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## RESEARCH ARTICLE

# ***Myo*-inositol as a main metabolite in overwintering flies: seasonal metabolomic profiles and cold stress tolerance in a northern drosophilid fly**

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### SUMMARY

Coping with seasonal changes in temperature is an important factor underlying the ability of insects to survive over the harsh winter conditions in the northern temperate zone, and only a few drosophilids have been able to colonize sub-polar habitats. Information on their winter physiology is needed as it may shed light on the adaptive mechanisms of overwintering when compared with abundant data on the thermal physiology of more southern species, such as *Drosophila melanogaster*. Here we report the first seasonal metabolite analysis in a *Drosophila* species. We traced changes in the cold tolerance and metabolomic profiles in adult *Drosophila montana* flies that were exposed to thermoperiods and photoperiods similar to changes in environmental conditions of their natural habitat in northern Finland. The cold tolerance of diapausing flies increased noticeably towards the onset of winter; their chill coma recovery times showed a seasonal minimum between late autumn and early spring, whereas their survival after cold exposure remained high until late spring. The flies had already moderately accumulated glucose, trehalose and proline in autumn, but the single largest change occurred in *myo*-inositol concentrations. This increased up to 400-fold during the winter and peaked at 147 nmol mg<sup>-1</sup> fresh mass, which is among the largest reported accumulations of this compound in insects.

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Key words: chill coma recovery, cold tolerance, cryoprotectant, cold acclimation, reproductive diapause, seasonality.

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### INTRODUCTION

Adaptation of ectothermic organisms to seasonally fluctuating environments typically leads to the evolution of seasonal phenotypic plasticity, including phenotypes that can resist harsh ambient conditions (Danks, 1987). A short growing season and low and highly variable ambient temperatures during winter likely represent the most important challenges limiting species' distributions, especially at high latitudes (Andrewartha and Birch, 1954; Chown and Gaston, 2000). In insects, exposure to low temperatures can result in several types of injuries, depending on the duration and severity of the cold exposure (Lee, 2010). Freezing injury is linked to the formation of ice crystals in body fluids (Muldrew et al., 2004), and also causes, besides mechanical injury, dehydration and accumulation of solutes in the body fluids (Lee, 2010). Direct chilling injury results from cold shock (a brief exposure to relatively severe cold, without freezing) and leads to membrane lipid phase transitions and dissociation of multimeric proteins (Drobnis et al., 1993; Tsai et al., 2002). Indirect chilling injuries accumulate over relatively long exposures to relatively mild cold and may cause various disturbances in homeostatic processes (Hochachka, 1986; Košťál et al., 2004; MacMillan and Sinclair, 2010).

Multiple physiological mechanisms have evolved to enable insects to cope with low temperatures (for a review, see Denlinger and Lee, 2010). Entering a state of diapause or quiescence is a common part of winter adaptation in many insects in temperate and polar environments (Danks, 2006) and it often represents a basic prerequisite for full expression of subsequent cold-acclimation

processes (Denlinger, 1991; Slachta et al., 2002). Seasonal accumulation of cryoprotectants is probably the most widely occurring adaptive trait that helps organisms, from plants to animals, prevent injuries caused by low temperatures (Storey and Storey, 1991). Low molecular weight compounds, including polyhydric alcohols (polyols), sugars and free amino acids, form an important class of cryoprotectants, which play multiple roles in stabilization of protein and membrane structures, reduction of transmembrane water fluxes, and maintenance of osmotic balance and cell volumes (Storey and Storey, 1991; Bale, 2002; Zachariassen et al., 2004). They also help to keep the body fluids unfrozen, i.e. supercooled, at sub-zero temperatures (Storey and Storey, 1988).

Tropical and subtropical drosophilids (particularly *Drosophila melanogaster*) are often used as models in the studies on insect adaptation to various environmental stressors (Hoffmann, 1991; Gibbs, 1997; Misener and Chen, 2001; Sejerkilde et al., 2003; Overgaard et al., 2007). Only minor attention, however, has been paid to the species inhabiting high latitudes, even though they offer a good opportunity to study naturally evolved mechanisms for successful overwintering. Therefore, we focus in this paper on *Drosophila montana*, one of the northernmost species of the *Drosophila* genus. This species has a circumpolar distribution range (Throckmorton, 1982) and faces a 6-month-long unfavorable winter season in the northern parts of its distribution. Females overwinter in photoperiodically controlled reproductive diapause during which the development of their ovaries is halted in the pre-vitellogenic stage (Lumme, 1978; Watabe, 1983). We maintained female and

male flies originated from a northern Finland (66°N) population in gradually changing ambient temperature and photoperiod conditions mimicking the conditions from autumn through spring in their location of origin. Samples of flies were collected during the autumn, winter and spring periods of the artificial year in order to trace seasonal changes in their cold tolerance and to identify possible correlations with changes in concentrations of metabolites with potential cryoprotective functions: sugars, polyols, free amino acids, organic acids and free fatty acids. We found that the cold tolerance of *D. montana* flies improves markedly towards the winter and remains at a high level until the late spring. Glucose, trehalose and proline started to accumulate during autumn, whereas *myo*-inositol was the most prominent compound associated with winter season. Concentration of *myo*-inositol increased more than 400-fold during the winter and reached average concentrations as high as of 147 nmol mg<sup>-1</sup> insect fresh mass.

