Enzyme Activity, Cold Hardiness, and Supercooling Point in Developmental Stages of *Acrosternum arabicum* (Hemiptera: Pentatomidae)

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**Abstract**

Several species of pentatomid bugs feed on pistachio fruits in Iran. *Acrosternum arabicum* Wagner (Hemiptera: Pentatomidae) is one of the most important pests of pistachio in Rafsanjan, Iran. This study was carried out to investigate the carbohydrase activities, supercooling points, and cold hardness profiles of different developmental stages of *A. arabicum* under laboratory conditions. The midgut amylolytic of *A. arabicum* showed an optimal pH at 7.0. The highest amylolytic activity was found in the female adults (35.41 ± 0.90 nmol/min/gut). The mean amylolytic activity measured in first instar nymph was 6.75 ± 0.54 nmol/min/gut. Midgut α- and β-glucosidase showed an optimal activity at pH 5 and 7, respectively. These activities increased from first (83 ± 5 and 54 ± 5 nmol/min, respectively) to fifth (881 ± 17 and 237 ± 14 nmol/min, respectively) instar nymphs. The enzyme activities increased in the adults. Midgut α- and β-galactosidase showed an optimal activity at pH 5. α- and β-galactosidase activities were low in the first instar nymphs (73 ± 5 and 21 ± 3 nmol/min, respectively). The level of α- and β-galactosidase activities in the female adults (533 ± 18 and 246 ± 6 nmol/min, respectively) was higher than the nymphs. The lowest supercooling points (−19 and −18.2°C, respectively) and the highest cold hardness (22 and 18% following 24 h exposure at −20°C, respectively) were recorded for the eggs and adult females.

**Key words:** *Acrosternum arabicum*; carbohydrase; stage specific digestion; super cooling point

Pistachio as a major agricultural product in Iran attacks by several pests. Different species of hemipteran bugs (e.g., *Acrosternum heegeri*, *A. millieri*, *A. arabicum*, *Apodipsus amygdali*, *Brachynema germari*, *Brachynema segetum*) have been reported as pistachio pests throughout pistachio producing regions of Iran (Mehrnejad 2001). *A. arabicum* Wagner (Hemiptera: Pentatomidae) is one of the key pests of pistachio since attacks nuts from early spring to the time of harvest, causing severe losses in production as well as affecting the product quality (Mehrnejad et al. 2013). Control of this pest is thoroughly dependent of chemical application. However, many problems are associated with using the pesticides (Subramanyam and Hagstrum 1995, Hagstrum and Subramanyam 1996, Regnault-Roger et al. 2012, Macfadyen et al. 2014, Guedes et al. 2016), and it is necessary to use alternative methods for control of pentatomid pests.

The digestive tract of insects is an excellent target for control agents that in general are not toxic to other organisms (Nauen et al. 2001, Lwalaba et al. 2010). Therefore, for developing alternative methods of insect control, it is essential to understand the function and physiology of digestive enzymes during on-growing of insect pest (Kazzazi et al. 2005).

Carbohydrases are widespread in nature, being found in animals, microorganisms, and plants (Franco et al. 2000). Activity of carbohydrases such as α-amylase, glucosidases, and galactosidases has been demonstrated and described in the gut of a number of hemipteran insects (Zeng and Cohen 2000, Kazzazi et al. 2005, Oliveira et al. 2006, Swart et al. 2006, Vatanparast et al. 2011, Mehrabadi et al. 2012, Ghamari et al. 2014).

Cold hardness has been defined as the ability of an insect to resist injury during long or short term exposure to low temperature. Several factors such as developmental stage, genetic potential, season, duration of exposure, and nutritional status may influence the cold hardness of insects. Cold hardness is a graded response in which capacity of insect to tolerate harsh environmental conditions is proportional to the intensity of the environmental stimulus. Some physiological adaptations (e.g., cryoprotectants accumulation) may be associated with cold hardiness. Insects at temperate zones usually utilize different strategies to avoid temperature below 0°C. These
strategies describe how insects resist temperatures where its body fluids might be expected to freeze (Lee 2010, Storey and Storey 2012, Sinclair et al. 2015).

