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SEASONAL VARIATION OF TREHALOSE AND GLYCEROL CONCENTRATIONS IN WINTER SNOW-ACTIVE INSECTS

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Abstract

Different kinds of molecules were identified as antifreezing agents in the body fluids of cold tolerant invertebrates: sugars, polyols and proteins. While none of the active arthropods were so far reported to accumulate polyols, these compounds are present in the haemolymph of species that hibernate in a passive stage such as diapause. In this work we investigated insect species that are active during winter and we demonstrated the ability of the mecopteran *Boreus hiemalis* (Mecoptera, Boreidae), the wingless fly *Chionea* sp. (Diptera, Limoniidae) and cantharid larvae (Coleoptera, Cantharidae) to accumulate sugars in their haemolymph to survive during winter. We report, for the first time, that for snow-active insects, trehalose comprises an important haemolymph component, its concentration changing as a function of the season, suggesting that the same adaptive strategies against cold conditions have evolved both in winter active and winter diapausing insects.

Keywords: Supercooling point, trehalose, *Boreus*, *Chionea*, Cantharidae

INTRODUCTION

Cold is one of the major challenges that insects must overcome to survive in temperate and polar climates. Three general strategies have emerged in insects and in invertebrates generally: long distance migration, overwintering with different physiological cold hardiness strategies and ovewintering in protected microhabitats (13). Most overwintering insects, spend the cold season in a “latent” stage (eggs, diapausing larvae, pupae or adults); only few species are active during winter. Several winter active arthropods exploit a specific micro-climate under the snow cover suitable for their life (2, 3, 4, 5). In this subnivean space, the temperatures are rather constant, near 0°C, independent of the external air variations (12, 21, 39). Arthropod cold tolerance was investigated in species living in polar and in temperate regions. Within the polar species particular attention was devoted to springtails, acari (7, 24, 42) to the wingless fly *Anatalanta aptera* (Diptera, Sphaeroceridae) (47) and to the midge *Belgica antarctica* (25).

Previous studies on the winter activity of non polar arthropods resulted in records of winter active species, information on their life history, thermal limits of cold tolerance and ecology (1, 6, 27, 44, 45). In contrast to the widely studied cold tolerant species, only limited

information is available on the physiological or biochemical adaptation of winter active species with most attention has being given to spiders and springtails (26, 41).

Winter active insects in temperate and cold regions perform activities such as walking, mating and feeding, both in the subnivean space and on the snow cover, at temperature below the freezing point of water. Different kinds of molecules have been identified as antifreeze agents in the body fluids of cold hardy invertebrates: sugars, polyols and proteins (15, 16, 40, 43). While none of the winter active arthropods have so far been reported to accumulate polyols, these compounds are present in the haemolymph of species that hibernate in a passive state such as diapause (22, 30, 31, 35, 38). Among the sugars, trehalose, that in insects constitutes a store of glucose for energy (18), seems to play in the survival of several types of stress, like cold (38), hypoxia (9), and desiccation (11). Trehalose concentration in the haemolymph of insects overwintering in latent stages undergoes changes with season: it increases during autumn and decreases at the end of the winter (22, 31, 35).

In this work we have investigated the ability of *Boreus hiemalis* (Mecoptera, Boreidae) and other snow active insects to accumulate sugars in their haemolymph and to maintain activity during winter. We report, for the first time, that, in snow-insects, trehalose is an important haemolymph component whose concentration changes as a function of the sampling period, suggesting a role in adaptative strategies against the cold season.

MATERIALS AND METHODS

Active adults of the wingless cranefly *Chionea* sp., the mecopteran *Boreus hiemalis* and cantharid larvae were collected by an entomological potter from the snow surface, during the winters of 2004-05 and 2005-06 (a first sample in December, a second one in March) in the Venetian Pre-Alps and in the Dolomites, between 1100-1500 m a.s.l . (Table 1) (46). The living specimens were stored in a vial with moist paper, at 5°C during transport to the laboratory (24-36 h) and then used for the analytical determinations. From the specimens collected in March 2005, a sub-sample of 12 individuals of *B. hiemalis* was stored for 8 days at 16°C in Petri-dishes with moss taken from the collection site of the insects. The same culturing technique was also used with 8 cantharid larvae.