## MATERIALS AND METHODS

### Insects and experimental conditions

*Drosophila montana* Stone, Griffen & Patterson 1941 flies were collected in Oulanka, northern Finland (66°N, 29°E), during summer 2008. Twenty isofemale strains consisting of progenies of single field-inseminated females were set up and maintained in constant light (to prevent diapause) at 19±1°C. The mass-bred population was established by combining the progenies of these isofemale strains in the F3 generation. Three of the original strains were maintained further and used in one part of this study (see below).

The 7.5-month-long experiment started in June 2009, when the flies had been kept for approximately eight generations in the laboratory. The experimental conditions in the climate chamber (Sanyo MLR-351H, Sanyo, San Diego, CA, USA) were set to mimic the basic trend in progressive seasonal conditions that the flies encounter in their natural habitat in northern Finland. The temperatures in the chamber were based on air temperature data collected by Oulanka Research Station (University of Oulu). The winter temperature was set to +4°C, slightly higher than the 0°C expected under snow cover, to ensure that a sufficient number of flies survived over the winter and that samples could also be obtained in the spring. The day and night temperatures (thermoperiod) and photoperiod were gradually changing, as depicted in Fig. 1. Humidity was kept constant (60±10%) for most of the experiment, except during the winter period (constant darkness), when it was 30%. Flies from the mass-bred population were transferred to the experimental conditions within 1 day of eclosion; females and males were

transferred to separate vials containing malt-yeast medium (10–13 individuals per vial). The first set of flies was transferred into the chamber and taken out for analyses 21 days after the transfer, in conditions corresponding to the month of July. This sample served as a non-diapause reference for all other samples (Fig. 1). At least in the Kemi population, which is approximately 100 km south of Oulanka, the few females that emerge during early July are known to develop their ovaries (Aspi et al., 1993). The second set of flies was transferred into the experimental conditions at a photoperiod of 16 h:8 h light:dark, corresponding to August, when the emerging females of the wild population enter diapause in northern Finland (Aspi et al., 1993; Tyukmaeva et al., 2011). In addition to the flies from the mass-bred population, we used two sets of males from three isofemale strains (see above). The first set of males was maintained in constant light and 19°C for 14 days before they were transferred into the experimental chamber under August conditions, whereas the second set of males was placed in the chamber within 1 day of eclosion (in August conditions). Samples of both sets of males were analyzed during mid-winter and named Winter 1 (first set) and Winter 2 (second set). The Winter 1 sample was collected to test whether the photoperiodic conditions during the first 2 weeks after eclosion affect the later accumulation of metabolites; this sample was used only in comparison with the Winter 2 sample.

### Cold tolerance

We measured the cold tolerance of the flies of summer, autumn and spring samples using two approaches: (1) the time needed for the recovery from chill coma (David et al., 1998) and (2) survival after a cold exposure. Chill coma recovery (CCR) times were measured in flies that had been kept at -6°C for 16 h. After chilling, the flies were brought to room temperature (21–22°C) and placed in separate compartments (4×3×2 cm) with lids. A fly was considered to have recovered from the chill coma when it was able to stand up on its legs. Both females and males in approximately a 1:1 ratio were used in the tests with the following total numbers of flies used for each treatment: 40 (July), 70–80 (August and October), 50 (April) and 20–30 (early and late May). After measuring the CCRs, the ovaries of all females were examined and classified into three groups: diapausing (small, transparent ovaries, no yolk), intermediate (some yolk accumulated, egg chambers visible, no eggs) or vitellogenic (mature eggs in the egg chambers).

