

On the Question of Parameters for Evaluating Cold Hardiness of Freeze Tolerant Insects

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Abstract

The super cooling point (SCP) is the best documented parameter for describing the ability of insects to survive freezing but its value as a predictor of overwintering survival or for estimation of the cold hardiness potential, is limited. According to this study, the SCP is influenced by such factors as polyol and protein concentration in the hemolymph; therefore it can be lower in winter than it is in spring. Nevertheless the quality of ice nucleating process is higher in winter insects due to which insects survive the lowest winter temperatures. The depletion of polyols in spring causes increase of SCP. Unlike in the autumn, when an increase in SCP is associated with development of freeze tolerance, spring changes in SCP of the insects occur simultaneously with loss of cold tolerance. The present study on the freeze-tolerant *Upis ceramoides* inhabiting central Yakutia (Eastern Siberia, Russia) shows that SCP itself is not a sufficiently appropriate parameter for evaluation of insect cold hardiness. For these purposes it should be used together with other criteria, such as specific ice nucleating activity and profile of ice nucleating activity.

Keywords: Cold hardiness; Supercooling point; *Upis ceramoides*; Specific ice nucleating activity; Profile of ice nucleating activity; Yakutia

Introduction

For insects that inhabit cold environments, resistance to subzero temperatures is a crucial feature because it allows them to occupy temperate and extremely cold biotopes [1,2]. By now, a large literature list has been developed regarding two basic cold adaptation strategies: freeze-avoidance and freeze-tolerance [3-8]. The freeze avoiding species, which die if frozen, depend on super cooling of their body fluids. This strategy involves removal or inactivation of all components which may trigger freezing. It also includes accumulation of a huge amount of polyols. Alternatively, a second strategy is evolved by the freeze tolerant species that are able to tolerate freezing of their extracellular body fluids [4,9,10]. In many insects which tolerate freezing this is achieved by means of potent ice nucleating agents which are present in the hemolymph during the cold seasons and initiate controllable damage-free freezing of extracellular body fluid [11].

The measure of insect cold hardiness is a supercooling point (SCP) that is the temperature at which freezing takes place [12]. The SCP seems to be the best documented parameter for describing insect cold hardiness [13]. For most freeze tolerant insects the SCP lies in the range from -7 to -12°C, while for freeze avoiding ones: below -20°C. High SCP's of freeze tolerant insects are linked with adoptive extracellular ice nucleators that are polypeptide's aggregates with a specific structure [11,14]. The most potential ice nucleator seems to be produced by insects inhabiting extreme cold regions such as Yakutia [11]. By producing of ice nucleators with high SCP's, freeze tolerant insects establish a protective extracellular freezing before the nucleation temperature in the cells is reached. Thus, the higher the SCP is, the higher is probability of freeze tolerant insects to be resistant to freezing.

The SCP is influenced by such factors as polyols and ice nucleating agents, the concentrations of which undergo to seasonal changes [11,12]. Therefore, there are certain fluctuations within the SCP's values during a year. According to my earlier study on warm acclimation of *Aporia crataegi* caterpillars, decrease of glycerol and protein content in the hemolymph during acclimation is associated with an increase in SCP [11]. Although warm acclimation of *A. crataegi* induced an increase in the SCP, it was not correlated with resistance to freezing.

Obviously, ice nucleation at this temperature was non-specific and therefore likely had no adaptive importance. The data testifies that the SCP does not reflect the real process associated with cold hardiness in the overwintering insects. Moreover, developing researches associated with different aspects of insect's cold hardiness leads to understanding that "SCP is no longer deemed an adequate predictor of overwintering survival for many species" [15]. It is also limited in estimating of the cold hardiness potential [11].

In this study, freeze tolerant beetles *Upis ceramoides* inhabiting central Yakutia (Eastern Siberia) were used to investigate how seasonal changes in the physical and chemical situation in the insect hemolymph influence their SCP to better understand the role of SCP in estimating of insect cold hardiness.

