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Changes in biochemical contents and survival rates of two stored product moths under different thermal regimes

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ABSTRACT

Plodia interpunctella (Hübner) and *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae) are two destructive pests of stored products. Survival and physiological changes of last instar larvae were investigated under different thermal regimes [i.e. control (optimal temperature), cold-acclimation (CA), fluctuating-acclimation (FA) and rapid cold-hardiness (RCH)]. Our results indicate a relationship between supercooling points, carbohydrate contents, enzyme activities and survival rates of the larvae under different thermal regimes. Glycogen content was greatest for control and RCH regimes whereas the greatest trehalose level was recorded for the CA regime. The supercooling points of control larvae of *P. interpunctella* and *E. ceratoniae* were − 14 and − 10 °C, respectively, and decreased to − 16 and − 18 °C under the CA regime. Thermal regimes had no significant effect on the survival of the larvae after 24 h exposure at 0 °C, but when larvae exposed to − 5 °C for 24 h, the highest and lowest survival rates were recorded in the CA and RCH regimes, respectively. Protein phosphatases 1 and cAMP-dependent protein kinase (AMPK) were found to have the highest enzyme activity. The activity of AMPK varied between different thermal regimes and was greatest under the CA regime. CA considerably increased lower lethal times of the larvae compared with the control regime. RCH showed the highest impact on lower lethal temperature limits of the larvae. Our results indicated a characteristic enhancement of the survival rates of the larvae of *P. interpunctella* and *E. ceratoniae* under the CA regime. This enhancement is likely related to elevated contents of low molecular weight carbohydrates under the CA regime.

1. Introduction

The Indianmeal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) is a destructive pest of stored products distributed in a wide range of climates throughout the world. The carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae) is another serious pest of stored products with a nearly cosmopolitan distribution (Perez-Mendoza and Agulera-Pena, 2004; Grieshop et al., 2006; Nay, 2006). Control of both pests relies mostly on synthetic pesticides which have several drawbacks [e.g. toxicity to non-target organisms, resistance development, resurgence of stored pests and environmental contamination (Sahaf et al., 2008; Stejskal, 2015)]. Therefore, alternatives to synthetic pesticides, such as phytosanitary temperature treatments, that are both effective and non-residual methods, may be useful as an alternative to synthetic pesticides (Subramanyam and Hagstrum, 1995).

As ectotherms, insects have behavioral or physiological thermoregulatory mechanisms. Variation in environmental temperature directly influences the fitness of insect species and their physiological processes (e.g. metabolic rates) (Chidawanyika et al., 2017). Extreme temperature, as an adverse environmental condition, may affect insect fitness and survival (Fields, 1992; Watanabe et al., 2002). The impact of the extreme temperature depends on insect species and developmental stage, diapause development, relative humidity, cold acclimation, temperature severity and duration of exposure. So, these variables must be considered if the thermal treatment is applied as a method for the control of insect pests (Fields, 2001; Beckett et al., 2007; Mohammadzadeh and Izadi, 2018).

Cold hardiness or cold tolerance is the capacity of insects to survive long or short-term exposure to low temperatures. This capacity is proportional to the strength of the cold shock and depends on some intrinsic (e.g. age, sex, feeding status and ontogeny of insects) and extrinsic (duration, type, magnitude, and timing of stress and cooling rate) traits (Marais et al., 2009; Nyamukondiwa and Terblanche, 2009; Scharf et al., 2014; Sgro et al., 2016; Andreadis and Athanassiou, 2017; Mohammadzadeh and Izadi, 2018). In addition, insect cold tolerance may also be associated with physiological adaptations (e.g.

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accumulation of polyols and low molecular weight carbohydrates, which may act as cryoprotectants) (Andreadis and Athanassiou, 2017; Mohammadzadeh and Izadi, 2016).

Exposure to low temperature may lead to non-freezing or freezing injuries. Non-freezing injury is a consequence of rapid or long-term cold exposure and usually results in a phase change of the membrane lipids and loss of ion balance. Freezing injury involves numerous simultaneous changes, i.e. dehydration and mechanical injury of the cells (Morris and Watson, 1984; Lee, 1991; Wang and Kang, 2005). Insects, as ectotherms, have evolved a series of physiological adaptations (e.g. accumulation of low molecular weight carbohydrate that act as a cryoprotectants) to mitigate detrimental effects of cold on cell organelle and membrane injuries. Cryoprotectants have a series of functions, i.e. prevention of intracellular volume from falling below a critical minimum, stabilization of the membrane bilayer structure and lowering the supercooling point (SCP) of some insect species (Storey, 1997; Holmstrup et al., 2002; Wang and Kang, 2005; Bemani et al., 2012; Heydari and Izadi, 2014; Mohammadzadeh and Izadi, 2016).

The supercooling point is the temperature at which an insect's body fluids spontaneously freeze and ice forms in tissues. Experimentally, the SCP is defined as the temperature immediately before the exothermic release of latent heat upon body fluid crystallization (Hahn et al., 2008; Andreadis et al., 2014; Sinclair et al., 2015). The SCP may also be affected by some intrinsic and extrinsic factors, such as developmental stage, age, body size, feeding status, ambient temperature and cooling rate (Andreadis et al., 2012; Andreadis and Athanassiou, 2017; Mohammadzadeh and Izadi, 2018). Investigations on insect cold tolerance usually begin with the preliminary measurement of the SCP (Sinclair et al., 2015). Measurement of the SCP is a standard procedure for describing the cold hardiness strategy of insects (Ditrich, 2018). Cold tolerance strategy describes how an insect withstands temperatures at which its body fluid is expected to freeze (Sinclair et al., 2015). Based on the SCP, Sinclair et al. (2015) divided the insect's cold tolerance strategies into three main categories: freeze-intolerant or freeze-avoidant (mortality occurs at the SCP), freeze-tolerant (mortality occurs below the SCP), and chill-intolerant or chill-susceptible (mortality occurs above the SCP).

From an applied perspective, low temperatures can have a detrimental effect on insects with no harmful effect on commodities or equipment. Accordingly, low temperature treatment they may be employed successfully in the control of stored product pests (Ditrich, 2018). Because of the economic importance of *E. ceratoniae* and *P. interpunctella* for stored products, the main goal of the current study was to determine the effect of acclimation on the cold tolerance, supercooling point and cryoprotectant contents of the pests.

