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Dual roles of glucose in the freeze-tolerant earthworm *Dendrobaena octaedra*: cryoprotection and fuel for metabolism

Sofia Calderon[1,2], Martin Holmstrup[1], Peter Westh[3] and Johannes Overgaard[1,2,*]

[1]National Environmental Research Institute, University of Aarhus, Department of Terrestrial Ecology, Vejlsøvej 25, PO Box 314, DK-8600 Silkeborg, Denmark, [2]Department of Zoophysiology, Institute of Biological Sciences, University of Aarhus, Building 540, DK-8000 Aarhus, Denmark and [3]NSM, Research Unit for Functional Biomaterials, Roskilde University, Roskilde, Denmark

*Author for correspondence (e-mail: biojo@biology.au.dk)

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SUMMARY

Ectothermic animals inhabiting the subarctic and temperate regions have evolved strategies to deal with periods of continuous frost during winter. The earthworm *Dendrobaena octaedra* is freeze tolerant and accumulates large concentrations of glucose upon freezing. The present study investigates the roles of glucose accumulation for long-term freeze tolerance in worms kept frozen at –2°C for 47 days. During this period, worms were sampled periodically for determination of survival and for measurements of glucose, glycogen, lactate, alanine and succinate. In addition we performed calorimetric measurements to assess metabolic rate of frozen and unfrozen worms. Long-term freezing was associated with a gradual depletion of glucose and worms that succumbed during this period were always characterised by low glucose and glycogen levels. The anaerobic waste products lactate and alanine increased slightly whereas succinate levels remained constant. However, it is argued that other waste products (particularly propionate) could be the primary end product of a continued anaerobic metabolism. Calorimetric measures of the metabolic rate of frozen worms were in accord with values calculated from the reduction in glucose assuming that most (~90%) glucose was metabolised anaerobically. Both estimates of metabolic rate demonstrated a 10-fold metabolic depression associated with freezing. Thus, in addition to the suspected role of glucose as cryoprotectant, the present study demonstrates that glucose accumulation is vital to ensure substrate for long-term anaerobic metabolism in frozen worms. On the basis of the estimated metabolite levels, we calculate that the combined effect of metabolic depression and large glucose stores enables a projected 3 months survival of freezing at –2°C of the 'average' *D. octaedra*. Such conditions are very likely to occur in the northern distribution ranges of this stress-tolerant earthworm.

Key words: cryoprotectant, glucose, anoxia, anaerobic metabolism, freeze survival.

INTRODUCTION

Ectothermic animals inhabiting the temperate and polar regions have evolved physiological and behavioural strategies to endure occasional or permanent exposures to sub-zero temperatures. One such strategy is termed freeze-tolerance where a number of both vertebrate and invertebrate species have evolved the ability to endure freezing of their extracellular fluids (Zachariassen, 1985; Storey and Storey, 1988). Most freeze-tolerant species produce cryoprotectants in relation to frost as this limits both the amount and speed of ice formation in the extracellular fluid, and some species may also produce ice-nucleating agents that help to initiate freezing of the extracellular fluid at relatively high temperatures where freezing occurs slower and is therefore a more controlled process (Zachariassen, 1985; Storey and Storey, 1988).

In earthworms, freeze tolerance is particularly prominent in the two northerly distributed species, *Eisenia nordenskioldi* and *Dendrobaena octaedra*, and individuals of *D. octaedra* may tolerate freezing to temperatures as low as –20°C (Holmstrup and Overgaard, 2007). Extracellular freezing of these species is associated with a rapid and substantial accumulation of glucose, derived from glycogen stores. In some individuals of adult *D. octaedra* glucose can constitute more than ~20% of dry mass less than 24 h after freezing has commenced. Glucose is the only cryoprotectant that accumulates in response to freezing in *D. octaedra* (Bundy et al., 2003; Rasmussen and Holmstrup, 2002; Holmstrup, 2003) and similarly glucose has also been found to be the most predominant cryoprotectant in many freeze-tolerant amphibians (Schmid, 1982; Storey and Storey, 1992; Storey and Storey, 1996). In *D. octaedra* it seems that the rapid accumulation of glucose is triggered by small temperature changes below 0°C (Overgaard et al., 2007) but accumulation is also induced by rapid changes in osmotic pressure such as those following extracellular ice formation (Rasmussen and Holmstrup, 2002; Holmstrup, 2003; Overgaard et al., 2007).