Materials and Methods

Insects

A large (up to 340 mg) tenebrionid beetles, *U. ceramoides* (Coleoptera: Tenebrionidae) were collected under loose bark of dead standing birch in the vicinity forest of Yakutsk city. Beetles to be used in the experiments were collected in 20th December, 2nd March, 4th April and 10th July. In its habitat of overwintering that is situated above snow line, the beetles can be exposed to winter temperatures as low as -55°C. The beetles collected in winter and early spring were kept at +4°C until they started to move. Summer specimens were placed at +4°C for 4 hours before experiments get started. This procedure was necessary for evacuating of ice nucleating agents from the gut.

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Supercooling point

The supercooling point of the beetles was measured by using a thin copper constantan thermocouple placed in close contact with the dry body surface. A layer of thin adhesive tape was wrapped around the thermocouple probe to prevent it from scratching the surface of the beetles and thus affecting the SCP. The thermocouple was connected to a computer, and the temperature was recorded every 10 s. Specimens with the thermocouple attached were cooled inside a Binder climatic chamber (TC-G-180, Tuttlingen, Germany) at a rate of about 1°C/min. Initiation of freezing was detected as a sudden temperature increase due to the release of heat of fusion from body water being transformed to ice, and the lowest temperature recorded prior to the temperature increase was taken as the SCP.

Ice nucleating activity

Samples of hemolymph (0.5 µL) collected from beetles were added to 4.5 µL of 0.9% NaCl solution in thin glass capillaries and cooled concomitantly in contact with the thermocouple until all samples were frozen. SCPs of the hemolymph were recorded and profile of the specific ice nucleating activity of the hemolymph was determined by isovolumetric technique of sample dilution at which each sample was diluted by the same factor from the same stock solution. This method was first described in details by Zachariassen et al. [16].

Hemolymph osmolality

The hemolymph osmolality of the beetles was measured by determining the melting point on a Clifton Nanolitre Osmometer. Tiny samples of hemolymph were sucked into thin glass capillaries by means of the capillary forces, where after the capillaries were closed by melting one end and centrifuged to remove hemocytes. The hemolymph osmolality could be read directly by placing 50 ml samples of hemolymph into the sample holder filled with paraffin oil, freezing the sample and gradually increasing the temperature. The temperature at which the last tiny ice crystal disappeared was taken as a melting point [17].

Protein concentration

The concentration of protein in the hemolymph collected from beetles was estimated by the method of Lowry using bovine serum albumin (BSA) as a protein standard [18].

Determination of cold hardiness potential of *U. ceramboides*

In January, beetles were taken from their hibernation sites outside and put into freezer for incubation for 1 hour at -85°C. After that the beetles were stored in a Sanyo freezer (MDF-136, Japan) at -28°C overnight followed by +4°C for 3 hours. For the next step they were brought up to a room temperature and checked for their ability for active movement at +22°C during 30 min. This research was carried out only with non-acclimated caterpillars situated in the leaf nests.

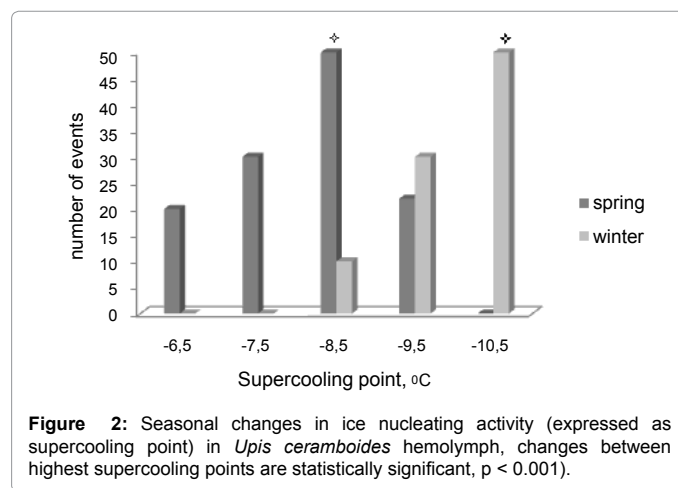
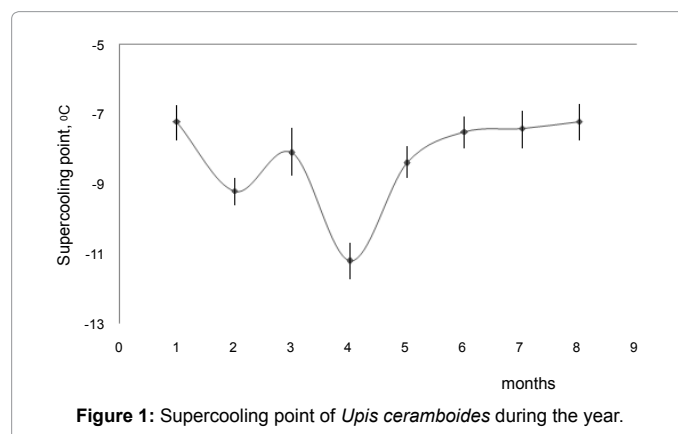
Beetles with certain degree of acclimatization were tested by placing them into freezer at -22°C for 30 min (cooling rate was approximately 1°C/min). After that they were also tested for their ability for active movement at room temperature.