The supercooling points of individual insects were measured by using a copper/constantan thermocouple (0.2 mm) which was kept in contact with the animal's cuticle. The temperature gradient during cooling, obtained with a Peltier system under PC control, was -1°C min^{-1}. The initiation of freezing was indicated by the rapid temperature increase due to the liberation of the latent heat of fusion of the body water.

Individual specimens were weighed on a Sartorius balance (sensitivity 0.1 mg) to measure their fresh weight (FW) and, after drying for 72 h at 60°C, also their dry weight (DW). The water content was calculated as (FW- DW).

The expected cryoscopic decrease was calculated for the different species from the sum of the contribution of each sugar and polyol assuming the value of -1.86° C·Kg·osm^{-1} for water.

The extraction, derivatization and analytical procedures (gas chromatography coupled to mass spectrometry) for the sugar and polyol determinations were performed as described in Kostal et al., 2007 (31). The number of specimens analysed is given in Table 1. Values are presented as mean ± SD. Tests between means were conducted with a one-way ANOVA, using a Bonferroni test for multiple comparisons.

Table 1. Number of specimens analysed for sugars and polyols (N); season of collection: early winter (December) (EW), late winter (March) (LW); field specimens (W) and specimens maintained at 16°C for 8 days (R),

Taxon	N	season	Field/Cultured
Cantharidae larvae	8	LW	W
Cantharidae larvae	3	LW	R
Chionea sp.	5	EW	W
Boreus hiemalis	16	EW	W
Boreus hiemalis	9	LW	W
Boreus hiemalis	7	LW	R

RESULTS AND DISCUSSION

Several atmospheric and environmental factors interact with sampling during winter often resulting in a limited number of specimens being collected: this was experienced in the present study.

The animals were collected on the snow surface at air temperatures of between +4 and -4°C and at high levels of humidity (90-100%). Most of the insects were active, walking or mating (*B. hiemalis*), on the snow surface when the air temperature was above -2°C. No insects were active on the snow surface when the air temperature was lower than -4°C. In days with rapid and repeated temperature changes (alternation of sun and shade depending on atmospheric conditions), specimens of *Boreus* and *Chionea* spp. were not active on the snow cover in contrast to cantharid larvae and Collembola (springtails). Collembola were not analysed in the present study. *Boreus* and *Chionea* specimens sampled frozen on the snow surface did not recover mobility when the temperature returned above 0°C. In contrast, all cantharid larvae collected in the frozen state in the field recovered full activity. These observations indicate that resistance to thermal injury (in this case cold) may be different in these species.

We reproduced such field observations by freezing the animals below their supercooling point (SCP) and measuring their survival after warming them to 5°C at a rate of +1°C min^{-1} (Table 2). The SCPs measured ranged from -6.8±1.1°C for the cantharid larvae to -11.1 ± 2.0°C for *B. hiemalis* specimens, which is significantly different ($P < 0.05$). No difference was observed between the SCPs of *B. hiemalis* males and females (data not shown); therefore the data for all specimens of *B. hiemalis* were pooled. After freezing, all the cantharid larvae recovered activity, whereas no survival was observed with *B. hiemalis*. The individuals of the latter species fall into a "reversible chill-coma" between -6 and -7° C, but are unable to survive freezing (Table 2).

To investigate the different responses to low temperature in winter-active snow insects, the presence of polyols and sugars as antifreeze compounds were examined. The most abundant sugars detected in the specimens were trehalose, glycerol and glucose. Arabinitol, fructose and myo-inositol were present in trace amounts in winter-sampled specimens and their concentration increased in the *B. hiemalis* specimens collected in March (data not shown). The specimens of *B. hiemalis* collected during early winter had higher concentrations of trehalose and glycerol than those collected in March (Fig. 1). The trehalose concentration

decreased from 8.0±0.6 and 3.8±1.8 μg mg^{-1}FW (December 2004 and 2005, respectively), to 0.3±0.2 and 0.4±0.2 μg mg^{-1}FW (March 2005 and 2006) ($P < 0.01$).