2. Materials and methods

2.1. Chemicals

All chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

2.2. Insect rearing

Indianmeal and carob moths were maintained for 2 years in the Laboratory of Entomology, Vali-e-Asr University of Rafsanjan, Rafsanjan, Iran. Larvae were fed pistachio nuts and maintained within an environmental chamber at 28 ± 1 °C with 65 ± 5% RH and a photoperiod of 14:10 (L:D) h. Larvae were placed in translucent plastic cups (2 ml) with pistachio nuts during cold acclimation treatments unless otherwise noted.

2.3. Cold acclimation treatments

Fifth instar larvae of *E. ceratoniae* and *P. interpunctella* were divided into four groups: control (C), cold-acclimation (CA), fluctuating-acclimation (FA) and rapid cold-harding (RCH). For the control treatment, larvae were maintained at standard rearing conditions. For the CA treatment, larvae were cooled in a programmable refrigerator from rearing conditions to 12 °C at a rate of 0.5 °C min^{-1} and kept at 12 °C for 8 days. Thereafter, the temperature was lowered to 5 °C at the same rate and the larvae were kept at this temperature for another 15 days. For the FA treatment, larvae were cooled in a programmable refrigerator from rearing conditions in a cycle of 240 min at 5 °C followed by 20 min at −10 °C followed by 240 min at 5 °C followed by 940 min at 33 °C. This cycle repeated over 10 consecutive days. For the RCH treatment, larvae were directly transferred to a programmable refrigerator at 0 °C and kept for 4 h. Surviving larvae were used for subsequent experiments.

2.4. Determination of the supercooling point

To measure the SCP of the individual larva ($n = 15$ for the Indianmeal moth; $n = 15$ for the carob moth), thermocouples (NiCr-Ni probes) connected to a Testo 177-T4 temperature recorder (Testo, Germany), were fixed to the larvae with adhesive paper. The specimens were cooled from rearing conditions at a rate of 0.5 °C min^{-1} in a programmable refrigerated test chamber (Gotech, GT-7005-A, Taiwan). The SCP was defined as the lowest temperature recorded before the exotherm caused by the latent heat of crystallization (Sinclair et al., 2015).

2.5. Cold tolerance assay

To assess the cold tolerance of larvae at −5, −10 and −15 °C for 24 h, five replicates of 15 larvae were used at each treatment and temperature point. To estimate cold tolerance, larvae from each acclimation treatment (i.e. control, CA, FA and RCH) were kept in a programmable refrigerated test chamber as temperature was lowered at a rate of 0.5 °C min^{-1} from rearing conditions to the desired treatment temperatures (i.e. 0, −5, −10, and −15 ± 0.5 °C). The larvae maintained at an optimum growth temperature for 24 h to check for survival. The larvae showing no movement in their appendages were considered to be dead (Mohammadzadeh and Izadi, 2016).

2.6. Survival after chronic low-temperature exposure

To estimate the effect of chronic cold exposure, 5th instar larvae were exposed to −5 °C, and survival was recorded every 6 h to calculate Lt_{30} (time at which 30% of larvae died), Lt_{50} (time at which 50% of larvae died) and Lt_{95} (time at which 95% of larvae died). This experiment was repeated five times.

2.7. Survival after acute low-temperature exposure

The lower lethal temperatures (LLTs) of fifth instar larvae were determined using acute exposure (1 h) to subzero temperatures. Groups ($n = 5$) of 15 fifth instar larvae (< 24 h old) were held at separate test temperatures [i.e. 0 to −25 °C (resulting in mortality from 0% to 100%) for 1 h]. Temperatures were recorded with thermocouples (NiCr-Ni probes) connected to a Testo 177-T4 temperature recorder (Testo, Germany). Larval survival was assessed after 24 h at each temperature. Finally, $LT_{80-1\,h}$ was calculated as the lowest temperature at which 80% of the larvae died after 1 h exposure (Sinclair and Rajamohan, 2008).

2.8. Energy analysis

The whole-body glycogen and polyol content of five larvae from each acclimation regime were measured. Glycogen content was estimated using a modified anthrone method as described in Heydari and Izadi (2014). Briefly, larvae were weighed and homogenized in 200 µl

of 2% Na_2SO_4 and 1300 µl of chloroform-methanol (1:2) was added to the homogenate. The homogenate was centrifuged for 10 min at 7150 ×*g* and the pellet washed in 400 µl of 80% methanol. An aliquot of 250 µl of distilled water was added, and the solution heated for 5 min at 70 °C. A 1 ml aliquot of anthrone was added to 200 µl of the solution and heated for 10 min at 90 °C. Optical density was measured at 630 nm with a spectrophotometer (T60U, Harlow Scientific, USA), and the glycogen concentration of samples determined from a glycogen standard curve.

Sugar alcohol content was measured in vitro using high-performance liquid chromatography (HPLC) (Knauer, Berlin, Germany) equipped with a carbohydrate column with 4 µm particle size (250 mm × 4.6 mm, I.D., Waters, Ireland) as described by Heydari and Izadi (2014). Briefly, larvae were weighed and homogenized in 1.5–2 ml of 80% ethanol and centrifuged for 15 min at 12000 ×*g*. The supernatant was evaporated at 40 °C in vacuum drying oven and re-suspended in 1 ml of HPLC grade water.

2.9. Enzyme activity assays

The activity of four enzymes, AMP-activated protein kinase (AMPK) and three major subunits of serine/threonine-specific protein phosphatase (i.e. PP1, PP2A and PP2C). Enzyme activity was measured with five replicates of larvae and expressed per gram fresh mass.

2.9.1. AMPK

Larvae were weighed and homogenized 1:10 (w/v) in ice-cold potassium phosphate buffer (20 mM; pH 6.8), X-mercaptoethanol (15 mM) and EDTA (2 mM), using a pre-cooled homogenizer (Teflon pestle, 0.1 mm clearance), with a few crystals of phenylmethylsulfonyl fluoride (PMSF) added. The homogenate was centrifuged at 13000 ×*g* at 5 °C for 3 min. The supernatant was recovered and stored on ice for enzymatic assay. The activity of AMPK was assayed according to the method described by Pfister and Storey (2002b). The reaction was started by incorporating of ^{32}P from ^{32}P-ATP onto Kemptide (LRRASLG), a synthetic phosphate-accepting peptide, in the presence of 0.1 mM adenosine 30, 50-cyclic monophosphate. One unit of AMPK activity was defined as the amount of enzyme required to catalyze the incorporation of 1 nmol ^{32}P into substrate per minute at 23 °C.

