassicae* (L.) and *S. exigua*, respectively, during cold hardening. In this study, sorbitol content increased with a decrease in glycogen until February. It decreased with an increase in glycogen in March, but their correlation was not significant, suggesting an interconversion between them (Goto and Honma, 2001; Cho *et al.*, 2005). The synthesis of sorbitol (the second most frequent polyol present in insects) occurs when the level of ATP is reduced. Pullin *et al.* (1991) have shown a close relationship between lowered metabolic rate and production of sorbitol in diapausing pupae of *P. brassicae*. Sorbitol has previously been reported as the most abundant cryoprotectant in several insect species, including *Dendroides canadensis* Leconte (Horwath and Duman, 1984), *Pyrrhocoris apterus* L. (Kostal *et al.,* 2001), *Ips typographus* (L.) (Kostal *et al.,* 2007), and *Tipula trivittata* Say (Duman *et al.*, 1985).

In January, the trehalose concentration reached its highest level (11.73 mg/g). Atapour and Moharramipour (2009) and Atapour (2017) reported 4 and 10 mg/g trehalose in overwintering *Ch. suppresalis* and *P. brassicae,* respectively, in January. Trehalose is a disaccharide found in the fat body and hemolymph of most insects. It plays an important role in insect physiology, especially in stressful situations like cold stress (Clarke, 1966). Decreasing temperature in December, January, and February likely induced elevation of this disaccharide as the correlation between this compound and ambient temperature was negative and significant. Maybe there was an interconversion between trehalose and glycogen, too, as the highest level of trehalose coincided with the lowest level of glycogen but their correlation was not significant. Trehalose can also actively repair the structure of proteins (Kandror *et al.,* 2002; Elbein *et al.,* 2003) and interact with chaperones to help re-fold partially demonstrated proteins after thermal stress (Viner and Clegg, 2001). Some cryoprotectants, such as trehalose, could also serve as potent free radical scavengers, thus protecting cells from oxidative damage (Benaroudj *et al.,* 2001). Carbon polyols do not readily cross membranes, which is one of the major reasons for their value as osmoprotectants in insect systems (Durak *et al.*, 2021). Trehalose has been reported as a cryoprotectant in some insect species (Storey and Storey, 1991; Kostal and Simek, 1995; Simek *et al.,* 1998; Goto *et al.,* 1998; Khani *et al.*, 2007; Han *et al.*, 2008).

Compounds such as glucose and sucrose are precursors in synthesizing other polyols (Kostal *et al.,* 2004). Glucose is the main cryoprotectant in some insects, for example, in overwintering pupae of *Hyphantria cunea* (Drury, 1773) (Li *et al.*, 2001), and can play an important role in the process of stabilizing proteins and membrane lipids (Durak *et al.*, 2021). According to the decrease in glucose concentration during cold months (5.74 mg/g in January), which coincides with increased trehalose, sorbitol, and myo-inositol concentrations, it can be hypothesized that glucose has been used as a precursor of these compounds. Atapour and Moharramipour (2009) reported a decrease from 12 to 1 mg/g in glucose concentration of overwintering *C. suppresalis* from August to January. Khani *et al.* (2007) reported 5.9 mg/g of glucose in the overwintering larvae of *C. pomonella* in January. Atapour (2017) also demonstrated a decrease in glucose concentration of overwintering pupae of P. brassicae, reaching 4 mg/g in January. Myo-inositol is accumulated during diapause and overwintering in many beetles, flies, and in hemolymph in planthoppers, where it could play a role in flight energy and also thermos-or cryoprotectant for changes in physiological conditions (Kostal and Simek, 1996; Watanabe and Tanaka, 1999; Moriwaki *et al.*, 2003; Vesala *et al.,* 2012). In this study, the concentration of myo-inositol compared to the other polyols and sugars was the lowest but peaked in January (0.65 mg/g), which was the coldest month. Atapour (2017) reported 0.2-0.5 mg/g of myo-inositol in the overwintering pupae of *P. brassicae* during January and February.