Supercooling point (SCP) is the temperature at which freezing begins, and ice formation will usually then proceed from the site of nucleation to other parts of the insect’s body. The SCP is experimentally determined by detecting the released latent heat of fusion as body water freezes (Bemani et al. 2012). Many investigations of insect cold tolerance begin with preliminary measurements of the SCP because this provides an anchor point about which the cold tolerance strategy can be determined (Sinclair et al. 2015).

Our investigations focus on the presence and activity level of digestive carbohydrates (α-amylase, glucosidases, and galactosidases) in different instar nymphs and adults of *A. arabicum* and developmental changes in SCPs and cold hardiness of *A. arabicum*, to gain a better understanding of the developmental physiology of the insect.

**Material and Methods**

**Insect Culture**

Adults of *A. arabicum* were collected from a pistachio garden in Kerman, Iran. A colony of different developmental stages was fed on *Salsola kali* L. and maintained at 26 ± 1°C, relative humidity of 65 ± 5% and a photoperiod of 16:8 h (L:D cycle), for two generations.

**Preparation of Samples**

Adults and different instar nymphs were sampled 1 d after molting when an active feeding behavior of insect was observed. Nymphs of different instars and adults were dissected following the procedure described by Cohen (1993). In brief, insect midguts were removed in 0.15 mM NaCl solution from nymphal and adult’s stages previously immobilized on ice. For each sample, 10 midguts were homogenized in 1.0 ml of cooled distilled water (at 4°C) and then centrifuged at 15,000 × g for 15 min at 4°C (Borzouei et al. 2015). The supernatant was recovered and frozen (−20°C) for enzymatic assays. In these conditions, enzyme activity remained constant for at least 1 month.

**Determination of Luminal pH of Midgut**

Appropriate optimal pH for α-amylase, glucosidases, and galactosidases was determined in starved adult females by monitoring RDA activity at various pH ranging from 3.0 to 10.0. Buffer used were 100 mM glycine HCl (pH 3.0–4.0), sodium acetate (pH 5.0–6.0), and sodium phosphate (pH 7.0–10.0). All experiments were replicated three times.

**Amylolytic Activity Assay**

Amylolytic activity was carried out as described by Bernfeld (1955). The reaction mixture consisted of 40 μl of 1% freshly prepared starch solution, 500 μl of 20 mM phosphate buffer (pH 7), and 20 μl of enzyme extract. After incubating for 30 min at 37°C, the reaction was stopped by adding 100 μl of 3,5-dinitro salicylic acid reagent, and heating in boiling water for 10 min. Absorbance of the samples was read in optical density units at ABS405nm against a blank in which the enzyme extract was replaced with deionized water. A standard curve of absorbance against amount of maltose released was constructed to enable its calculation during amylolytic assay. All experiments were replicated three times.

**Glucosidases and Galactosidases Assay**

Glucosidases and galactosidases activities were determined as described by Nakonieczny et al. (2006) and Ghamari et al. (2014). The substrates were p-nitrophenyl-α-D-glucopyranoside (5 mM, for α-glucosidase), p-nitrophenyl-β-D-glucopyranoside (5 mM, for β-glucosidase), p-nitrophenyl-α-D-galactopyranoside (5 mM, for α-galactosidase), and p-nitrophenyl-β-D-galactopyranoside (5 mM, for β-galactosidase). Reaction was started by the addition of 5 μl substrate and stopped 30 min later by adding 100 μl of NaOH (2 M) and released p-nitrophenols were determined at the ABS405nm after 5 min. In the blank, the enzyme extract was added to the reaction mixture after NaOH treatment. All experiments were replicated three times.

**Determination of SCPs**

The SCPs of individual egg, nymph, and adult (n = 6) were measured using a thermocouple (NiCr–Ni probe) connected to an automatic temperature recorder, Testo 177-T4 (Testo, Germany), so that the cooling could be recorded at 0.5 min. intervals and the data were read using Comsoft 3 Software. The specimens were attached to the thermocouple by adhesive tape and placed inside a programmable refrigerated test chamber (Gotech, GT-7005-A, Taiwan). The temperature was lowered at a rate of 0.5°C/min, starting at 20°C and ending at −30°C. The temperature at which an abrupt increase occurred with the liberation of the latent heat of freezing was taken as the SCP (Khani and Moharamipour 2010).