Although it seems clear that accumulation of glucose is an essential component of freeze survival in *D. octaedra*, previous experiments have not conclusively demonstrated that high glucose accumulation correlates with short-term freeze survival. There was, for example, no significant correlation between the average amount of glucose mobilized and tolerance to low temperature when this was examined in a common garden experiment comparing freeze tolerance of different *D. octaedra* populations from Denmark, Canada, Sweden, Finland, Poland and Greenland (Holmstrup et al., 2007). Instead, the study by Holmstrup et al. (Holmstrup et al., 2007) demonstrated that the magnitude of the glycogen reserves prior to freezing correlated with population freeze tolerance. Moreover, several studies have found large individual differences in glucose accumulation that do not necessarily relate to freezing survival of individuals. Thus, individuals that accumulate relatively low amounts of glucose may also survive short-term freeze-tolerance tests (Rasmussen and Holmstrup, 2002; Holmstrup et al., 2007;

Overgaard et al., 2007). A similar relationship is also seen when considering interspecific differences since some species of earthworms, such as *Aporrectodea caliginosa*, show some freeze tolerance although their cryoprotectant accumulation response is limited (Holmstrup and Overgaard, 2007). Given the lack of a straightforward relationship between glucose concentration and freeze tolerance in *D. octaedra* we hypothesized that another primary role of glucose accumulation is to serve as a well-distributed energy resource during long-term frost.

On a broader comparative scale, freeze-tolerant species of earthworms share a number of features with freeze-tolerant amphibians and, to some extent, reptiles. Thus, all groups have representatives that tolerate freezing of more than 50% of body water and they all primarily use glucose as cryoprotectant. Species from these diverse groups also share the use of convective transport to sustain gas exchange as well as the risk of inoculative freezing when hibernating. However, in reptiles, when glucose levels are low, the duration of freeze tolerance is rarely above 1 week and in anuran amphibians, where glucose is accumulated to high but heterogeneous levels, there are no reports of freezing survival of more than a month (Storey and Storey, 1988; Layne and Kefauver, 1997; Storey, 1990; Voituron et al., 2002a; Costanzo et al., 2006). Thus, from a comparative perspective it seems that *D. octaedra* is much more tolerant to long-term freezing and this may be linked to the much larger and more uniform accumulation of glucose in these animals (M. Tolarova, personal communication) which enables sustained metabolism of all organs during freezing.

In this study we test the hypothesis that glucose accumulation is beneficial for long-term cold tolerance because it provides a reliable energy resource during long periods in a frozen state. Under such conditions there will be no, or very limited, convective transport of fermentable energy resources in the extracellular fluid and sufficient energy resources must, therefore, be distributed prior to freezing or before the freezing process is completed. Owing to the impeded convective transport and because oxygen diffuses extremely slowly through ice it is probable that frozen worms rely primarily on anaerobic metabolism during the extended periods with subzero temperatures. Given the inefficiency of ATP production per glucose molecule under anaerobic conditions we hypothesize that long-term freezing must be associated with a substantial mobilization of glucose, but also with a considerable metabolic depression, reducing glucose depletion during the winter period in these worms.

Here we report, for the first time, that long-term freezing tolerance is an environmentally realistic phenomenon since adult *D. octaedra* from Greenland survived Arctic winter conditions in frozen soil for at least 3 months. The relationship between glucose mobilisation, metabolic rate and long-term freezing survival in *D. octaedra* was examined further using a similar and highly freeze-tolerant population of *D. octaedra* from Finland showing a gradual depletion of glucose stores during long-term frost. In addition, it was investigated how anaerobic metabolism and general metabolic depression contributes to the long-term freeze tolerance of *D. octaedra*.

MATERIALS AND METHODS

Field survival

Field mortality of adult *Dendrobaena octaedra* Savigny was tested during long-term frost in a population collected at Arctic Station, Godhavn, Disko, west Greenland (latitude 69 deg. N, longitude 53 deg. W). Seventy adult worms were collected locally and 10 specimens were then placed in each of seven small cylindrical containers (10 cm diameter and 20 cm length) with each end closed by gauze to allow ventilation and water exchange but preventing escape of the worms. The containers were placed vertically in the soil in the same area where the worms were collected in July 2006 and the worms were therefore only able to move between 0 and 15 cm soil-depth. Two data loggers were placed next to the containers so that temperature was continuously monitored at 5 min intervals (Tiny tag; Gemini Data loggers, Chichester, UK). Survival status of the worms was recorded approximately 11 months later in June, 2007.