The survival of the flies after a cold exposure at -11°C for 24 h was scored after recovery at a room temperature for 24 h in vials containing malt-yeast medium. A fly was considered alive if it was

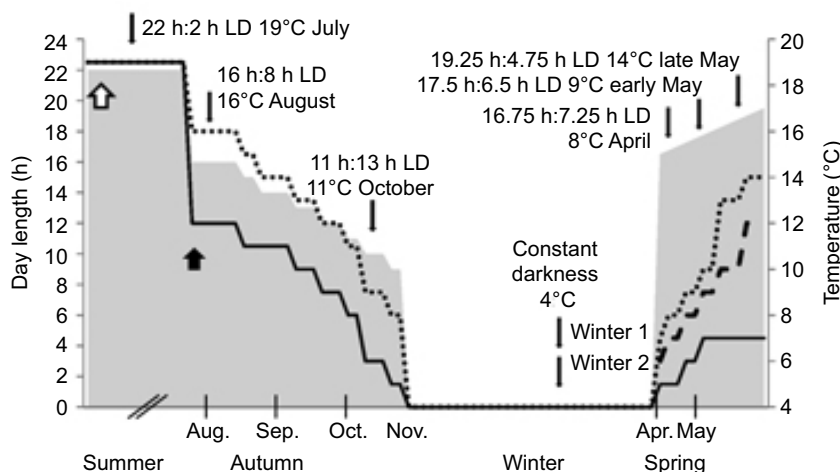


Fig. 1. Experimental conditions in the climate chamber mimicking the temperature and photoperiodic conditions in summer, autumn, winter and spring (x-axis) in northern Finland. Gray-shaded areas mark the day lengths (hours of light). Note that the changes in photoperiod were gradual. The temperature conditions (°C) also fluctuated on a daily basis: solid line, night temperature; dotted line, day temperature; dashed line, temperature during dawn/dusk in the spring. The thin arrows mark the times of sample collection with information on the exact photoperiod and day temperature at the time of sampling. The white thick arrow marks the time point when the first set of flies (summer flies) were placed in the chamber. These flies were sampled in July and they served as non-diapause controls. The black thick arrow marks the time point when the second set (all remaining flies) was placed in the chamber in diapause-inducing conditions. LD, light:dark.

able to respond to a slight agitation with forceps. The tests were made for 60 to 70 females and males in the autumn samples, and 30–40 in the spring sample.

### Metabolite analysis

Summer, autumn and spring samples involved three male and three female samples each (with 10 flies per sample), whereas both of the winter samples involved three male samples (with nine flies per sample; three males per isofemale strain). The flies were immersed in liquid nitrogen and stored at  $-84^{\circ}\text{C}$  until analyzed (approximately 1 year later). The samples were weighed using a Mettler Toledo XS204 analytical balance with a precision of 0.1 mg and processed and analyzed as described in Kostál et al. (Kostál et al., 2011a; Kostál et al., 2011b). Briefly, low molecular weight sugars and polyols were quantitatively determined in ethanolic extracts after *o*-methyloxime trimethylsilyl derivatization and subsequent analysis by gas chromatography coupled to mass spectrometry (GC/MS). Other metabolites were analyzed by a combination of GC/MS and liquid chromatography/MS techniques in the same ethanolic extracts after their treatment with ethyl chloroformate under pyridine catalysis and simultaneous extraction into chloroform. All concentrations are expressed in  $\text{nmol mg}^{-1}$  fresh mass (FM).

### Statistical analyses

The data on fly chill coma recovery times did not meet the requirement for homoscedasticity (regardless of transformations) and were therefore analyzed using a non-parametric Kruskal–Wallis test for multiple comparisons and a Mann–Whitney *U*-test for comparing the sexes. The data on fly survival were analyzed using the chi-square test. Bonferroni-adjusted alpha levels were used to correct for multiple testing. The metabolite data were first explored using principal component analysis (PCA) to determine which metabolites show the highest contributions to variation among the samples. Based on the PCA results, the most promising candidate metabolites were tested using the Kruskal–Wallis test for multiple comparisons and pairwise comparisons were performed with Dunn's post test following the Kruskal–Wallis test. The two winter samples were compared with a Mann–Whitney *U*-test. All analyses were conducted using PASW Statistics 18.0 (SPSS, IBM, Armonk, NY, USA).

## RESULTS

### Seasonal changes in cold tolerance

The CCRs were similar in males and females and a significant difference between the sexes was found only in the late May sample (Mann–Whitney *U*-test:  $U=513$ ,  $N=53$ ,  $\text{d.f.}=1$ ,  $P=0.003$ ). Pooled data for females and males revealed substantial seasonal variation in this trait (Kruskal–Wallis:  $K=483.7$ ,  $N=601$ ,  $\text{d.f.}=5$ ,  $P<0.001$ ; Fig. 2A). The mean recovery time of the July flies was 18 min on average, that of the August flies was 6.5 min, and the minimum recovery times of 1–2 min were reached during October and April. In the last sample of late May, the recovery times increased again, and reached similar levels as in August.

The survival after cold exposure also showed a clear seasonal variation (chi-square:  $\chi^2=347.3$ ,  $\text{d.f.}=5$ ,  $P<0.001$  for combined data of the sexes, which did not differ from each other; Fig. 2B). Although only 19% of flies survived the cold exposure in July, 89% of females and 98% of males survived in October. In contrast to the seasonal pattern observed in the CCR data, survival after cold exposure also remained at 100% in spring samples.