2.9.2. PP1

Larvae were weighed and homogenized 1:3 (w/v) in ice-cold Buffer A [Tris–HCl (20 mM; pH 7.4), EGTA (2 mM), EDTA (2 mM), X-mercaptoethanol (15 mM)] containing protease inhibitors: TPCK (0.1 mM), PMSF (1 mM), aprotinin (1 mg/ml) and benzamidine (5 mM), using a pre-cooled homogenizer. The homogenate was centrifuged at 1000 ×*g* at 5 °C for 3 min. The supernatant was recovered and used immediately to assay PP1 activity. Estimation of PP1 activity at physiological levels of modulating proteins and other factors was done based on the assay of concentrated extracts, as described by Toth et al. (1988). The activity of PP1 was assayed by monitoring ^{32}P cleavage from ^{32}P-labeled phosphorylase at 23 °C (Pfister and Storey, 2002b). One unit of PP1 activity was defined as the amount of enzyme required to release 1 nmol of phosphate per minute at 23 °C.

2.9.3. PP2

The extraction of the larva was done as for PP1 except for a 1:10 (w/v) dilution. The homogenate was centrifuged at 13000 ×*g* at 5 °C for 20 min. The supernatant was recovered and desalted by centrifugation at low-speed for 1 min (at room temperature) through 5 ml Sephadex G-25 columns equilibrated in ice-cold Buffer A. The eluant was collected, passed through a second, fresh column and stored on ice for subsequent use. The activities of PP2A and PP2C were assayed according to the method described by Cowan et al. (2000). The activity of PP2A was measured as activity in the presence (blank) versus absence of okadaic acid (2.5 nM). Reaction was started by addition of 10 µl of enzyme extract to the reaction mixture containing peptide RRA(pT)VA (150 mM), EGTA (0.2 mM), X-mercaptoethanol (0.02%), and imidazole (50 mM), pH 7.2, and stopped 40 min later by addition of 50 µl of malachite green dye solution [ammonium molybdate (10%) and malachite green dye (2%), both in HCl (4 N) mixed 1:3 v/v and diluted 2:3 v/v with distilled, deionized water, Tween 20 (0.05%) and Triton X-100 (0.05%)] (Ekman and Jaeger, 1993). The absorbance was read at 595 nm. Appropriate blanks, which substrate was added after malachite green dye solution, were prepared for each treatment. The activity of PP2C was identical as PP2A, except for the presence of okadaic acid (2.5 nM) and incubation of the reaction mixture for 90 min; PP2C was detected as the different activities in the presence (blank) versus absence of $MgCl_2$ (10 mM), as described by Cowan et al. (2000).

2.10. Statistical analysis

All data were examined for normality using Kolmogorove-Smirnov tests (PROC GLM; SAS Institute, 2009). Because data were normally distributed, data transformation was unnecessary. Larval weight, supercooling points and cold hardiness of insects were analyzed by one-way analysis of variance (ANOVA) using SAS ver.9.2 program (PROC GLM; SAS Institute, 2009) with *post-hoc* Tukey's tests.

3. Results

3.1. Effect of the thermal regimes on carbohydrate content, supercooling points and cold hardiness

There was a significant relationship between carbohydrate content, enzyme activity and survival rates under the different thermal regimes in *P. interpunctella* and *E. ceratoniae*. In both species, glycogen content was highest in the control and RCH regimes and lowest in the CA and FA regimes (Fig. 1A, Fig. 2A). Changes in low molecular weight carbohydrates were inversely proportional to the changes in glycogen content (Fig. 1B-D, Fig. 2B-D). The amount of trehalose, the main low molecular carbohydrate, was greatest among the CA regime (Fig. 1B, Fig. 2B).

The SCP of the fifth instar larvae of *P. interpunctella* was approximately −14 °C and decreased to −16 °C under the CA regime (Table 1). For *E. ceratoniae*, the SCP decreased to – 18 °C under the CA regime from −10 °C. The RCH regime had no effect on the SCP of larvae of both species. Exposure to 0 °C for 24 h had no effect on larval survival, but the CA (98.3%) and FA (88.0%) regimes improved survival after exposure to −5 °C for 24 h in *P. interpunctella* larvae. In the CA regime, survival rate of *E. ceratoniae* fifth instar larvae was highest (100%) after 24 h exposure to −5 °C. The lowest survival rate was observed in the RCH regime in *P. interpunctella* and *E. ceratoniae*. Exposure to −15 °C for 24 h resulted in 100% mortality in all the regimes other than CA. In the CA regime, at −15 °C/24 h survival rates of *P. interpunctella* and *E. ceratoniae* were 17.3% for 30.7%, respectively (Table 1).

3.2. Effect of thermal regimes on lethal time

In control larvae of *P. interpunctella*, the time necessary to reach 30%, 50% and 90% mortality was 10.5, 18.7 and 76.6 h, respectively (Table 2). Cold acclimation increased the time to 30%, 50% and 90% mortality to 42.8, 53.9 and 94.7 h, respectively. Lethal times (lower, median and upper) in FA and RCH regimes were also significantly greater than the control. In control larvae of *E. ceratoniae* lower, median and upper lethal times were 16.3, 25.3 and 74.1 h, respectively. Cold-acclimated larvae had the greatest lower, median and upper lethal times: 48.9, 60.2 and 100.1 h, respectively. There was no effect of RCH on lethal time. The time necessary to reach 90% mortality in *P. interpunctella* and *E. ceratoniae* remained approximately the same.