Statistical methods

Comparison of means between samples was made with ANOVA/ Tukey's test using the statistical package Statistica v6.0. Mean ± SD is presented throughout.

Results and Discussion

Figures 1 and 2 illustrate the seasonal pattern of SCP changes. Supercooling points declined from a summer average of -7,2°C to a winter value of about -9,4°C, indicating that this species is freezing tolerant (Figure 1). Physiological mechanism of adaptation of *U. ceramboides* to extreme cold includes production of extracellular ice nucleators [19]. In this study, Figure 2 firmly demonstrates the distribution of nucleation temperatures of ice nucleators from the 0.5 µL samples of haemolymph in 4, 5 µL of 0.9% NaCl according to which most of ice nucleators with -8.5°C activity were presented in the spring samples while a notable amount of ice nucleators with -10.5°C activity were found in the “winter” beetles’ hemolymph. Such changes in ice nucleating activity are associated with seasonal variations in the chemical and physical situation in the hemolymph of insects. Analysis of hemolymph osmolality has shown that sizeable changes in its value occurred from summer to winter (Table 1). In this study, increasing



Season	SCP, °C	Osmolality mOsmol	Protein concentration, mg/ml	Specific ice nucleating activity, °C/mg	Survival at -85°C, %	Survival at -22°C, %
Winter	-10,5 ± 1,2	550 ± 25	117 ± 19	-0,089 ± 0,02	50	98
Spring	-8,5 ± 0,8	310 ± 18	106 ± 11	-0,08 ± 0,015	0	93
Autumn	-7,7 ± 0,2	150 ± 14	45 ± 26	-0,17 ± 0,085	0	77
Summer	-7,8 ± 0,15	432 ± 10	56,8 ± 15	-0,14 ± 0,066	0	0

Table 1: Seasonal variations in the SCP, osmolality, protein concentration, specific ice nucleating activity in the hemolymph of *Upis ceramboides* and survival of beetles at different freezing temperatures.

solute concentrations in winter depressed the temperature at which biological INAs induce freezing (Figure 3). This is why the hemolymph of *U. ceramboides* was characterized by a moderate nucleating activity in winter (Figure 2). During winter the beetles are resistant to as low temperatures as -85°C (Table 1). 50% of the tested beetles tolerated this temperature for at least 1 hour. In this period the hemolymph of *U. ceramboides* can be diluted by a factor of up to 10³ without any significant reduction in ice nucleating activity (Figure 6a). Thus, within this concentration's range the nucleating activity forms a plateau where it is not significantly affected by variations in the nucleator's level. Zachariassen has demonstrated in early studies that various freeze tolerant organisms differ by range of plateau. Thus, the plateau appears to be specific for the various types of ice nucleators [14].

