

Short communication. Impact of the amino acid proline on the cold hardiness of honey bee, *Apis mellifera* L.

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Abstract

Like many insects, honey bee can increase its cold tolerance through freeze avoidance, using antifreeze proteins (AFPs) to lower its supercooling point (SCP). Proline is the most dominant amino acid in honey bee hemolymph, which can be obtained by the insect through feeding. In the current study the antifreeze activity of this amino acid was evaluated on worker honey bees, immediately before the start of cold season. The experiment was established on four treatments including three different concentrations of proline (1%, 3% and 4.35%) diluted in 1:1 water sucrose syrup, and the syrup without proline (control). Newly emerged worker honey bees were fed on the mentioned diets for 2 weeks, under cage condition, and then 20 bees from each treatment (cage) were selected randomly for determination of cold hardiness inside a cooling bath. Using a CHY data logger, equipped with a K100 sensor attached to the bee's gaster, the SCP, the amount of released heat and the rate of this release as measures of insect cold hardiness were recorded. Proline significantly reduced honey bees' SCP. The lowest point, $-7.67 \pm 0.2646^{\circ}\text{C}$, was observed in the concentration of 1% proline. The amount of released heat and the rate of this release were not significantly different across the treatments.

Additional key words: antifreeze protein; apiculture; cold tolerance; diet manipulation; supercooling point.

Insects exposed to temperatures below the melting point of their body fluid are in danger of being killed by a lethal freezing of that fluid (Salt, 1961; Baust, 1973; Block, 1995). Over cold seasons, many insects utilize a combination of microhabitat selection and physiological alterations (the synthesis of cryoprotectants) to increase cold tolerance. Selecting an appropriate microhabitat improves low temperature exposure and cryoprotectants lower their supercooling point (SCP), the temperature at which their body's water freezes (Zachariassen, 1985; Duman, 2001). SCPs provide a basic indication as to what the coldest temperature extreme is that an insect could survive (Salt, 1961).

The SCP of an insect can be lowered by a number of means, including production of glycerol and other antifreeze compounds, dehydration of the insect, changes in fatty acids and/or ingestion of certain substances (Somme, 1982). There are substantial

evidences of some amino acids impacts on the enhancement of SCP in insects (Denliger & Lee, 1998; Mei & Li, 2006; Bandani, 2011). These amino acids called "antifreeze proteins" (AFPs) or "thermal hysteresis proteins" do not affect the melting point of insect hemolymph while reducing the freezing point of it. Thereupon, AFPs keep the freezing point some degrees (5-6°C) under the melting point, and increase the organism tolerance in lower temperatures (Bandani, 2011).

Producing thermal hysteresis is a unique diagnostic characteristic of AFPs for their presence. The rate of thermal hysteresis depends on the specific activity and concentration of AFPs, and in some cases the presence of enhancers of AFP activity (Duman, 2001; Duman & Serianni, 2001). AFPs lower the freezing point by a non-colligative mechanism whereby the AFPs adsorb onto preferred surfaces of potential seed ice crystals (Raymond & DeVries, 1977; Brown & Sonnichsen,

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Abbreviations used: AFP (antifreeze protein); CRD (completely randomized design); SCP (supercooling point).

2002). Consequently, growth of the crystal (addition of water molecules to the crystal surfaces) can only occur between the adsorbed AFPs and in high radius of curvature fronts (high surface free energy), rather than the preferred low radius of curvature fronts (low surface free energy). Therefore, according to the Kelvin effect, the temperature must be lowered below the colligative melting point for growth to proceed (Duman *et al.*, 2004).

AFPs have been identified in numerous terrestrial arthropods including spiders (Duman, 1979; Husby & Zachariassen, 1980), mites (Block & Duman, 1989; Sjørnsen & Somme, 2000), centipedes (Tursman *et al.*, 1994; Tursman & Duman, 1995), and of course insects (Duman, 1977, 2001). Most terrestrial arthropods which produce AFPs are freeze avoiding. While AFPs are more often present in freeze avoiding species, they are also found in certain freeze tolerant (able to survive freezing) species (Tursman & Duman, 1995; Duman, 2001). While recrystallization inhibition by AFPs does occur in these situations, additional AFP functions are likely and, consequently, their function is not well understood in freeze tolerant species. So far some insect species, including Coleoptera, Collembola, Plecoptera, Orthoptera, Hemiptera, Mecoptera, Lepidoptera, Diptera and Hymenoptera, have shown to use AFPs, usually through the presence in the hemolymph of the distinctive thermal hysteresis activity. Honey bee (*Apis mellifera* L.) is also a freeze-tolerant insect when overwintering, surviving ice formation inside its body (Zachariassen, 1985).

There are many apiculture industries in Zanjan, Iran, suffering bees cold lose. Despite this, rare works have been done on the improvement of these colonies' cold tolerance by diet manipulation. Knowledge of SCP would indicate the coldest possible temperature extremes that *A. mellifera* could survive and provide insights about optimum hive utilization in the cold situations. The aims of this study were to determine (i) the supercooling point of *A. mellifera*, and (ii) the impact of the amino acid, proline on honey bee cold hardiness.