**Survival at Low Temperature**

Eggs, nymphs, and adults of *A. arabicum* were transferred (n = 7–10) to a programmable refrigerated test chamber. The temperature was lowered at a rate of 0.5°C/min, from 20°C to the desired treatment temperature (0, −5, −10, −15, and −20°C). The eggs, nymphs, or adults were held at these temperatures for 24 h and then slowly (0.5°C/min) heated to 25°C and held at that temperature for 24 h. Live and dead eggs, nymphs, and adults were counted. The blacked eggs and the nymphs or adults showing no movement were considered as dead (Khani and Moharamipour 2010).

**Statistical Analysis**

Statistical analysis were performed using one-way analysis of variance (ANOVA) followed by a post-hoc Tukey’s test (P = 0.05). Data were initially tested for normality (Kolmogorov–Smirnov test) and homoscedasticity (Levene’s test) before subjecting them to ANOVA. A non-parametric test (Mann–Whitney U test and Kruskal–Wallis test) was used to test for differences in cold hardiness data.

**Results**

**Effect of pH on the Enzymes Activity**

The pH values of α-amylase (Fig. 1), α- and β-glucosidase (Fig. 2), and α- and β-galactosidase (Fig. 3) were detected in the midgut of *A. arabicum* adults. All carbohydrases showed optimal pH values in the range 5.0–7.0.

**Digestive α-Amylase Activity During Development of *A. arabicum***

The midgut amylolytic activity of *A. arabicum* showed an optimal pH of 7.0 against the substrates starch (Fig. 1). Table 1 shows changes in amylolytic activity in different stages of *A. arabicum*. 
The activity of α-amylase showed a steady-state increase from the first instar nymph to adults ($F = 126.35$, $P < 0.0001$, $df = 6$, 14). The α-amylase activity per individual was at the highest level in the female adults ($35.41 \pm 0.90$ nmol/min/gut) (Table 1). The mean activity measured in the first instar nymph was $6.75 \pm 0.54$ nmol/min/gut and reached to $27.56 \pm 1.68$ nmol/min/gut in the fifth instar nymph. The activity of the enzyme in adult male was significantly lower than female (Table 1).

Glucosidases and Galactosidases Activities During Development of A. arabicum

Midgut α- and β-glucosidase showed an optimal activity at pH 5 and 7, respectively (Fig. 2). Table 1 show changes in values of α- and β-glucosidase activities contained within the nymphs and adults midgut ($F = 358.34$, $P < 0.0001$, $df = 6$, 14 and $F = 25.85$, $P < 0.0001$, $df = 6$, 14). The activity of glucosidases increased with nymphal development as expected. These activities increased from first ($83 \pm 5$ and $54 \pm 5$ nmol/min, respectively) to fifth ($881 \pm 17$ and $237 \pm 14$ nmol/min, respectively) instar nymph. Also, in the adult stages the enzyme’s activities increased. The results showed that α- and β-glucosidase activities in female adults ($1081 \pm 24$ and $290 \pm 23$ nmol/min, respectively) were higher than male adults ($736 \pm 35$ and $234 \pm 25$ nmol/min, respectively) (Table 1).

The comparison of galactosidases activities from the different stages of the A. arabicum is shown in Fig. 3 ($F = 158.89$, $P < 0.0001$, $df = 6$, 14 and $F = 107.29$, $P < 0.0001$, $df = 6$, 14). Alpha- and β-galactosidase activities were low in the first instar nymph 73 ± 5 and 21 ± 3 nmol/min, respectively and steadily increased as the nymph grew and the enzyme activities reached a maximum in the fifth instar nymphs (481 ± 12 and 230 ± 11 nmol/min, respectively). In this insect, the levels of α- and β-galactosidase activities in female adults (533 ± 18 and 246 ± 6 nmol/min, respectively) were higher than in nymphs (Table 1).