Collection and maintenance of *D. octaedra* used for laboratory experiments

Specimens of *D. octaedra* were collected in Konnevesi, Finland (latitude of 62 deg. N, longitude of 25 deg. E) in September 2006 and brought to Denmark in plastic beakers with moist vegetation from the location. Here the animals were maintained at 10±1°C, in 11 plastic beakers each containing 10 individuals and 600–700 g moist soil. The worms were fed every 4 weeks by the addition of a soil/cow dung mix (40 g dry soil, 10 g cow dung and 55 ml water). The animals used for experiments were adults or large juveniles with a dry mass ranging from 5 to 75 mg. Worms used for long-term frost exposure were placed individually in 10 ml plastic vials containing moist soil and they were acclimated at 2±1°C for 4 weeks prior to the start of the experiment in February 2007. Worms used for calorimetrical measurements were placed singly in containers with soil and kept at 5°C for 2 weeks prior to the start of the experiment in January 2008. Worms used for these experiments were from the F1 generation that had developed under similar conditions as mentioned above.

Long-term freezing

After acclimation, the worms were split into a control group, which remained at 2±1°C, and a frozen group, which was moved to a programmable freezing cabinet for 24 h, in which the temperature could be controlled and gradually lowered to –2°C (–0.042°C h^{-1}). A small piece of ice was added to each plastic vial when the temperature reached –1°C to initiate freezing of soil and worms; the worms were subsequently left at –2°C throughout the remainder of the experiment. Previous experiments have shown that worms freeze when using this procedure (Bindesbøl et al., 2005). After 3, 6, 10, 13, 20, 26 and 47 days, approximately 15 worms, respectively, were thawed at room temperature and cleaned of soil and debris. The survival status of each individual worm was noted, after which they were rapidly frozen at –80°C for later assessment of glucose, lactate, succinate, alanine and glycogen content (see below). Pilot studies showed that earthworms could be considered alive if they responded to tactile stimuli, showed normal locomotive activity and had no visible signs of freezing damage, and this status could usually be scored within 2 h of thawing. To test for effects of time, untreated control worms were taken from 2°C after 0, 13 and 47 days. Each control sampling consisted of eight worms, which were quickly cleaned and frozen at –80°C for subsequent biochemical analysis.

Measurements of glucose, glycogen, lactate, alanine and succinate

Worms were taken from –80°C and immediately freeze dried for 24 h after which the dry mass of each individual was determined. Worms with a dry mass exceeding 10 mg were cut in pieces and randomly split between two Eppendorf tubes to allow for determination of glycogen and metabolites (glucose, lactate, alanine and succinate), respectively. Glycogen was not measured for worms smaller than 10 mg dry mass (dm). Glycogen was extracted for 3 h

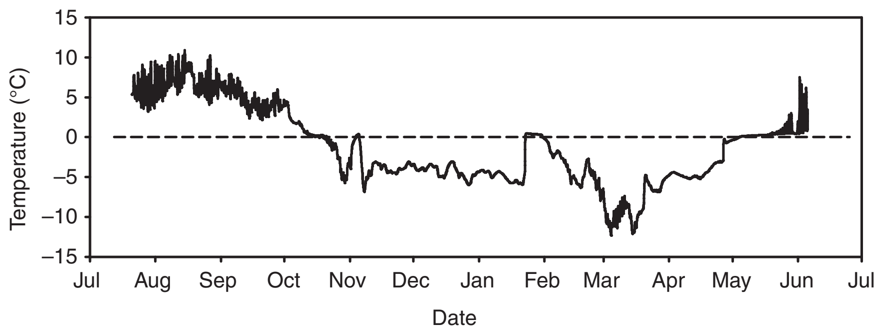

Fig. 1. Soil temperature at 5 cm depth, Disko Island, Greenland (July 2006–June 2007). Several adult worms survived the entire experimental period in field containers placed next to the temperature loggers. The brief increase to 0°C in late January is uncommon at Disko where the soil usually remains frozen for nearly 6 months.

at 80°C in 1 ml 1 mol l^{-1} NaOH after which the extract was stored at –80°C. Samples for glucose, lactate, succinate and alanine measurement were treated with 600 µl of 6% perchloric acid (PCA) and homogenized on ice using an Ultra-Turrax T8 Homogenizor (IKA-Werke Gmbh and Co. KG, Staufen, Germany). The samples were left on ice for 10 min and then slowly neutralized by the addition of 160 µl 2 mol l^{-1} K_2CO_3. The neutralised extract was left for 15 min on ice before the samples were centrifuged at 10,000 ***g*** for 10 min, after which the supernatant was stored at –80°C until further analysis.