Studies were conducted at University of Zanjan (36.4108N 48.2424E, 1586 m asl) in the first days of autumn (immediately before the start of cold season). Three combs containing worker honey bee pupae were selected from a colony reared in this university. To produce cohorts with similar age, the

pupae were allowed to hatch and 4 groups of 1-3 days old emerged bees (each group included 50 bees) were separated and put into 4 cages to establish the experiment treatments.

The dimensions of experimental wooden cages, with transparent mesh walls, were 15 cm × 10 cm × 15 cm. Each cage was provided with a vial of water and a piece of wax comb on the underside of the cage lid. The cages were held in the dark in an incubator at $33 \pm 2^\circ\text{C}$ and $50 \pm 5\%$ RH, during the experiment.

The treatments consisted of three different concentrations of proline (1%, 3% and 4.35%) diluted in 1:1 water sucrose syrup, and the syrup without proline (control), as bees' diet. Honey bees with their diet (in a vial) were put inside the cages, and 15 cm³ of each food was added daily to its related cage for 14 days. Every day, dead individuals were removed from cages, and at last day (day 14), 20 bees from each cage were captured to assess their cold hardiness according to the method provided by Jones *et al.* (2008).

The cold hardiness of honey bees were determined in a cooling bath in which temperature was reduced by a rate of $0.5^\circ\text{C min}^{-1}$. A CHY data logger, equipped with a K100 sensor (with accuracy of 0.1°C), was used to track temperature changes. The thermal sensor was attached to the gaster of worker honey bee and the insect was put in the cooling bath inside a test tube with 1-cm in diameter. The data logger was interfaced with a computer, and utilizing its software, the SCP, the amount of released heat and the rate of this release as measures of insect cold hardiness were recorded.

The experiment was performed in a completely randomize design (CRD), and the analysis of variance was performed on supercooling point, released heat and the rate of this release. If treatments were significant at $p < 0.05$, then differences between means were determined using the Turkey's HSD test at 95% confidence level.

Mean SCPs of each treatment are shown in the Table 1. Honey bees' average SCPs in the three proline-containing treatments were significantly lower than the amount of control cohort. The lowest point ($-7.67 \pm 0.2646^\circ\text{C}$), which was significantly different from the control and the two other treatment cohorts, was observed in 1% proline. Mean SCPs recorded in the concentrations 3% ($-6.79 \pm 0.1552^\circ\text{C}$) and 4.35% ($-6.85 \pm 0.2197^\circ\text{C}$) were statistically equal. There was

Table 1. Averages (\pm standard errors) of supercooling points (SCPs), released heats and rates of these releases, related to the four treatments of the experiment

Measured item	Proline concentration (%)			
	0	1	3	4.35
SCP ($^{\circ}\text{C}$)	$-5.25 \pm 0.3163^{\text{a}}$	$-7.67 \pm 0.2646^{\text{b}}$	$-6.79 \pm 0.1552^{\text{c}}$	$-6.85 \pm 0.2197^{\text{c}}$
Released heat ($^{\circ}\text{C}$)	$1.99 \pm 0.600^{\text{ns}}$	$1.82 \pm 0.333^{\text{ns}}$	$1.72 \pm 0.209^{\text{ns}}$	$1.36 \pm 0.235^{\text{ns}}$
Heat releasing rate ($^{\circ}\text{C s}^{-1}$)	$0.0153 \pm 0.0080^{\text{ns}}$	$0.0095 \pm 0.0024^{\text{ns}}$	$0.0145 \pm 0.0046^{\text{ns}}$	$0.0097 \pm 0.0021^{\text{ns}}$

^{a-c} Means within a row with different superscripts are significantly different ($\alpha = 0.05$). ns: not significant

no significant difference between the four treatments of experiment in the amount of released heat and the rate of this release.

To our knowledge, this is the first study of SCP in relation with the amino acid proline on honey bee. Supercooling ability can often be attributed to the accumulation of cryoprotectant chemicals and/or the absence on ice nucleating agents (Lee & Denlinger, 1991). In the current study we found that a change in the proline concentration in diet can alter honey bee's SCP, so proline could be considered as an important antifreeze agent in its body.

On the other hand, the lowest mean SCP was observed in the lowest concentration of proline and it was significantly different from the three other means. A possible explanation to this process could be the impact of high level concentrations of proline on the insect feeding rate. Thereupon, it is predicted that a concentration threshold exists, above which the feed rate and so the proline acquisition decreases and below which, by increasing the amino acid in the diet, supercooling point decreases.

It has been demonstrated that proline is the most dominant amino acid in honey bee hemolymph (Mullins, 1985), which the insect can obtain through feeding on pollen, the main proline source for honey bee (Lipp, 1991; Funck *et al.*, 2012; Hossain *et al.*, 2012). Our results with regard to the existence of this amino acid in bees' hemolymph and natural diet warrant further studies on dietary manipulation of the proline concentration in the insect body for a more successful overwintering.

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