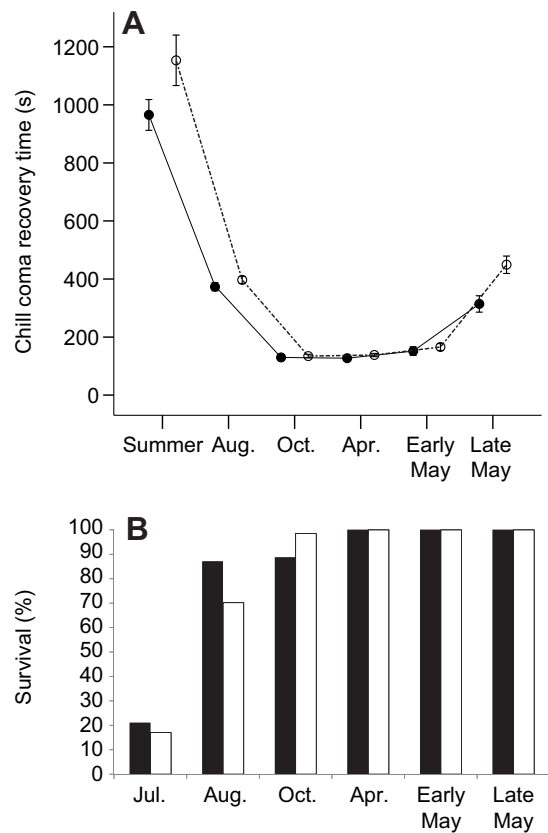


Fig. 2. Seasonal changes in (A) chill coma recovery time (means  $\pm$  s.e.m.) and (B) survival after cold treatment in *Drosophila montana* flies that were overwintering in experimental conditions and sampled as shown in Fig. 1. Filled circles/bars, females; open circles/bars, males.

All females in the July sample had developed ovaries, as expected, whereas the females of the second set of flies, which had been transferred into the experimental chamber in short day conditions (August), had entered diapause (i.e. they had small, pre-vitellogenic ovaries). In April, all these females still had undeveloped ovaries, but in the early May 19% of females had intermediate ovaries and one female fully developed ovaries. In the late May sample, most females had fully developed ovaries (73%).

### Seasonal changes in metabolomic profiles

Seasonal patterns in metabolomic profiles were similar in females and males, so the data were pooled for the analyses. Based on the PCA, changes in the metabolite concentrations were explained by nine principal components with eigenvalues  $>1$ . The first principal component (PC 1) explained 29.4% and the second PC (PC 2) explained 19.6% of the variation in the data set, whereas other PCs contributed less (PC 3 contributed 10%, all remaining PCs  $<4.0\%$  each). When plotting the samples according to the first two PCs (Fig. 3), PC 1 clearly separated the July (summer) sample from the other samples. The remaining samples showed distinct groupings along PC 2, with the two winter samples being clearly separated from the autumn and spring samples. Eleven metabolites [eight amino acids, ribose, lactate and oleic acid (C18:1n6)] were associated with the July sample, whereas three metabolites, namely *myo*-inositol, palmitoleic acid (C16:1n7) and linoleic acid (C18:2n6), were found to discriminate the rest of the samples. These metabolites

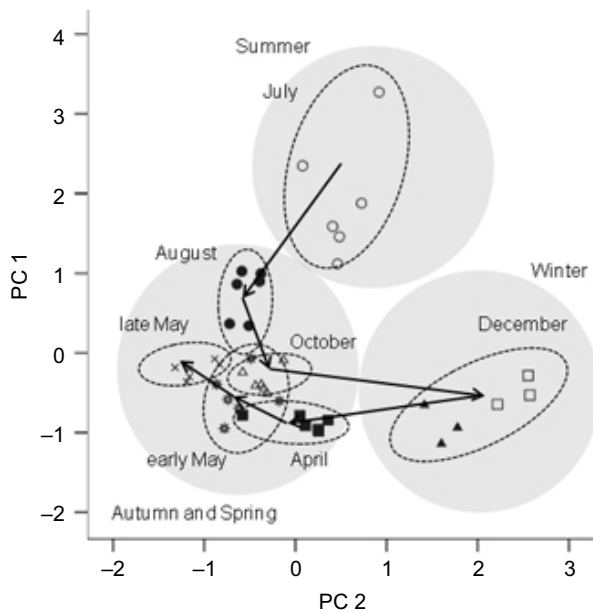


Fig. 3. Principal component analysis (PCA) plot of metabolomic data of *D. montana* adults that were sampled as shown in Fig. 1. The first and second principal components (PC1 and PC 2) contributed 29.4 and 19.6% of the total variance in the data, respectively. The data points belonging to one sampling time are encircled with ellipses and the ellipses are connected with arrows representing the chronological course of the experimental seasons. Three shaded circles represent three main clusters in the data (summer, autumn–spring and winter).

were statistically analyzed in detail to determine their seasonal patterns. Trehalose, glucose and proline were also added to this statistical analysis as data from the literature suggest their importance in cold acclimation of insects (Table 1, supplementary material Table S1). We used only the Winter 2 sample (not Winter 1) for the analysis because the flies from this sample had experienced the same initial conditions as all remaining flies.