SCPs and Cold Hardiness of the Different Developmental Stages

Data presented in Table 2 indicate that the lowest SCPs were recorded for egg, fifth instar nymph, and adult stages. There were no significant differences between the SCPs of these stages, but the SCPs of first to fourth instar nymphs were significantly lower than those of eggs, fifth instar nymphs, and adults. The highest SCPs with −7 and −8.4°C were recorded for first and second instar nymphs, respectively. The least cold hardiness was found in the first instar nymphs with a survival rate of 26% following exposure at −5°C/24 h. At this temperature, survival rate was directly proportional to developmental stage and reached to 100% in fourth instar nymphs. Survival rate of eggs was 100% following exposure at −5°C/24 h. The survival rates of the first to third instar nymphs decreased following exposure at −10°C/24 h but no changes were found in survival rates of other stages. The cold hardiness decreased following exposure at −15°C/24 h. At this temperature, the highest survival rates were recorded for the eggs, fifth instar nymphs, and female adults. There were no significant differences between SCPs of the male adults and first to fourth instar nymphs. At −20°C/24 h survival rates of eggs (22%) and female adults (18%) were at the highest levels and significant differences between cold hardines of male and female adults were detected (Table 2).

Discussion

The major objectives of this study were to determine the characteristic of digestive carbohydrates, SCPs and cold hardiness different developmental stages of A. arabicum. Slightly acidic optimal pH for the assayed digestive carbohydrates in A. arabicum was similar to the ones stated for the majority of the examined Pentatomids.
Optimal pH for starch hydrolysis may have a broad range [e.g., from 3 to 6 in B. germani (Kolkenati) (Hemiptera: Pentatomidae)] (Ramzi and Hosseininaveh 2010), but this was not the case for A. arabicum.

On the contrary, glucosidases and galactosidases in the A. arabicum had the highest activity in slightly acidic conditions. In other Pentatomids, the optimal pH for these enzymes ranges between at 4 and 7 (Bigham and Hosseininaveh 2010, Ramzi and Hosseininaveh 2010, Ghamari et al. 2014).

The presence of amylase in the midgut of other phytophagous heteropterans has been reported in several researchers (Zeng and Cohen 2000, Boyd et al. 2002, Boyd 2003, Kazzazi et al. 2005). For all the other studied enzymes, A. arabicum exhibited enzymatic activity since the early stages of development as reported for other Hemipteran species (Kazzazi et al. 2005, Ghamari et al. 2014). Early instars are crucial periods for establishment of feeding sites on plants and subsequent feeding damage (Zalucky et al. 2002). In this study, we showed that early instars of A. arabicum have lower levels of α-amylase activity, which gradually increase until the fifth instar.

Midgut α-amylase level was observed to be highest at female adults and decreased in the male adults than the fifth instar nymphs (Table 1). It seems that maximum food intake occurs in the female adults and insect feeding in male adults and nympha! stages is lower than female adults. Alfonso et al. (1997) reported that midgut α-amylase activity was at the maximum level when food intake was high.

The results of this study based on a quantitative measurement showed that the midgut glucosidases activity in A. arabicum was gradually increased from first to the fifth nympha! instar. As reported by Dastranj et al. (2013), α- and β-glucosidase activities were lower in the first instar larvae and progressively increased as the larvae grew and the enzyme activities reached a maximum in the fifth instar larvae. In this study, the activity of these enzymes was higher in female adults than that in the male adults and nymphal instars. The higher activity of glucosidases as well as the larger size of female adults supports the idea of a great ability of A. arabicum female adults to ingest food, a feature that has been also reported for Podisus maculiventris (Say) (Hemiptera: Pentatomidae) (Ghamari et al. 2014).

Alpha- and β-galactosidase activities detected in the midgut extract of A. arabicum nymphs and adults indicate their presence in the midgut tissue. Galactosidase activities were higher in fourth and fifth instar nymphs than in the last instar. Such an increase in galactosidic enzyme activities may result from the greater food intake by older instars. Our results show that the galactosidases are important in the final phases of food digestion. This agrees with the finding that it is the main carbohydrase in pest species (Saberi Riseh et al. 2012, Dastranj et al. 2013, Ghamari et al. 2014).