In this study seasonal changes in the profile of hemolymph ice nucleating activity were observed. Measurements were made in different periods of acclimatization (December, March, July and October). As seen on the graphs, seasonal acclimatization induces dropping of ice nucleating activity since early spring and leads to qualitative changes in the profile of ice nucleating activity that is apparently related to a decline in the amount of active nucleators (Figure 6). In particular, (Figure 6a) shows that are volumetric dilution of the hemolymph (early spring samples) leads to a rapid drop of ice nucleating activity. Within this period 93% of the beetles endured 30 min at -22°C. With progressive acclimatization of the insects that is accompanied by polyols and proteins declines (Table 1) the active plateau in the profile of ice nucleating activity has disappeared. By summer, the changes in the profile became dramatic and were perhaps caused by structural modifications of ice nucleators (Figure 6b). These changes in quality of ice nucleation in the hemolymph were associated with a loss of survival of *U. ceramboides* at freezing temperatures (-22°C is according to Table 1). Increase of protein concentration and osmolality of the hemolymph in autumn seems to lead to qualitative changes in structure of ice nucleators (Figure 6b). Although there was no plateau in the profile of ice nucleating activity, ice nucleation was an adoptive process and 77% of tested beetles have survived at -22°C.

Thus, although warm acclimatization of *U. ceramboides* induces an increase of the SCP, ice nucleation at this temperature is non-specific (summer type of insects) and is therefore not likely to have adaptive importance. Hence, warm acclimatization is accompanied by a loss of adaptive ice nucleators (although incidental ones remain), associated with a drop in the cold hardiness potential of the insects. The nucleation temperature itself does not reflect the character of ice nucleating process

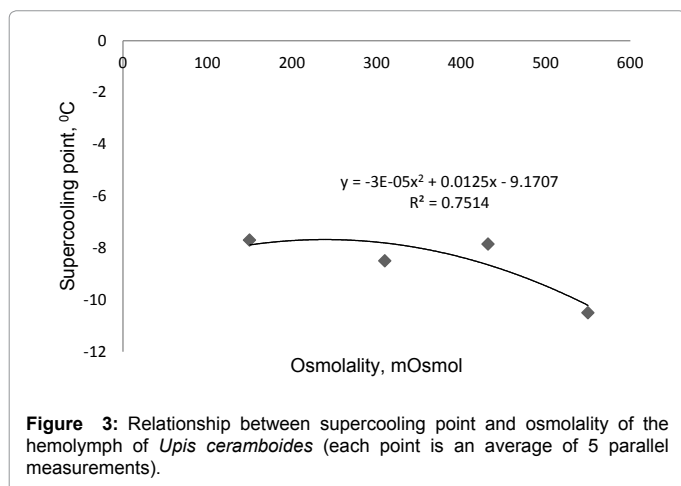


Figure 3: Relationship between supercooling point and osmolality of the hemolymph of *Upis ceramboides* (each point is an average of 5 parallel measurements).