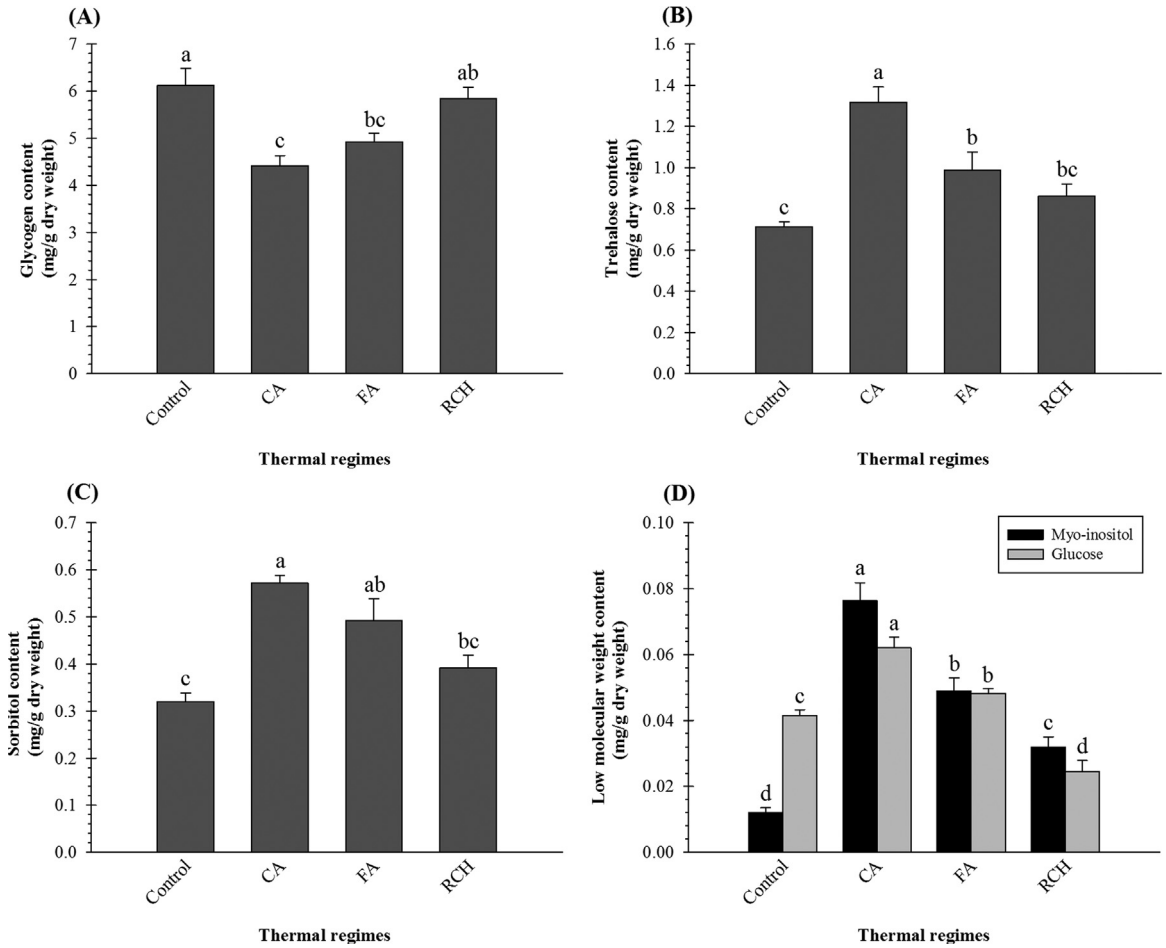

Fig. 1. Carbohydrate contents of *Ectomyelois ceratoniae* after exposure to different thermal regimes. Each point is average of five replications. Mean values followed by different letters are significantly different (Tukey's test, $P < 0.05$). **CA:** cold-acclimated, **FA:** fluctuating-acclimated, **RCH:** rapid cold-hardened.

3.3. Effect of thermal regimes on lethal temperature

After exposure of control larvae of *P. interpunctella* and *E. ceratoniae* to zero and subzero temperatures mortality was first detected at 0 °C, followed by a sharp decline in survival with decreasing temperature. Larvae had the highest cold hardiness in RCH treatment. In RCH treatment, the lower lethal temperatures (LLT) (temperature at which 100% of the larvae died) for *P. interpunctella* and *E. ceratoniae* were approximately −23 and −25 °C, respectively. The LLTs for the control larvae of *P. interpunctella* and *E. ceratoniae* were approximately −16 and −15 °C, respectively. For *P. interpunctella*, the $LT_{80-1\,h}$ for control, RCH, FA and CA treatments was approximately −15, −22, −18 and −16 °C, respectively. For *E. ceratoniae*, the $LT_{80-1\,h}$ was approximately −13, −24, −19 and −16 °C for control, RCH, FA and CA treatments, respectively (Fig. 5).

3.4. Effect of thermal regimes on enzyme activity

Two enzymes, PP1 and AMPK, were found to be the most active forms of the tested enzymes (Figs. 3 and 4). In control larvae of *P. interpunctella*, the activities of PP1 and AMPK were 25.0 and 19.9 units/g respectively. Interestingly, the activity of AMPK varied considerably between thermal regimes and was greatest in cold-acclimated larvae (39.0 units/g). However, while the activities of AMPK in the FA and RCH regimes were higher than that of the control, this effect was not significant. In control larvae of *E. ceratoniae*, PP1 was more active than AMPK. The activity of PP1 substantially varied between different thermal regimes and was greatest in cold-acclimated larvae (38.0 units/g) (Fig. 3). The activity of PP1 in larvae of *P. interpunctella* was highest in under FA (14.9 units/g) and lowest in the CA regime (10.0 units/g) (Fig. 4). RCH had no significant effect on the activity of PP1 (Fig. 3, Fig. 4). In larvae of *E. ceratoniae*, the highest (19.1 units/g) and lowest (7.1 units/g) activities of PP1were recorded for control and CA regimes, respectively (Fig. 3).

4. Discussion

4.1. Effect of thermal regimes on carbohydrate content, supercooling points and cold tolerance

The results of the current study demonstrate that thermal regimes (CA, FA, and RCH) affect the physiology and survival of last instar larvae of *P. interpunctella* and *E. ceratoniae*. Moreover, the results suggested a strong relationship between carbohydrate contents and survival rates of the larvae. Out of three different thermal regimes, CA had the greatest impact on carbohydrate contents, enzyme activities, SCPs, survival rates and lethal times of the larvae. An inverse relationship between glycogen content and low molecular weight carbohydrate (e.g. trehalose) content and a positive relation between low molecular weight carbohydrate contents and survival rates were also revealed. However, these effects were greatest for CA larvae relative to the control and other thermal regimes (FA and RCH). RCH had the greatest impact on lower lethal temperature. The lowest glycogen and the highest trehalose contents were detected in cold-acclimated larvae of both species. These increases were coincident with the lowest SCPs and the highest survival rates of the larvae. In addition, the activity of the