As it is evident from our results, SCPs of the eggs, fifth instar nymphs, and adults with no significant differences ranged between −19.0 and −16.4 °C. That means the overwintering stage (adults) of the bug relies mostly on extensive SCPs to avoid freezing of their body fluid. The SCPs increased with increase in developmental stages from first instar nymphs onward. If the SCP of female adults was slightly lower than males, but, the difference was not significant. The eggs were found to have the lowest mean SCP. In agreement with our results Vernon and Vannier (1996) studied the supercooling capacity of different developmental stages of Anatalanta aptera Eaton (Diptera: Sphaeroceridae) and found the

### Table 1. Mean (±SE) carbohydrases activity of different developmental stages of A. arabicum.

| Developmental stage | α-amylase (nmol/min/gut) | α-glucosidase (nmol/min) | β-glucosidase (nmol/min) | α-galactosidase (nmol/min) | β-galactosidase (nmol/min) |
|----------------------|--------------------------|--------------------------|-------------------------|---------------------------|---------------------------|
| First instar         | 6.75 ± 0.54e             | 83.0 ± 5.0g              | 54.0 ± 5.0d             | 73.0 ± 5.0c                | 21.0 ± 3.0c                |
| Second instar        | 9.11 ± 0.53de            | 186.0 ± 13.0f            | 99.0 ± 10.0cd           | 180.0 ± 8.0d               | 49.0 ± 5.0c                |
| Thirds instar        | 13.24 ± 0.27cd           | 300.0 ± 15.0e            | 154.0 ± 9.0bc           | 343.0 ± 18.0c              | 129.0 ± 12.0b              |
| Fourth instar        | 17.63 ± 0.78c            | 558.0 ± 9.0d             | 216.0 ± 16.0ab          | 468.0 ± 14.0ab             | 241.0 ± 12.0a              |
| Fifth instar         | 27.56 ± 1.68b            | 881.0 ± 17.0b            | 237.0 ± 14.0a           | 481.0 ± 12.0ab             | 230.0 ± 11.0a              |
| Adult (female)       | 35.41 ± 0.90a            | 1081.0 ± 24.0a           | 290.0 ± 23.0a           | 533.0 ± 18.0a              | 246.0 ± 6.0a               |
| Adult (male)         | 24.94 ± 1.08b            | 736.0 ± 35.0c            | 234.0 ± 25.0a           | 456.0 ± 12.0b              | 207.0 ± 7.0a               |

The means followed by different letters in the same column are significantly different (Tukey’s test, P < 0.05).

### Table 2. Relationship between low temperature survival rate and SCPs of different developmental stages of A. arabicum

| Developmental stage | SCPs (°C) | Survival rate (%) |
|---------------------|-----------|-------------------|
|                     | 0 °C/24 h | −5 °C/24 h | −10 °C/24 h | −15 °C/24 h | −20 °C/24 h |
| Egg                 | −19.0 ± 0.7a | 100.0 ± 0.0a | 100.0 ± 0.0a | 100.0 ± 0.0a | 54.0 ± 5.1a | 22.0 ± 3.7a |
| First instar        | −7.0 ± 0.7d  | 100.0 ± 0.0a | 26.0 ± 5.1d  | 0.0 ± 0.0c   | 0.0 ± 0.0d  | 0.0 ± 0.0c  |
| Second instar       | −8.4 ± 0.5d  | 100.0 ± 0.0a | 42.0 ± 3.7c  | 0.0 ± 0.0c   | 0.0 ± 0.0d  | 0.0 ± 0.0c  |
| Third instar        | −11.4 ± 0.5c | 100.0 ± 0.0a | 72 ± 3.74b   | 46.0 ± 5.1b  | 0.0 ± 0.0d  | 0.0 ± 0.0c  |
| Fourth instar       | −14.2 ± 0.6b | 100.0 ± 0.0a | 100.0 ± 0.0a | 100.0 ± 0.0a | 22.0 ± 3.7c | 0.0 ± 0.0c  |
| Fifth instar        | −17.0 ± 0.4a | 100.0 ± 0.0a | 100.0 ± 0.0a | 100.0 ± 0.0a | 40.0 ± 4.5ab| 10.0 ± 4.5bc|
| Adult (female)      | −18.2 ± 0.6a | 100.0 ± 0.0a | 100.0 ± 0.0a | 100.0 ± 0.0a | 48.0 ± 3.7ab| 18.0 ± 7.3ab|
| Adult (male)        | −16.4 ± 0.5ab| 100.0 ± 0.0a | 100.0 ± 0.0a | 100.0 ± 0.0a | 34.0 ± 5.1bc| 6.0 ± 2.4c  |
| F value             | F<sub>7,32</sub> = 62.10 | F<sub>7,32</sub> = 0.00 | F<sub>7,32</sub> = 135.96 | F<sub>7,32</sub> = 652.81 | F<sub>7,32</sub> = 40.68 | F<sub>7,32</sub> = 11.68 |
| Probability         | P < 0.0001 | P < 0.0001 | P < 0.0001 | P < 0.0001 | P < 0.0001 | P < 0.0001 |