The most significant seasonal variation was observed in the concentration of *myo*-inositol, which accumulated only during the winter (Fig. 4A). It increased from  $0.2 \text{ nmol mg}^{-1} \text{ FM}$  in July to  $147 \text{ nmol mg}^{-1} \text{ FM}$  in December (>400-fold change in concentration) and decreased back to July levels in early May (Table 1). The two sugars, glucose and trehalose, also showed relatively distinct seasonal patterns (Table 1), beginning to accumulate in August and reaching high levels already in October compared with July. Concentrations of both sugars remained high until the spring.

Several free amino acids showed a decrease in concentration towards the winter (cysteine, glycine, histidine, isoleucine, leucine, lysine, methionine and serine), whereas only one (proline) showed a winter maximum (Fig. 4B depicts the examples of both trends and Table 1 contains the detailed data). Seasonal variation in proline was characterized with an early start of accumulation during autumn and threefold higher concentrations in December than in July. In addition, lactate showed a specific trend of continuous decrease from July to late May (Fig. 4C). Three unsaturated fatty acids could be divided into summer-related (oleic acid) and winter-related (linoleic acid and palmitoleic acid) metabolites based on their seasonal concentrations.

The two types of overwintering males (Winter 1 and 2) clustered close to each other but were still clearly separable in the PCA (Fig. 3). The males of the Winter 1 sample accumulated significantly smaller amounts of the main winter compound *myo*-inositol

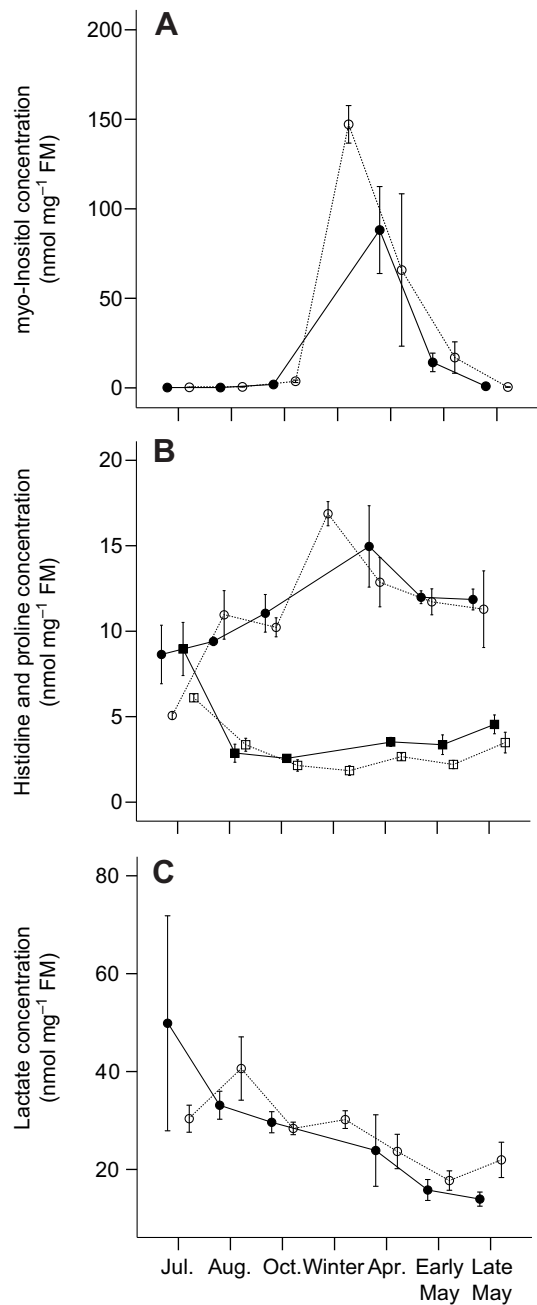


Fig. 4. Seasonal changes in concentrations (means  $\pm$  s.d.) of (A) *myo*-inositol, (B) histidine and proline and (C) lactate in *D. montana* adults representing seasonal samples shown in Fig. 1. The winter concentrations are given for only the Winter 2 sample (see Materials and methods for explanation). Filled symbols, females; open symbols, males. In B, circles represent proline and squares represent histidine. FM, fresh mass.