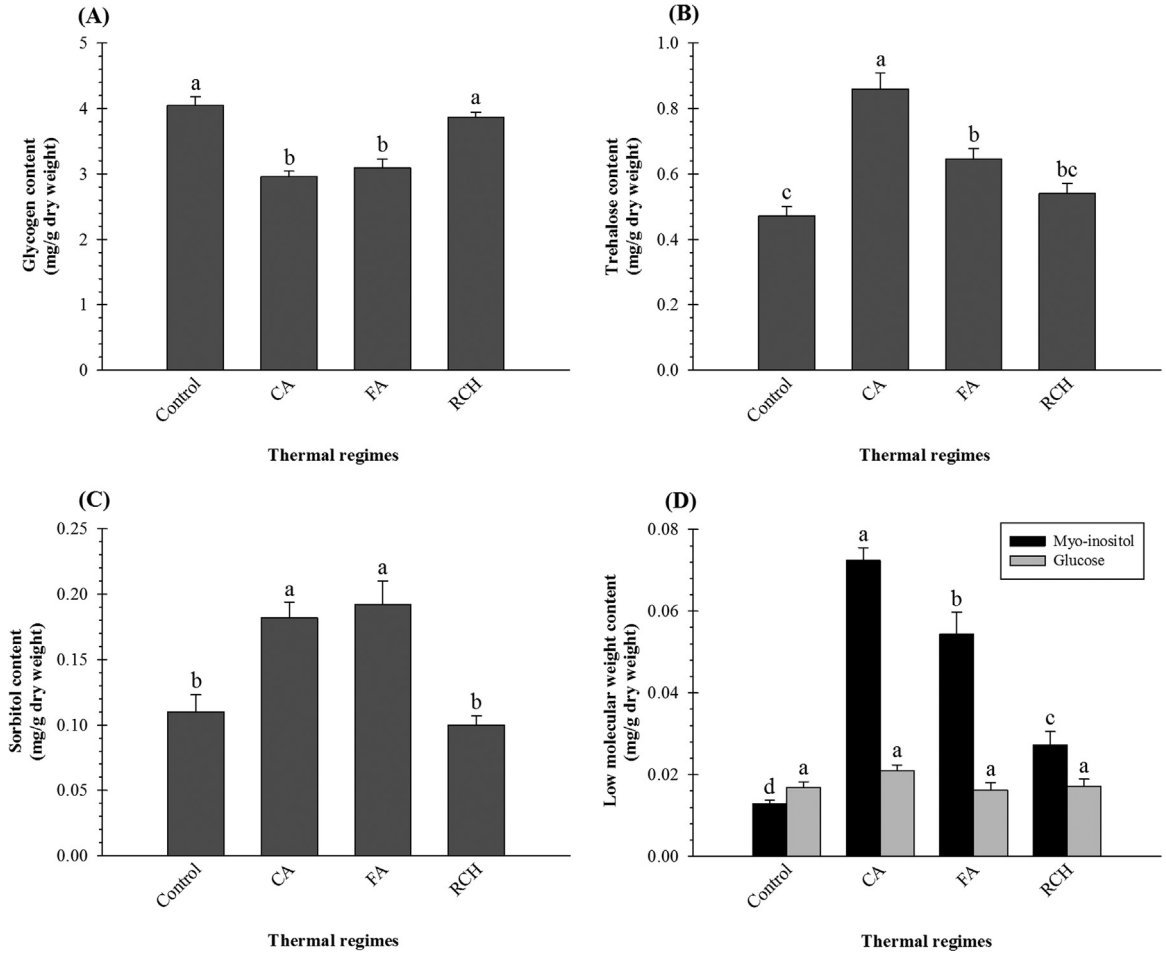

Fig. 2. Carbohydrate contents of *Plodia interpunctella* after exposure to different thermal regimes. Each point is average of five replications. Mean values followed by different letters are significantly different (Tukey's test, $P < 0.05$). **CA:** cold-acclimated, **FA:** fluctuating-acclimated, **RCH:** rapid cold-hardened.

AMPK was highest in the CA regime.

In both *P. interpunctella* and *E. ceratoniae*, glycogen content under the CA regime was significantly lower than that of the control. Whereas, trehalose content in the CA was significantly higher than the control larvae. This finding suggests that there is a conversion of glycogen into trehalose and other cryoprotectants under cold acclimation treatment. Synthesis of cryoprotectants involves the conversion of glycogen to low molecular weight carbohydrates or sugar alcohols (Storey and Storey, 2012). Trehalose as a cryoprotectant plays a vital role in decreasing SCP and increasing survival rate and this is a probable reason for the lower SCP and the higher survival rate of *P. interpunctella* and *E. ceratoniae* 5th instar larvae in CA regime. Interestingly, RCH had no effect on glycogen, low molecular weight carbohydrate contents, SCPs and survival rates of the larvae. Therefore, it could be concluded that in cold-acclimated last instar larvae of *P. interpunctella* and *E. ceratoniae*, elevation of cold hardiness is a function of a decrease in SCP and an increase in polyols and low molecular weight carbohydrates synthesis and accumulation. On the other hand, in cold-acclimated larvae, acclimation had a trend towards lower SCP and subsequently higher survival rate. Mohammadzadeh and Izadi (2018) reported a significant increase in the carbohydrate content and cold tolerance of the last instar larvae of *Trogoderma granarium* Everts (Coleoptera: Dermestidae) under a CA

Table 1
Relationship between low temperature survival rate and supercooling points of 5th instar larvae of two stored-product moth following different thermal regimes.

Treatments	SCP (°C)	Survival rate (%)			
		0 °C/24 h	-5 °C/24 h	− 10 °C/24 h	− 15 °C/24 h
Plodia interpunctella					
Control	11.4 ± 0.4 b	90.7 ± 1.6 a	53.3 ± 2.1c	10.7 ± 3.4c	0.0 ± 0.0 b
Cold acclimation	16.3 ± 0.7 a	98.6 ± 1.3 a	89.3 ± 3.4 a	46.7 ± 3.0 a	17.3 ± 2.7 a
Fluctuating acclimation	14.5 ± 0.7 a	97.3 ± 1.6 a	88.0 ± 2.5 a	30.7 ± 1.6 b	0.0 ± 0.0 b
Rapid cold-hardening	11.8 ± 0.5 b	89.3 ± 4.5 a	68.0 ± 3.9 b	16.0 ± 3.4c	0.0 ± 0.0 b
Ectomyelois ceratoniae					
Control	10.5 ± 0.7c	93.3 ± 3.0 a	57.3 ± 2.7 BCE	0.0 ± 0.0c	0.0 ± 0.0 b
Cold acclimation	18.6 ± 0.7 a	100.0 ± 0.0 a	100.0 ± 0.0 a	62.7 ± 3.4 a	30.7 ± 2.7 a
Fluctuating acclimation	15.7 ± 0.5 b	100.0 ± 0.0 a	70.7 ± 3.4 b	34.7 ± 2.5 b	0.0 ± 0.0 b
Rapid cold-hardening	12.3 ± 0.6c	94.7 ± 2.5 a	65.3 ± 4.4c	8.0 ± 2.5c	0.0 ± 0.0 b

The means followed by different letters in the same columns are significantly different (Turkey's test, $P < 0.05$).