The means (±SE) followed by different letters in the same row are significantly different (Tukey’s test, P < 0.05).
lowest mean SCPs of $-28.0$ and $-17.6^\circ$C in eggs and pupae, respectively. In the red imported fire ant, Solenopsis invicta Fabricius significant differences were found in the SCPs of different castes and developmental stages. The lowest SCP ($-13.6^\circ$C) was recorded for worker pupae (Hao-Tao et al. 2009). Carrillo and Cannon (2005) investigated factors affecting SCPs of the Indian meal moth, Plodia interpunctella Hubner and found that the differences between the mean SCPs of eggs, first instar larvae, pupae, and adults were negligible (ranged between $-24.4$ and $-22.2^\circ$C and significantly lower than that of other larval instars ($-14.4$ to $-11.6^\circ$C). The highest survival rates following $24$ h exposures to $-20^\circ$C were recorded for eggs and female adults and the lowest survival rates were recorded for immature stages. This study revealed that although eggs and adult females of A. arabicum could be supercooled below $-15^\circ$C, few of them could survive extended exposure to $-20^\circ$C. In this study, the eggs and female adults were considered as the most tolerant stages followed by fifth instar nymphs, male adults and first to fourth instar nymphs. In agreement with our results, Jensena et al. (2007) reported large variation in cold hardness among different developmental stages of Drosophila melanogaster Meigen. The most tolerant stages were found to be eggs followed by adults, pupae, and larvae. No significant differences were found in the cold tolerance of the genders. Andreadis et al. (2012) investigated cold hardness and SCPs profiles of the Mediterranean flourmoth, Euphestia kuehniella Zeller and resulted that there were no significant differences between mean SCPs of pupae ($-23.3^\circ$C) and adults ($-21.6^\circ$C) but the SCPs of early and late instar larvae ($-16.1$ and $-19.5^\circ$C, respectively), were significantly lower than that for pupae and adults. Pupae and adults showed the highest cold-tolerance capacity, followed by late and early instar larvae and eggs.

Based on our and other investigator results (Vernon and Vannier 1996, Jensena et al. 2007, Hao-Tao et al. 2009, Andreadis et al. 2012) two points may come to the conclusion: (1) different developmental stages of insects inherently have different cold hardness capacities and SCPs and (2) among immature stages of insects inactive ones (i.e., eggs and pupae) have lower SCPs and higher cold tolerance.

According to Sinclair et al. (2015) insects could be divided into three major groups based on their cold tolerance strategies. If most mortality occurs at the SCP, the insect is freeze-intolerant, below the SCP it is freeze-tolerant, and above the SCP it is chill-intolerant. Based on the above, A. arabicum nymphs and adult males were found to be chill-intolerant whereas eggs and female adults may be classified as freeze-intolerant.

In conclusion, it was found that the carbohydride activities increased with insect growing and reached to the highest levels in the fifth instar nymphs and female adults and the enzyme activities in the male adults were less than the female adults. The eggs showed the lowest SCP and the highest cold hardness. There was a direct relationship between the nymphs growth and survival rate.

References Cited

Alfonso, J., F. Ortego, R. Sanchez-Monge, G. Garcia-Casdo, M. Pujol, P. Castanera, and G. Salcedo. 1997. Wheat and barley inhibitors active towards a mylamy and trypsin-like activities from Spodoptera frugiperda. J. Chem. Ecol. 23: 1729–1741.

Andreadis, S. S., P. A. Eliopoulos, and M. Savopoulou-Soultani. 2012. Cold hardness of immature and adult stages of the Mediterranean flour moth, Euphestia kuehniella. J. Stored Prod. Res. 48: 132–136.

Bemani, M., H. Izadi, K. Mahdian, A. Khani, and M. A. Samih. 2012. Study on the physiology of diapause, cold hardness and supercooling point of overwintering pupae of the pistachio fruit hull borer, Arismaria comaroffii. J. Insect Physiol. 58: 897–902.

Bernfeld, P. 1955. Amylases, z and b. Methods Enzymol. 1: 149–158.