Table 2: Mean SCPs, survival after freezing (%) and expected cryoscopic decrease of the body fluids (Δ_{cr}, see materials and methods)

Taxon (number of specimens used for SCP determination; number of specimens for the antifreeze determination)	SCP °C (mean±SD)	% survival after freezing	Δ_{cr} (°C)
Cantharidae Larvae (5, 5)	-6.8 (1.1)	100	-0.282
Chionea sp. female (1, 5)	-11.2	0	-0.152
Boreus hiemalis[1] (4, 6)	-11.1 (2.0)	0	-0.148
Boreus hiemalis[2] (16, 4)	-10.2 (3.1)	0	-0.026
Boreus hiemalis[3] (7, 7)	-7.4 (1.8)	0	-0.017

[1] specimens collected in December (2004-05); [2] specimens collected in March (2005-06); [3] specimens collected in March and cultured at 16°C for 8 days.

Glycerol, the second most abundant molecule (2.5±4.0 μg mg^{-1}FW at the beginning of winter 2004) showed a similar decrease. The concentration of glucose, remained almost constant during the first sampling season (2004-05) with a value of about 0.8 μg mg^{-1}FW (Fig. 1) and decreased during the 2005-06 sampling season. These differences in the sugar concentration between years, could be related to differences in environmental temperatures.

An elevated concentration of trehalose was measured also in the *Chionea* specimens collected in early winter, showing a value of 14.8±5.4 μg mg^{-1}FW.

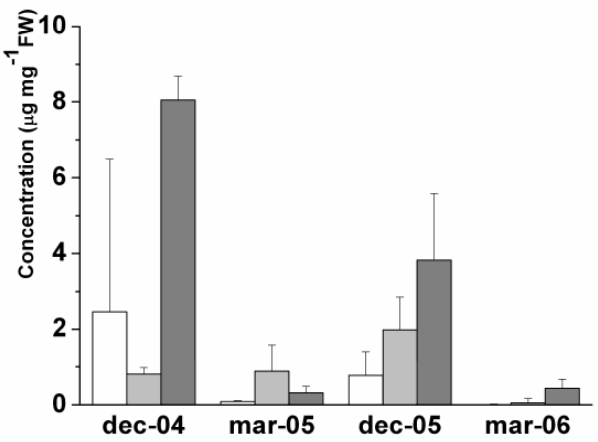

Figure 1. Glycerol (open bars), glucose (light grey) and trehalose (dark grey) concentrations in *Boreus hiemalis* specimens sampled at different times from snow surfaces.

In insects, an increase in trehalose concentration has been reported during cold acclimation in diapausing species and has been attributed an antifreeze role (19, 32). However, this molecule as well as other sugars and polyols could have, in winter-active insects, other functions, e.g. enzyme and protein protection (10, 30) in the response to low temperatures, rather than an antifreeze role. These considerations are supported by the observation that the concentration of sugars and polyols *per se* does not fully account for the cryoscopic decrease (Δ_{cr}) of the body fluids. In fact, the Δ_{cr}, expected from their molality, can be estimated from the water, sugar and polyol contents on the basis of the -1.86°C freezing point depression of a

1 osm/kg aqueous solution. These values, listed in Table 2, are significantly different from the mean SCPs for the specimens. The cryoscopic decrease (Δ_{cr}) of the body fluids calculated was less than 0.2°C in all the insects collected during winter, whereas the measured mean SCPs ranged between almost -6 and -11°C. However, the differential effect of the sugar concentration on the cryoscopic decrease (Δ_{cr}) of the body fluids caused by diversified storage both at cellular and tissue level cannot be excluded.

The important cryoprotective role of polyols at low concentrations other than a depression of SCP was suggested by Kostal & Simek (29) and demonstrated by Kostal, Slachta & Simek (30). The theory of "preferential exclusion" of solutes from proteins was proposed to explain the mode of action of polyols as demonstrated by Carpenter & Crowe (10). Moreover, Meyer-Fernandes, et al. (37) showed thermal stabilization induced by trehalose and other polyols on the enzyme glycogen phosphorylase B of the moth *Manduca sexta*, suggesting the presence of particular sites of interaction between proteins and trehalose which are present only in insect phosphorylase and not in vertebrates.