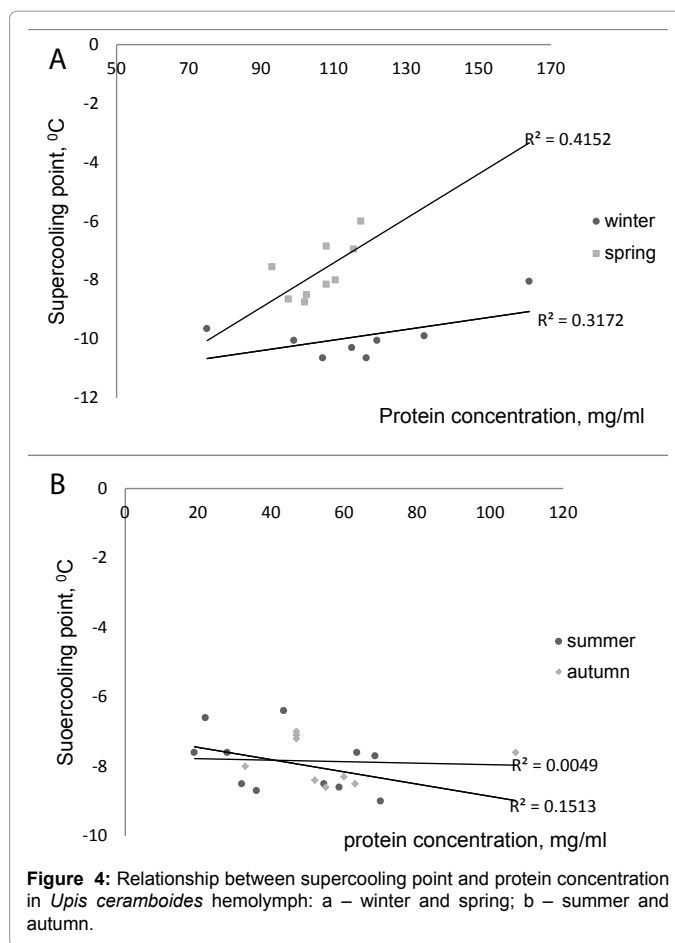
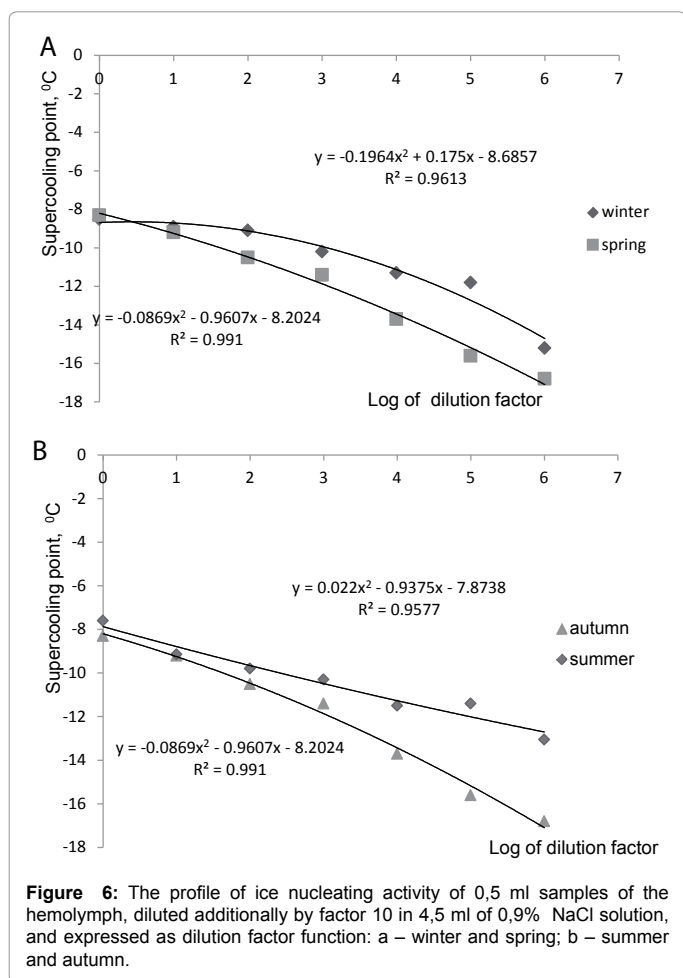
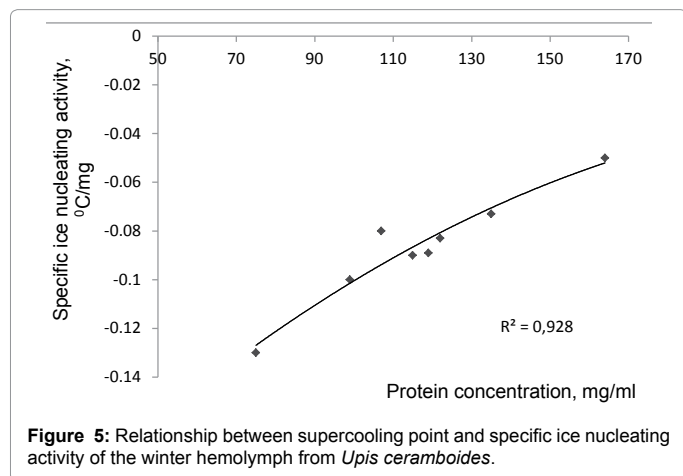


Figure 4: Relationship between supercooling point and protein concentration in *Upis ceramboides* hemolymph: a – winter and spring; b – summer and autumn.

in the insects. It merely indicates body liquid freezing temperature, while the profile of ice nucleating activity seems to determine the ice nucleation in the hemolymph as a feature of cold adaptation process.