Bigham, M., and V. Hossineinaveh. 2010. Digestive proteolytic activity in the pistachio green stink bug, Brachyema gennari Kolenati (Hemiptera: Pentatomidae). J. Asia Pacific Entomol. 13: 221–227.

Borzouei, E., B. Naseri, and F. Rahimi Namin. 2015. Different diets affecting biology and digestive physiology of the Khapra beetle, Trogoderma granarium Everts (Coleoptera: Dermestidae). J. Stored Prod. Res. 62: 1–7.

Boyd, D. W. 2003. Digestive enzymes and stylet morphology of Deraeocoris nigritulus (Uhler) (Hemiptera: Miridae) reflect adaptations for predatory habits. Ann. Entomol. Soc. Am. 96: 667–671.

Boyd, D. W., A. C. Cohen, and D. R. Alverson. 2002. Digestive enzymes and stylet morphology of Deraeocoris nobilis (Hemiptera: Miridae), a predatory plant bug. Ann. Entomol. Soc. Am. 95: 393–401.

Carrillo, M. A., and C. A. Cannon. 2005. Supercooling point variability in the Indian meal moth, Plodia interpunctella (Hubner) (Lepidoptera: Pyralidae). J. Stored Prod. Res. 41: 556–564.

Cohen, A. C. 1993. Organization of digestion and preliminary characterization of salivary trypsin-like enzymes in a predaceous heteropteran, Zelus renardis. J. Insect Physiol. 39: 823–829.

Dastranj, M., A. R. Bandani, and M. Mehrabadi. 2013. Age-specific digestion of Tenebrio molitor (Coleoptera: Tenebrionidae) and inhibition of proteolytic and amylolytic activity by plant proteinaceous seed extracts. J. Asia Pacific Entomol. 16: 309–315.

Franco, O. L., D. J. Riggen, F. R. Melo, C. Bloch, C. Silva, and M. F. Grossi de Sa. 2000. Activity of wheat $z$-amylase inhibitors towards bruchid amylases and structural explanation of observed specificities. Eur. J. Biochem. 267: 2166–2173.

Ghamari, M., V. Hosseininaveh, and A. Darvishzadeh. 2014. Carborydrases in the digestive system of the spined soldier bug, Podisus maculiventris (Say) (Hemiptera: Pentatomidae). Arch. Insect Biochem. Physiol. 85: 195–215.

Goudes, R. N. C., G. Smagge, J. D. Stark, and N. Desneux. 2016. Pesticide-induced stress in arthropod pests for optimized integrated pest management programs. Annu. Rev. Entomol. 61: 31–320.

Hao-Tao, C., L. Li-Zi, and J. Xiang-Fu. 2009. Determination of the supercooling points of various castes and developmental stages of the red imported fire ant, Solenopsis invicta (Hymenoptera: Formicidae) in mainland China. Acta Entomol. Sin. 52: 502–508.

Hagstrum, D. W., and B. Subramanyam. 1996. Integrated management of insects in stored products. Marcel Dekker, Inc., New York.

Jensena, D., J. Overgaard, and J. G. Sorensenb. 2007. The influence of developmental stage on cold shock resistance and ability to cold-harden in Drosophila melanogaster. J. Insect Physiol. 53: 179–186.

Kazzazi, M., A. R. Bandani, and S. Hosseibkhani. 2005. Biochemical characterization of $z$-amylase of Sunn pest Eurygaster integriceps. Entomol. Sci. 8: 371–377.

Khani, A., and S. Moharamipour. 2010. Cold hardness and supercooling capacity in the overwintering larvae of the codling moth, Cydia pomonella. J. Insect Sci. 10: 1–12.

Lee, R. E. 2010. A primer on insect cold-tolerance, pp. 3–34. In D.L. Denlinger and R.E. Lee (eds.), Low temperature biology of insects. Cambridge University Press, Cambridge, United Kingdom.

Lwalaba, D., K. H. Hoffmann, and J. Woodring. 2010. Control of the release of digestive enzymes in the larva of the fall armyworm, Spodoptera frugiperda. Arch. Insect Biochem. Physiol. 73: 14–29.

Macfadyen, S., J. E. Banks, J. D. Stark, and A. P. Davies. 2014. Using semifield studies to examine the effects of pesticides on mobile terrestrial invertebrates. Annu. Rev. Entomol. 59: 383–404.