Table 2
Lt_{30}, Lt_{50} and Lt_{90} values of 5th instar larvae of two stored-product moths after chronic low-temperature exposure.

Treatments	n[a]	χ^2	Slope ± SE	Lethal time (h)		
				LT_{30} (95% FL)	LT_{50} (95%FL)	LT_{90} (95%FL)
Plodia interpunctella						
Control	405	57.7	2.09 ± 0.27	10.5 (7.4–13.2)	18.7 (15.3–21.9)	76.6 (58.8–116.5)
Cold acclimation	675	70.7	5.23 ± 0.62	42.8 (38.0–46.4)	53.9 (50.3–57.2)	94.7 (85.4–110.8)
Fluctuating acclimation	540	72.2	4.08 ± 0.48	26.2 (22.1–29.4)	35.2 (31.8–38.2)	72.5 (64.1–87.3)
Rapid cold-hardening	450	64.5	4.29 ± 0.53	21.5 (18.0–24.3)	28.5 (25.5–31.1)	56.8 (50.2–68.3)
Ectomyelois ceratoniae						
Control	495	66.2	2.74 ± 0.33	16.3 (12.5–19.4)	25.3 (21.6–28.5)	74.1 (61.7–98.0)
Cold acclimation	810	138.4	5.80 ± 0.49	48.9 (45.0–52.2)	60.2 (56.9–63.3)	100.1 (93.1–110.2)
Fluctuating acclimation	630	100.0	4.34 ± 0.43	30.2 (26.4–33.4)	39.9 (36.5–42.9)	78.5 (70.8–90.5)
Rapid cold-hardening	540	80.4	3.52 ± 0.39	22.5 (18.7–25.6)	31.7 (28.8–34.7)	73.1 (63.8–89.3)

Lethal concentrations and 95% fiducial limits (FL) were estimated using logistic regression (SAS Institute 2002).
[a] The total number of larval used for bioassay test.

regime. They also reported a significant decrease in SCP and an increase in survival of the larvae under CA thermal regime. Hiiesaar et al. (2001) found that in *Leptinotarsa decemlineata* (Say) (Col.: Chrysomelidae) the SCP decreased from −10.5 °C in non-acclimated to −17.5 °C in cold-acclimated beetles. However, in disagreement with our results, Maes et al. (2012) reported that cold acclimation of *Macrolophus pygmaeus* (Hemiptera: Miridae) resulted in an increase in SCP of the predatory bugs. Spranghers et al. (2017) reported an increase in cold-acclimated prepupae of *Hermetia illucens* (L.) (Dip.: Stratiomyidae) but SCP of the prepupae was unaffected by cold acclimation. However, in disagreement with our results, SCP of *M. caliginosus* adults had no change in response to acclimation, but cold hardiness of the acclimated adults was significantly more than non-acclimated individuals (Hart et al., 2002). Cold acclimation of the predatory bug, *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae) (Hughes et al., 2009) and the parasitoid, *Eretmocerus eremicus* (Rose and Zolberowich) (Hymenoptera: Aphelinidae) (Tullett et al., 2004) had no significant effect on their SCP. Our results showed that in control as well as different thermal regimes most of the pest's mortality occurs near the SCP. Therefore, the last instar larvae of *P. interpunctella* and *E. ceratoniae* could be considered as

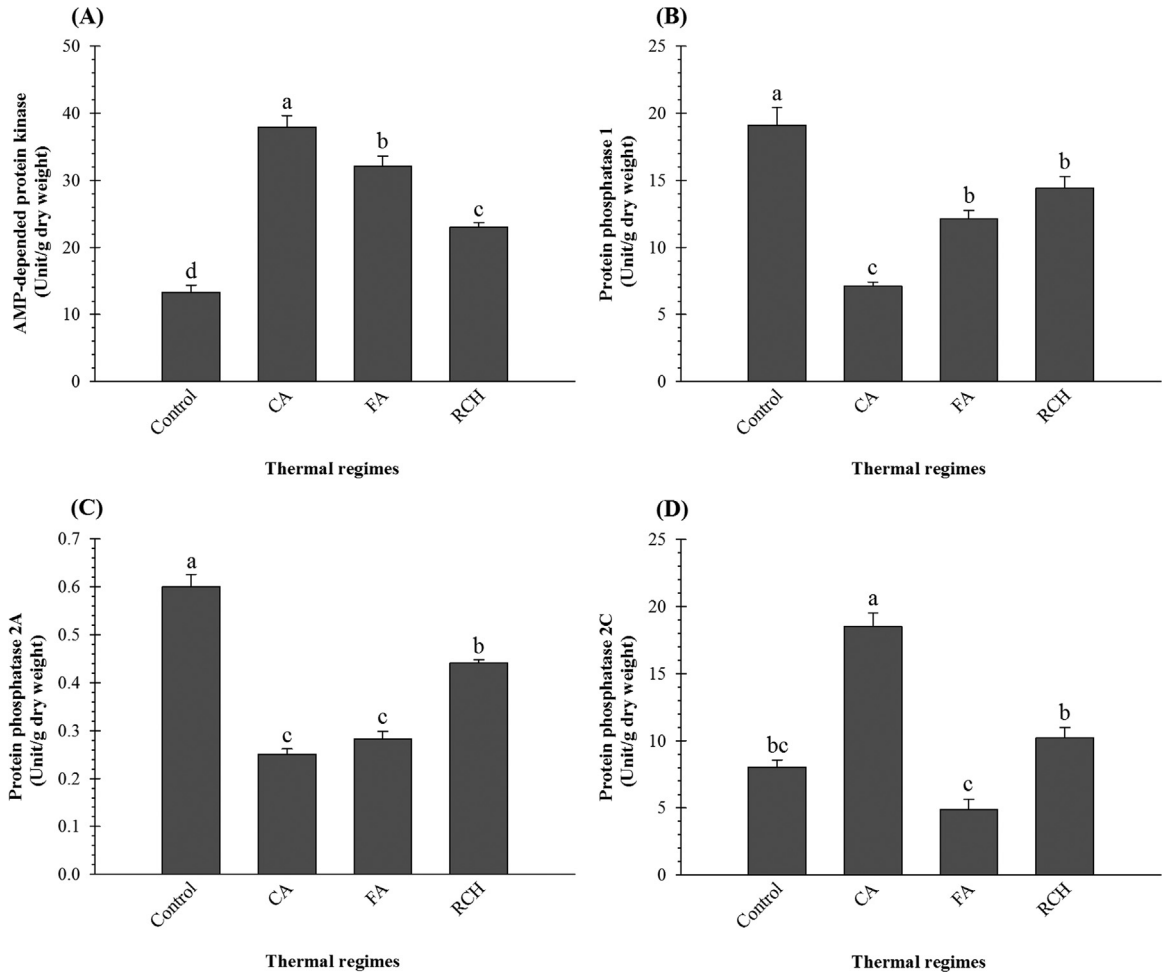

Fig. 3. Enzyme activities of *Ectomyelois ceratoniae* after exposure to different thermal regimes. Each point is average of five replications. Mean values followed by different letters are significantly different (Tukey's test, $P < 0.05$). **CA:** cold-acclimated, **FA:** fluctuating-acclimated, **RCH:** rapid cold-hardened.