( $94 \text{ nmol mg}^{-1} \text{ FM}$ ) than the males of the Winter 2 sample (Mann–Whitney *U*-test:  $U=9.00$ ,  $P=0.05$ ; Fig. 4A). Similar trends for smaller concentrations in the Winter 1 sample were observed also for glucose, trehalose and proline ( $U=9.00$ ,  $P=0.05$  for all). See supplementary material Table S1 for concentrations.

## DISCUSSION

In the present study, we show that the cold tolerance of overwintering *D. montana* flies improves during the late summer/autumn, prior to

Table 1. Concentrations (nmol mg<sup>-1</sup> fresh mass) of metabolites detected in *Drosophila montana* adults that were found to contribute to the seasonal variation in the principal component analysis

Metabolite	Seasonal samples							K
	July	August	October	Winter 2	April	Early May	Late May	
Sugars and polyols								
<b>Glucose</b>	33.93±3.57 <sup>a</sup>	43.03±3.41 <sup>a</sup>	57.53±2.36 <sup>b</sup>	56.65±6.08 <sup>b</sup>	46.73±6.66	54.87±2.48	56.99±4.70	<b>28.65***</b>
<b>Myo-inositol</b>	0.24±0.10 <sup>a</sup>	0.36±0.22 <sup>a,b</sup>	2.77±1.10 <sup>b</sup>	147±10.47 <sup>c</sup>	78.00±33.29	15.58±6.61	0.67±0.79	<b>33.97***</b>
Ribose	0.21±0.12 <sup>a</sup>	0.15±0.02 <sup>a</sup>	0.02±0.06 <sup>b</sup>	0.06±0.01 <sup>a,c</sup>	0	0	0	30.37***
<b>Trehalose</b>	0.42±0.17 <sup>a</sup>	1.11±0.37 <sup>a</sup>	4.23±2.13 <sup>b</sup>	2.66±1.29 <sup>b</sup>	3.81±1.49	4.88±2.77	2.98±1.71	<b>26.64***</b>
Free fatty acids								
C16:1 n7 cis	0.83±0.54 <sup>a</sup>	0.46±0.18 <sup>a</sup>	0.82±0.205 <sup>b</sup>	1.61±0.04 <sup>c</sup>	0.60±0.30	0.62±0.34	0.45±0.23	14.46**
C18:1n6	0.21±0.09 <sup>a</sup>	0.04±0.02 <sup>a,b,c</sup>	0.07±0.01 <sup>b,c</sup>	0.05±0.004 <sup>c</sup>	0.02±0.01	0.04±0.03	0.02±0.01	25.37***
<b>C18:2n6</b>	0.54±0.21 <sup>a</sup>	0.27±0.11 <sup>b</sup>	0.34±0.02 <sup>b</sup>	0.84±0.02 <sup>a</sup>	0.40±0.16	0.36±0.16	0.24±0.10	<b>28.23***</b>
Organic acids								
Lactate	39.07±16.8 <sup>a</sup>	32.15±13.0 <sup>a</sup>	25.74±9.64 <sup>a</sup>	30.18±1.81 <sup>a</sup>	21.73±7.26 <sup>b</sup>	14.83±5.44	15.39±7.20	30.883***
Free amino acids								
Cysteine	0.87±0.312 <sup>a</sup>	0.46±0.180 <sup>a,b</sup>	0.23±0.07 <sup>b</sup>	0 <sup>c</sup>	0.09±0.03	0.14±0.06	0.22±0.09	34.79***
Glycine	1.73±0.60 <sup>a</sup>	1.30±0.60 <sup>a</sup>	0.73±0.23 <sup>b</sup>	0.93±0.01 <sup>b</sup>	0.52±0.02	0.57±0.20	0.96±0.38	34.93***
Histidine	6.96±2.73 <sup>a</sup>	2.76±0.20 <sup>b</sup>	2.38±0.30 <sup>b,c</sup>	1.84±0.30 <sup>c</sup>	2.79±1.12	2.58±1.05	3.65±1.43	28.48***
Isoleucine	0.29±0.12 <sup>a</sup>	0.27±0.12 <sup>a</sup>	0.21±0.01 <sup>a,b</sup>	0.17±0.04 <sup>b</sup>	0.07±0.03	0.14±0.05	0.25±0.10	29.91***
Leucine	0.53±0.25 <sup>a</sup>	0.44±0.21 <sup>a</sup>	0.39±0.15 <sup>a,b</sup>	0.23±0.03 <sup>b</sup>	0.11±0.04	0.22±0.08	0.46±0.19	28.69***
Lysine	1.25±0.47 <sup>a</sup>	1.01±0.40 <sup>a,b</sup>	0.81±0.02 <sup>b</sup>	0.63±0.04 <sup>c</sup>	0.73±1.12	0.59±0.323	1.01±0.43	30.19***
Methionine	0.23±0.10 <sup>a</sup>	0.11±0.04 <sup>a,b</sup>	0.10±0.02 <sup>b,c</sup>	0.04±0.01 <sup>c</sup>	0.04±0.02	0.04±0.02	0.03±0.01	27.69***
<b>Proline</b>	6.43±2.76 <sup>a</sup>	8.84±3.64 <sup>a</sup>	9.50±3.48 <sup>a</sup>	16.87±0.05 <sup>b</sup>	12.60±4.51	10.44±4.10	10.24±4.10	<b>27.62***</b>
Serine	1.31±0.53 <sup>a</sup>	0.82±0.32 <sup>b</sup>	0.60±0.23 <sup>c</sup>	0.63±0.09 <sup>c</sup>	0.58±0.19	1.10±0.40	0.85±0.35	30.21***