The role of glycogen varies in different insects during overwintering. In temperate insects examined for seasonal changes in carbohydrate content, glycogen is rapidly depleted and converted to sugar alcohol and sugar in late autumn or early winter (Storey and Storey, 1986; Goto *et al.,* 1998). In this study, a decrease in ambient temperature from October to January is associated with a reduction of glycogen and an increase in trehalose, sorbitol, and myo-inositol contents. Hayakawa and Chino (1981, 1982) demonstrated a temperature-dependent interconversion between glycogen and trehalose in diapausing pupae of the saturniid moth, *Philosamia cynthia pryei* Donovan. Results obtained by Behroozi *et al*. (2012) suggested the interconversion between glycogen and trehalose in overwintering larvae of *Ocneria terebinthina* Strg. under field conditions. Shimada *et al.* (1984) reported a reverse trend between the concentrations of glycogen and trehalose in relation to the ambient temperature in overwintering larvae of *Leguminivora glycinivorella* (Mats.). Glycogen contents were significantly lower in larvae of *S. exigua* collected in February and March rather than overwintering larvae collected in November and December in the studies of Atapour and Moharramipour (2011, 2014).

The synthesis of cryoprotectants is only a part of a more complex mechanism of increasing cold hardiness. Other important components are synthesizing antifreeze proteins and removing ice-nucleating agents from the body (Somme, 1982; Duman, 2001). Low molecular weight sugar alcohols can enhance the cold hardiness of insects directly by increasing the hemolymph concentration and stabilizing membranes and proteins (Tian *et al.*, 2016), or they can improve the content of bound water or interact with proteins to protect metabolic systems and defend the insect from damage potentially caused by freezing during overwintering (Feng *et al.*, 2018). In this study, we have demonstrated, at least in part, some physiological adaptations for winter survival of *Z. pyrina* larvae. So far, no information has been reported regarding the overwintering of the leopard moth.

Conclusion

In conclusion, our results demonstrated at least some biochemical adaptations for winter survival in larvae of *Z. pyrina* in northwestern Iran. Trehalose, sorbitol, and myo-inositol contents in overwintering larvae reached near the highest level in January, the coldest month of the year. This result suggests that the accumulation of these compounds plays an important role in the harsh-season survival of *Z. pyrina.* The total cryoprotectants increased significantly during these months. Glycogen content was the highest in October and decreased significantly with decreasing ambient temperature.

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ترکیبات محافظ سرما در لاروهای زمستان‌گذران کرم خراط، *Zeuzera pyrina* (Lepidoptera: Cossidae) جمع‌آوری شده از شمال غرب ایران

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چکیده: کرم خراط یا پروانه فری با نام علمی *Zeuzera pyrina* L. (Lepidoptera: Cossidae) یکی از مهم‌ترین آفات چوب‌خوار است که خسارت آن با نفوذ لاروهای سن یک به سرشاخه‌های غیرچوبی شروع شده و با گذر زمان و افزایش سن لارو افزایش می‌یابد و در نهایت منجر به ضعف و خشکیدن گیاه می‌گردد. در سال‌های اخیر این آفت منجر به خسارت‌های فراوانی به درختان گردو در ایران شده است. این آفت زمستان را به‌صورت سنین مختلف لاروی درون شاخه یا تنه درختان سپری می‌کند. به‌منظور بررسی ترکیبات ضدیخ موجود در این آفت و تغییرات آن‌ها در طول پاییز و زمستان، لاروهای زمستان‌گذران به‌طور ماهانه از آبان ۱۳۹۹ تا فروردین ۱۴۰۰ از باغ‌های گردو شهرستان مراغه در شمال غرب ایران جمع‌آوری شدند. لاروهای زمستان‌گذران، سوربیتول، ترهالوز و مایواینوزیتول را در طی زمستان ذخیره کردند. در سردترین ماه‌ها میزان ترهالوز و سوربیتول به‌ترتیب ۱۱ برابر و ۷/۵ برابر افزایش یافت. میزان گلیکوژن در آبان ماه بیش‌ترین غلظت را داشت و با کاهش دمای محیط به‌طور معنی‌داری کاهش یافت. نتایج این تحقیق نشان داد که ذخیره سوربیتول، ترهالوز و مایواینوزیتول نقش مهمی را در بقاء *Z. pyrina* در شرایط سخت ایفا می‌کند.

واژگان کلیدی: سرماسختی، پلی‌ال، گلوکز، گلیکوژن

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