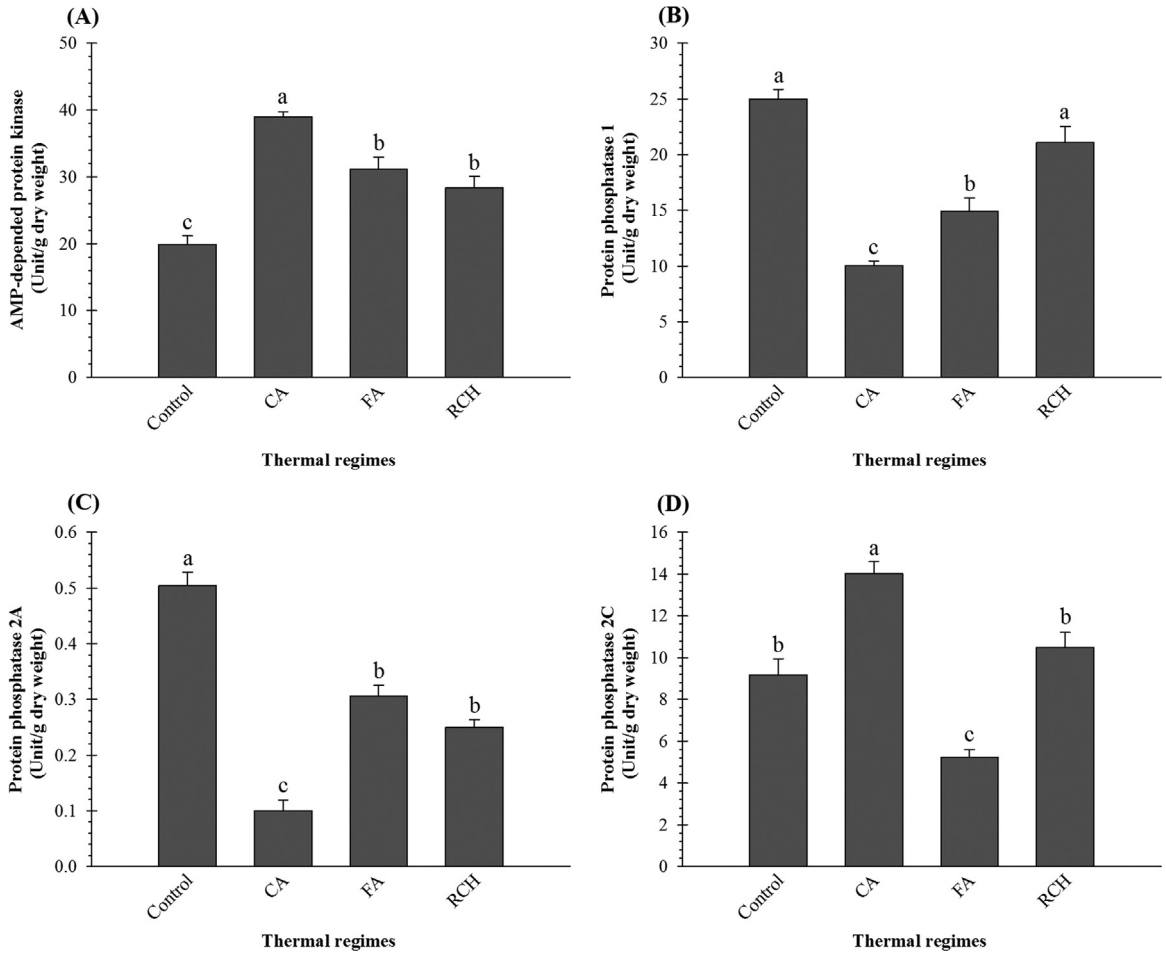

Fig. 4. Enzyme activities of *Plodia interpunctella* after exposure to different thermal regimes. Each point is average of five replications. Mean values followed by different letters are significantly different (Tukey's test, $P < 0.05$). **CA:** cold-acclimated, **FA:** fluctuating-acclimated, **RCH:** rapid cold-hardened.

freeze-intolerant.

4.2. Effect of thermal regimes on lethal time and temperature

The results of the current study indicate that the time required to kill 30%, 50% and 90% of the last instar larvae of *E. ceratoniae* was greater than *P. interpunctella*. However, in both species, lethal times for the thermal regimes were significantly longer than that of control. The differences between lethal times of the CA and two other regimes (FA and RCH) were also significant. The highest lethal times were recorded for the CA regime. In cold-acclimated larvae of *P. interpunctella*, the Lt_{30} was approximately 4-fold greater than that of control larvae. The Lt_{50} and Lt_{90} were 2.9 and 1.2 folds greater than that of control larvae, respectively. In cold-acclimated larvae of *E. ceratoniae,* the Lt_{30} was approximately 3-fold greater than that of control larvae. The Lt_{50} and Lt_{90} were 2.4 and 1.3 folds greater than that of control larvae, respectively. Together, these results suggest that cold acclimation has a greater impact on Lt_{30} than Lt_{90}. Koštál et al. (2011) found that cold acclimation considerably extended the lethal time and improved cold tolerance of third instar larvae of *D. melanogaster*. Wilches et al. (2017) demonstrated that acclimation significantly enhanced cold hardiness of diapausing and non-diapausing larvae of *T. granarium*.

RCH is the capacity to rapidly (within hours) develop protection against injury caused by low temperatures in insects. This capacity plays a crucial role in the insect survival in response to rapid decreases in ambient temperature (Saeidi et al., 2017). It is believed that RCH increases the tolerance to acute cold shock at both organismal and cellular levels (Lee et al., 2006). The results of this study show that RCH has the greatest impact on lower lethal temperature limits of larvae compare with other acclimation treatments. The temperature at which 80% of the larvae died after 1 h exposure was almost 1.5-fold lower in RCH larvae than that of the control larvae. In addition, RCH significantly increased the survival of the larvae of *P. interpunctella* and *E. ceratoniae*. The same results have been reported by Lee et al. (2006) on the larvae of an Antarctic midge, *Belgica antarctica* (Diptera, Chironomidae), Ju et al. (2011) on the sycamore lace bug, *Corythucha ciliata* (Say) (Hemiptera: Tingidae) and Saeidi et al. (2017) on the Russian wheat aphid, *Diuraphis noxia* (Kurdjumov).