Other molecules, with non-colligative depression of the freezing point, have to be present in the body of the studied insects. Antifreeze proteins have been reported and totally or partially characterized from winter inactive species belonging to the orders Coleoptera (17, 23, 36), Diptera (33) and Lepidoptera (14). Moreover in the mecopteran *Boreus vieswoodi* the presence of a thermal hysteresis agent was inferred by the haemolymph properties but was not further characterized (26).

The difference in sugar concentration within the same species sampled in different seasons could be regulated by temperature and/or photoperiod. To investigate if this decrease could be related to temperature, 12 specimens of *B. hiemalis* (from the March 2005 collection) were cultured at 16°C for 7 days. The mortality was 41%, whereas a control sample, stored at 5°C, did not show any mortality over the same period. Comparison of the mean SCPs measured after field collection and those measured after culturing at 16°C, revealed a significant difference: *B. hiemalis* specimens decreased their mean SCP from -10.2±3.1°C after collection to -7.4±1.8°C after culturing at 16°C. The trehalose concentration declined from 0.3±0.2 to 0.2±0.1 μg mg^{-1}FW ($p < 0.1$) and the glucose concentration reduced from 0.9±0.7 μg mg^{-1}FW to 0.2±0.0 μg mg^{-1}FW ($p < 0.05$). These decreases after culturing winter insects for 7 days at 16°C were confirmed in cantharid larvae in a parallel experiment to *B. hiemalis*. The amount of trehalose and glucose was 13.7 ±5.6 and 2.3±0.6 μg mg^{-1}FW respectively in specimens collected from snow, whilst after acclimation their concentrations were reduced to 6.8±5.6 and 1.1±0.2 μg mg^{-1}FW respectively ($p < 0.09$). By contrast, in cantharid larvae stored for 7 days at 4°C, the concentration of trehalose attained 20.5± 5.8 and glucose was 2.1±0.2 μg mg^{-1}FW. These results suggest an effect of temperature on trehalose and glucose concentrations. Furthermore, the data suggest that the change in mean SCP of *B. hiemalis* could be related to the decrease in the concentration of sugars. However the mortality of 41% during the experiment and the small number of specimens does not allow a firm conclusion to be drawn.

It has been shown that overwintering pupae of the moth *Phyllonorycter ringoniella* accumulate trehalose and develop a high level of cold hardiness during diapause development. In this species, trehalose decreased rapidly at the beginning of spring (35). Trehalose levels increased in diapausing pupae of *Hyphantria cunea* (34) and seasonal patterns of accumulation/depletion of sugars and polyols have been found in an overwintering spruce bark beetle, in laboratory strains of *Sitophilus granarius* and *Cryptolestes ferrugineus* (Coleoptera) (19, 31) as well as in the heteropteran *Pyrrochoris apterus* (30) and the cabbage root fly *Delia radicum* (Diptera) (28), suggesting an adaptative mechanism common to several overwintering insects, both diapausing and active, as the mecopteran reported here. Heat and cold shock conditions influence trehalose accumulation and the activity of enzymes

in trehalose metabolism in an entomopathogenic nematode (20). This suggests that trehalose may have an important role in thermal tolerance not only in insects but also in all the ecdisozoa.

The concentrations of polyols in winter snow-active insects reported in the present study were significantly lower than those measured in non-active overwintering insects (30, 31). Since an elevated concentration of polyols could affect the viscosity of the hemolymph, this condition may be incompatible with high metabolic rates. Thus, an elevated hemolymph viscosity will not affect the metabolic rate of a diapausing insect, but it becomes a limiting factor in active insects because the diffusion rate of solutes related to the viscosity of the solution (26).

In conclusion, although so far the presence of elevated trehalose and glycerol concentrations have been reported only in diapausing larvae of flies and in other insects in a latent stage (8, 30, 31, 32, 33, 34, 35), the present study reports for the first time the accumulation of trehalose and glycerol in snow-active insects during winter and their depletion in spring. The concentrations of sugars and polyols in the studied species suggests that the potential cryoprotective effect of these molecules is related more to their non-colligative functions.

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