Mehrabadi, M., A. R. Bandani, R. Mehrabadi, and H. Alizadeh. 2012. Inhibitory activity of proteinaceous a-amylase inhibitors from Triticale spp. against Eurygaster integriceps salivary a-amylases: interaction of the inhibitors and the insect digestive enzymes. Pest. Biochem. Physiol. 102: 220–228.

Mehreijad, M. R. 2001. The current status of pistachio pests in Iran, p. 31522. In B. E. Ak (ed.), XI GREMPA seminar on pistachios and almonds. Zaragoza: CIIIHEAM, 2001.(Cahiers Options Méditerranéennes; n. 56).
Mehrnejad, M. R., R. E. Linnavuori, and S. Hossein Alavi. 2013. Hemipteran bugs associated with pistachio trees and notes on major species. Zool. Ecol. 23: 29–40.

Nakonieczny, M., K. Michalczyk, and A. Kedziorski. 2006. Midgut glycosidases activities in monophagous larvae of Apollo butterfly, *Parnassius apollo* ssp. frankenbergeri. C. R. Biol. 329: 765–774.

Nauen, R., D. Sorge, A. Sterner, and D. Borovský. 2001. TMOF-like factor controls the biosynthesis of serine proteases in the larval gut of *Heliotis virescens*. Arch. Insect Biochem. Physiol. 47: 169–180.

Oliveira, J. A., M. G. A. Oliveira, R. N. C. Guedes, and M. J. Soares. 2006. Morphology and preliminary enzyme characterization of the salivary glands from the predatory bug *Podisus nigrispinus* (Heteroptera: Pentatomidae). Bull. Entomol. Res. 96: 251–258.

Ramzi, S., and V. Hosseininaveh. 2010. Biochemical characterization of digestive alpha-amylase, alphaglucosidase and beta-glucosidase in pistachio green stink bug, *Brachynema germani* Kolenati (Hemiptera: Pentatomidae). J. Asa Pacific Entomol. 13: 215–219.

Regnault-Roger, C., V. Charles, and J. T. Arnason. 2012. Essential oils in insect control: low-risk products in a high-stakes world. Annu. Rev. Entomol. 57: 405–424.

Saberi Risch, N., M. Ghadamyari, and B. Motamedinia. 2012. Biochemical characterisation of α- and β-glucosidases and α- and β-galactosidases from red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Col.: Curculionidae). Plant Protect. Sci. 48: 85–93.

Sinclair, B. J., L. E. Coello Alvarado, and L. V. Ferguson. 2015. An invitation to measure insect cold tolerance: methods, approaches, and work flow. J. Therm. Biol. 53: 180–197.

Storey, K. B., and J. M. Storey. 2012. Insect cold hardiness: metabolic, gene, and protein adaptation. Can. J. Zool. 90: 456–475.

Subramanyam, B., and D. Hagstrum. 1995. Resistance measurement and management, pp. 33198. In B. Subramanyam and D. Hagstrum (eds.), Integrated management of insects in stored products. Marcel Dekker Inc., New York.

Swart, C. C., L. E. Deaton, and B. E. Felgenhauer. 2006. The salivary gland and salivary enzymes of the giant waterbugs (Heteroptera: Belostomatidae). Comp. Biochem. Physiol. A. 145: 114–122.

Vatanparast, M., V. Hosseininaveh, S. M. Sajjadian, and S. Amiri, 2011. Glucosidases in the midgut and salivary glands of the pistachio red seed bug *Lygaeus pandurus* (Hemiptera: Lygaeidae). In Global Conference on Entomology, 5–9 March, 2011, Chiang Mai, Thailand.

Vernon, P., and G. Vannier. 1996. Developmental patterns of supercooling capacity in a subantarctic wingless fly. Experientia. 52: 155–158.

Zalucki, M. P., A. R. Clarke, and S. Malcolm. 2002. Ecology and behavior of first instar larval Lepidoptera. Ann. Rev. Entomol. 47: 361–393.

Zeng, F., and A. C. Cohen. 2000. Demonstration of amylase from the zoophagous anthocorid *Orius insidiosus*. Arch. Insect Biochem. Physiol. 44: 136–139.