4.3. Effect of thermal regimes on enzyme activity

Several previous studies demonstrated the effects of low temperatures on enzyme activity (Pfister and Storey, 2002a, 2002b; Mohammadzadeh and Izadi, 2018). Here, we investigated whether AMPK, PP1, PP2A, and PP2C could be involved in cold adaptation in the last instar larvae of *P. interpunctella* and *E. ceratoniae*. In control larvae of *E. ceratoniae* and *P. interpunctella*, activity of the enzymes could be rated as PP1 > AMPK > PP2C > PP2A. Interestingly, in cold-acclimated larvae of *P. interpunctella* and *E. ceratoniae* the activities of AMPK and PP2C substantially increased, whereas the activities of PP1 and PP2A significantly decreased. In control larvae of *P. interpunctella* AMPK activity was approximately 2-fold compared with larvae of the CA regime. Among control larvae of *E. ceratoniae* AMPK activity was 3-fold higher compared with larvae of CA regime. In the FA and RCH regimes AMPK activity was also greater than control larvae. PP1 and AMPK were found to be the predominant signal transduction enzymes

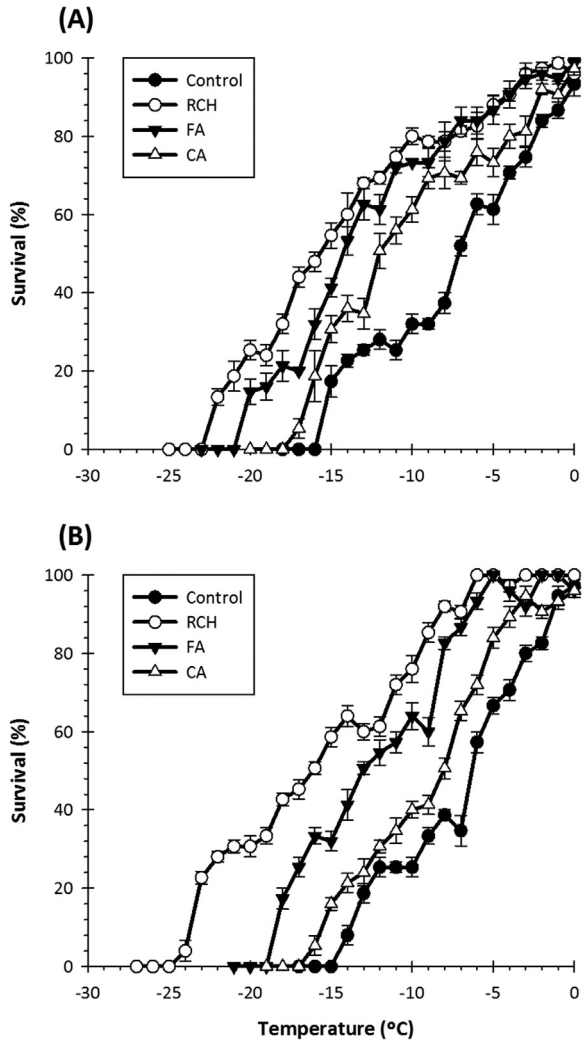

Fig. 5. Survival of larvae of two stored product moths after acute low-temperature exposure.

of *P. interpunctella* and *E. ceratoniae* last instar control larvae, but the thermal treatment of the larvae changed the activities of the enzymes. In the CA, FA and RCH regimes the activities of AMPK and PP2C were higher than PP1. Mohammadzadeh and Izadi (2018) reported that AMPK was the major signal transduction enzyme in *T. granarium* last instar larvae. AMPK is a downstream component of a kinase cascade that plays an important role in the regulation of glycogen, sugar, and lipid metabolism (Dzamko and Steinberg, 2009; Lim et al., 2010). Our results support a relationship between the activity of the AMPK, the conversion of glycogen into cryoprotectants (e.g. trehalose) supercooling point reduction and cold tolerance improvement. The survival of many insects in low temperature depends on cold tolerance adaptations (Rider et al., 2011; Storey and Storey, 2012). The most important adaptations are physiological, biochemical and metabolic alterations (e.g. biosynthesis and accumulation of cryoprotectants and overexpression of the genes encoding the enzymes) (Overgaard et al., 2007, 2014; Teets and Denlinger, 2013). Accordingly, the accumulation of low molecular weight carbohydrates and raising AMPK activity in last instar larvae of *P. interpunctella* and *E. ceratoniae* could be explained as the physiological adaptations in the CA regime. The results of the current study showed that in larvae of these pests, thermal reaction curves of AMPK and PP2C follows the same norm that is different from those of PP1 and PP2A. This finding agrees with the results of Mohammadzadeh and Izadi (2018).

From an applied perspective, the use of low temperatures for disinfection of store structures and products has been considered in recent years (Andreadis and Athanassiou, 2017). Chill-susceptible or chill-intolerant insects are killed by low temperatures without internal ice formation (Sinclair et al., 2015). Therefore, in this group of insects, cooling of the stored products such as seeds near the SCP of the stored pests for a defined period of time may be a suitable method for control (Phillips and Throne, 2010; Hagstrum and Phillips, 2017). Freeze-tolerant insects that survive internal ice formation (i.e. below their supercooling point) use a complex strategy to tolerate low temperatures. Freeze-intolerant or freeze-avoidant insects may depress their supercooling points by physiological and biochemical adaptations to avoid ice formation within their body fluids (Andreadis and Athanassiou, 2017). Thus, cooling products to temperatures near SCPs may not necessarily cause mortality in the freeze-tolerant and intolerant insects. However, phytosanitary temperature treatments may still substantially reduce their survival rate.

In conclusion, the current study demonstrated that enhanced cold tolerance of larvae of *P. interpunctella* and *E. ceratoniae* under CA regimes is closely related to a decreased SCP and elevated level of cryoprotectants (e.g. trehalose) and activity of enzymes (e.g. AMPK). Moreover, the results of this study suggest that RCH may play an important role in the survival of *P. interpunctella* and *E. ceratoniae* under acute low temperatures.

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