Seasonal variation among sampling points was tested using the Kruskal–Wallis test (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ), and the pairwise differences were analyzed using Dunn's *post hoc* test. Different superscripted letters indicate means ( $\pm$ s.d.) that are significantly different from one another. Compounds that were found to be winter-related are in bold. Note: pairwise comparisons were performed among July, August, October and Winter 2 samples.

a decline of ambient temperatures below the threshold for direct development (Loeb and Northrop, 1917). At least in females, the increased cold tolerance could be linked with a switch to diapause state. The highest level of cold tolerance, however, was achieved during the maintenance stage of diapause, and could be associated with shortening day length, decreasing temperature or both.

*Drosophila montana* can be regarded as a freeze-avoiding species, as at least non-acclimated flies of this species die before their super cooling point (SCP;  $-20^{\circ}\text{C}$ ) is reached (Lumme, 1980). CCR (an indirect measure of cold tolerance) and survival after cold exposure (a direct measure) varied according to the season in slightly different ways. Although the CCRs showed a seasonal minimum (maximum cold tolerance) during October–April, the survival after a cold exposure was the highest in spring. Such a difference may be explained, at least partially, by differences in the type of chill injury associated with two different treatments (Rako and Hoffmann, 2006). Our cold tolerance assays were based on such exposures ( $-6^{\circ}\text{C}$  for 16 h and  $-11^{\circ}\text{C}$  for 24 h), which are likely to cause both acute and chronic chill injuries in an unknown, and possibly different, ratio (Sinclair and Roberts, 2005; Lee, 2010). In addition, seasonal progression of diapause development likely influences CCR: the deeper the diapause (the higher the diapause intensity), the longer the recovery time. Intensity of diapause is usually highest during early phases of diapause development (corresponding to the late summer sample) and decreases during diapause termination (corresponding to autumn and winter samples) (Kostál, 2006).

*Myo*-inositol concentrations showed the most pronounced seasonal variation among all detected metabolites. It increased more than 400-fold from July to December and decreased back to prior-winter levels during spring. *Myo*-inositol is biosynthesized from D-glucose in three enzymatic steps and has several important functions, including regulation of cell development and growth (Loewus and Loewus, 1983). It is also an important precursor of inositol phospholipids, producing secondary messengers, the inositol

phosphates (Downes and Macphree, 1990). A seasonal trend with a high peak during the winter, as observed in *D. montana*, suggests a cryoprotective function for *myo*-inositol (Pio and Baust, 1988; Storey and Storey, 1991). Our data set did not show a simple and clear-cut association between high levels of *myo*-inositol and high cold tolerance. The highest levels of survival after cold exposure (24 h at  $-11^{\circ}\text{C}$ ) were observed in the spring, even though the concentration of *myo*-inositol had already decreased close to the seasonal minimum in May. The lack of clear correlation between *myo*-inositol level and survival after cold exposure could be partly because our test measures resistance to direct rather than indirect (or cumulative) chilling injuries, which accumulate over long periods spent even at relative high temperatures (i.e. well above SCP). Hence, the accumulated *myo*-inositol might be involved primarily in protection against indirect chilling injury, which may accumulate in overwintering *D. montana* flies spending months under the snow cover, where the temperature remains quite constantly close to  $0^{\circ}\text{C}$ . An alternative explanation would be that the high winter levels of *myo*-inositol represent a byproduct of high metabolic suppression during winter months (Pullin et al., 1991). Although we assume the adaptive explanation for *myo*-inositol accumulation, functional tests are required to directly test this hypothesis.