Ice nucleating activity is a function of the content of ice nucleating agents (INAs) that are proteins [14,19]. In this context, the relationships between ice nucleating activity and protein's concentration was studied to see how the SCP is associated with protein's fraction. As seen on (Figure 4a), there is a positive correlation between the SCP and protein's concentration in the hemolymph during winter ($R^2=0,317$) and spring ($R^2=0,415$). Relationship between SCP and protein concentration is more evident for spring samples of the hemolymph than winter ones that is apparently linked with certain physical and chemical situation in the hemolymph. There is no positive correlation between SCP and protein's concentration in the hemolymph during summer (Figure 4b) indicating that SCP's were not associated with protein's fraction. Because of the relationship between SCP and protein concentration, the nucleating activity represented by the ratio of nucleation temperature to protein concentration can be termed the specific ice nucleating activity (Table 1). Figure 5 shows that specific ice nucleating activity proportionally related to total protein's concentration.

Hence, two parameters should be used additionally to SCP for describing cold hardiness in freeze tolerant insects. First one is the specific ice nucleating activity that performs activity of ice nucleators in insect's hemolymph more realistically. Second parameter is the profile of ice nucleating activity reflecting the degree in which ice nucleation is a feature of cold adaptation process of insects.



References

- Baust J, Rojas R (1985) Review - insect cold hardiness: facts and fancy. *Insect Physiol* 31: 755-759.
- Margolin R, Schinner F (1999) Cold adapted organisms. Springer, Germany.
- Zachariassen KE (1985) Physiology of cold tolerance in insects. *Physiol Rev* 65: 799-832.
- Storey KB, Storey JM (1988) Freeze tolerance in animals. *Physiol Rev* 68: 27-84.
- Block W (1990) Cold tolerance of insects and other arthropods. *Phil Trans R Soc* 326: 613- 633.
- Duman JG, Wu Ding Wen, Xu Lei, Tursman Donald, Olsen Mark T (1991) Adaptations of insects to subzero temperature. *Quart Rev Biol*. 66: 387-407.
- Lee RE, Delinger DL (1985) Cold tolerance in diapausing and non-diapausing stages of the flesh fly *Sarcophaga crassipalpis*. *Physiol Entomol* 10: 309-315.
- Holmstrup M, Zachariassen K (1996) Physiology of cold hardiness in earthworms. *Comp Biochem Phys* 115: 91-101.
- Sinclair BJ, Addo-Bediako A, Chown SL (2003) Climatic variability and the evolution of insect freeze tolerance. *Biol Rev Camb Philos Soc* 78: 181-195.
- Lee RE, Delinger D (1991) *Insects at low temperature*. Chapman and Hall, New York.
- Ramløv H (2000) Aspects of natural cold tolerance in ectothermic animals. *Hum Reprod* 15 Suppl 5: 26-46.
- Li NG (2012) Relationships between cold hardiness, and ice nucleating activity, glycerol and protein contents in the hemolymph of caterpillars, *Aporia crataegi* L. *Cryo Letters* 33: 135-143.
- Zachariassen KE (1985) Physiology of cold tolerance in insects. *Physiol Rev* 65: 799-832.
- Turnock WJ, Fields PG (2005) Winter climate and coldhardiness in terrestrial insects. *Eur J Entomology* 102 : 561-576.
- Zachariassen KE (1992) *Ice nucleating agents in cold hardy insects*. Water and Life. Springer Verlag, Berlin.
- Renault D, Salin C, Vannier G, Vernon P (2002) Survival at low temperatures in insects: what is the ecological significance of the supercooling point? *Cryo Letters* 23: 217-228.
- Zachariassen KE, Baust JG, Lee RE Jr (1982) A method for quantitative determination of ice nucleating agents in insect hemolymph. *Cryobiology* 19: 180-184.
- Kristiansen E, Li NG, Averensky AI, Laugsand AE, Zachariassen KE (2009) The Siberian timberman *Acanthocinus aedilis*: a freeze-tolerant beetle with low supercooling points. *J Comp Physiol B* 179: 563-568.
- Jones A, Reed R, Weyers J (2003) *Practical Skills in Biology*. UK.
- Li NG (2011) Ice-nucleating activity of hemolymph of *Upis ceramoides* beetle inhabiting in central Yakutia. *Problems of cryobiology* 21: 34 - 36.