Seasonal accumulation of *myo*-inositol has been previously reported in some insect species, including three species of sub-Antarctic beetles (Block and Sømme, 1983) and the ladybird beetle *Ceratomegilla undecimnotata* (Kostál et al., 1996). A clear association between seasonal cold tolerance and *myo*-inositol concentration was observed in the lady beetle *Harmonia axyris*, in which both the SCP decreased and the chill tolerance improved along with an increase in *myo*-inositol concentration (Watanabe, 2002). In several other species, the *myo*-inositol levels have been found to correlate with some (but not all) of the cold tolerance measures. In the leaf beetle, *Aulacophora nigripennis*, the seasonal accumulation

of *myo*-inositol has been found to occur in synchrony with chill tolerance (Watanabe and Tanaka, 1999), whereas in the terrestrial isopod *Porcellio scaber*, the accumulation of *myo*-inositol correlates positively with the lower lethal temperature (LTT<sub>50</sub>), but not SCP (Tanaka and Udagawa, 1993). In the cotton bollworm, *Helicoverpa armigera*, the lowest observed SCP coincided with the highest amount of *myo*-inositol, suggesting that *myo*-inositol (together with glycerol) is the main compound decreasing SCP in this species (Liu et al., 2009). In an alpine beetle, *Patrobis septentrionis*, this metabolite was found to accumulate during cold acclimation, which also decreased the SCP (Bakken, 1985). *Myo*-inositol has also been found to accumulate when insects are exposed to fluctuating thermal regimes, for example in the eggs of the migratory locust, *Locusta migratoria* (Wang et al., 2006), and in the tropical beetle *Alphitobius diaperinus* (Lalouette et al., 2007). Although in most of the species a low temperature trigger was required for the accumulation of *myo*-inositol, in the house spider, *Achaearanea tepidariorum*, it was triggered by short day length, along with diapause induction (Tanaka, 1995). To our knowledge, the winter accumulation of *myo*-inositol detected in *D. montana* represents one of the largest seasonal changes in this metabolite observed in any insect species (concentrations of *myo*-inositol were converted in comparisons to nmolmg<sup>-1</sup>). Only in the 28-spotted potato ladybird (*Epilachna vigintioctomaculata*) did similar amounts accumulate in winter conditions (Hoshikawa, 1987).

Glucose, trehalose and proline emerged as other metabolites that could contribute to the seasonal improvement of cold tolerance in *D. montana*. They accumulated by autumn and stayed at high levels until the spring, thus following nicely along the seasonal pattern of cold tolerance. All three metabolites may exert cryoprotective functions through stabilizing cell membranes and protein structures (Withers and King, 1980; Storey and Storey, 1991). Direct evidence for the adaptive role of proline accumulation has been recently reported in a temperate drosophilid fly, *Chymomyza costata* (Kostál et al., 2011a), and winter- or cold-related accumulation of proline has been observed in many other insects (Storey et al., 1981; Morgan and Chippendale, 1983; Fields et al., 1998), including the fruit fly *D. melanogaster* (Kostál et al., 2011b). It has to be noted that in the present experiment the flies did not encounter sub-zero temperatures, which they are likely to encounter occasionally in nature, for example during autumn and the spring frost. As some of the cryoprotectants are known to require certain low temperatures to be accumulated, this may limit the findings in this study.

Among the other metabolites, seven amino acids, ribose and lactate were most abundant in the summer sample, which could be related to a higher feeding activity of the flies in summer. The decrease of free ribose, required for DNA and RNA synthesis, in the winter may reflect decelerated cell proliferation. However, high concentrations of lactate are surprising. Lactate may be produced as the end product of anaerobic metabolism (Grieshaber et al., 1994; Hobak and Stanley, 2001), but it is unlikely that our flies suffered from hypoxia or anoxia during the experiments. Thus, most lactate may come from the diet. Finally, three unsaturated fatty acids, linoleic acid, palmitoleic acid and oleic acid, showed a seasonal pattern, the first two having the highest concentration in the winter and the last in the summer. Increased levels of linoleic acid during cold acclimation have previously been observed in *D. melanogaster* (Overgaard et al., 2008). However, changes in the free fatty acid concentrations in *D. montana* were only minor and it is doubtful whether these changes contributed to the seasonal patterns in cold tolerance.

Interestingly, the concentrations of winter-related metabolites (*myo*-inositol, glucose, trehalose and proline) differed between the

two groups of overwintering males (Winter 1 and 2). Males that had been maintained the first 2 weeks of their life in continuous light (Winter 1) accumulated smaller amounts of winter-related metabolites than the males that had been maintained under short day length since their eclosion (Winter 2). These results indicate that the conditions experienced during early adult development may considerably affect the levels of metabolites that are expressed during the winter.

In conclusion, our study revealed clear seasonal changes in the cold tolerance of overwintering *D. montana* flies, accompanied by changes in several metabolites. This study is the first metabolic analysis of a drosophilid species performed in thermoperiods and photoperiods that mimic natural seasons. Nevertheless, in nature the flies are likely to encounter also sub-zero temperatures, which may trigger accumulation of other cryoprotectants that were not detected in this study. The overwintering strategy of this species seems to be linked with a marked increase in concentration of a single prominent metabolite, *myo*-inositol. The exact physiological role of this metabolite, including its putative cryoprotective function, needs further assessment.

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