Glucose and succinate were measured spectrophotometrically using standard kits (Glukose Gluc-DH, Diagnostic systems Gmbh, Holzheim, Germany and Succinic acid (succinate), Megazyme, Wicklow, Ireland). Alanine was measured spectrophotometrically according to the method of Lowry and Passonneau (Lowry and Passoneau, 1972) using a buffer solution consisting of Tris-HCl buffer (25 mmol l^{-1} Tris base, 25 mmol l^{-1} Tris-HCl), NADH (50–150 µmol l^{-1}), α-ketoglutarate (200 µmol l^{-1}), lactic dehydrogenase (2.0 µg ml^{-1}) and glutamic-pyrovic transaminase (100 µg ml^{-1}). Glycogen measurements were made by adding 100 µl of the glycogen extract to 900 µl acetate buffer (0.25 mol l^{-1}, pH 4.75) containing 40 mg l^{-1} amyloglucosidase (EC 3.2.1.3, Sigma-Aldrich Denmark A/S, Copenhagen). This solution was kept at 25°C for 2 h until the amyloglucosidase had cleaved all glycogen to glucose. Subsequently, glycogen content was determined using the same procedure as for glucose measurements. Concentrations of glucose, glycogen, alanine and succinate were all calculated relative to known standards that had been through the same extraction procedure as the samples.

Lactate was measured using a YSI 1500 SPORT lactate analyzer (YSI, Yellow Springs, Ohio, USA) which was calibrated with a lactate standard of 5.0 mmol l^{-1}.

Calorimetry

Calorimetry was used to assess cumulative anaerobic and aerobic metabolism for unfrozen worms at 10, 6 and 2°C and for frozen worms at –2°C. Frozen worms were placed at –2°C in a water bath in glass cells 6 days prior to the measurements. A small piece of ice was added to each cell to initiate freezing and ensure that the worms had been frozen prior to measurements of heat production. After each test the survival status was noted and only data for surviving worms were used. We applied a DSC 4207 (Hart Scientific, Provo, UT, USA) operated in the isothermal mode. Individual animals were placed in hermetically closed 700 ml steel cells mounted in the calorimeter and the heat flow was recorded over 3 h exposures to 10, 6, 2 and –2°C, respectively. The equipment had one reference cell and three cells for samples, and was therefore capable of simultaneous measurement of three individuals' heat production. The baseline offset was measured in separate trials using the same protocol and empty cells, and subtracted to quantify the heat production of the worm.

Statistical analysis

Effects of exposure time were based on linear regression analysis (SigmaStat 2.03) and an effect was considered significant at the $P<0.05$ level. Differences in metabolite levels between treatment groups (control, frozen survivors and frozen dead) were tested using a one-way ANOVA on Ranks and a *post-hoc* Dunn's test to separate groups that differed. All data are presented as mean ± s.e.m.

RESULTS

Field mortality during long-term frost exposure

Fig. 1 shows the temperature profile of the soil at a depth of 5 cm at Disko, Greenland. From this it is clear that continuous frost periods can last for at least 3 months and it should be mentioned that the brief thaw period observed in late January is a rare event at this location. Clearly, long-term frost is a recurring scenario for this northerly distributed earthworm species. Several of the worms that were placed in the containers next to the temperature logger survived the winter. Unfortunately most of the containers were raided by wild geese or foxes prior to collection and consequently it is impossible to assess a reliable survival estimate of the adult worms. Nevertheless these data clearly demonstrate that adult worms are able to survive extended periods of freezing while overwintering in the top soil layer.

Mortality and recovery in laboratory experiment

There was only a slight and insignificant increase in freeze mortality over the 47 day experimental freezing period (Fig. 2; $P=0.203$). Hence, 60–70% of the worms survived at the different times of collection. Recovery time was noted for the individual worms, but this did not change with the duration of frost exposure (data not shown). Thus, the average time to regain activity was approximately 60 min throughout the experimental period.

Metabolite concentrations in frozen and unfrozen worms

There were marked differences in glucose and glycogen between the control worms, worms surviving frost and worms that died during frost exposure. As seen in Table 1, control worms were characterised by high glycogen content and low concentrations of glucose. Both groups of frozen worms had the majority of their glycogen stores transformed to glucose. However, among the frozen worms, the survivors had a much higher average glucose concentration and glycogen content than the worms that did not survive freezing. Both groups of frozen worms had a tendency for increased levels of the anaerobic waste products lactate and alanine although this was not significantly different from control in all cases.

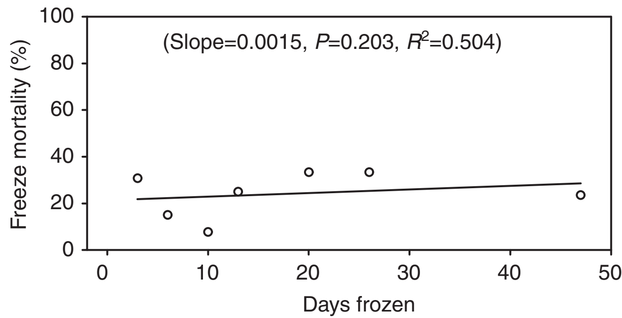

Fig. 2. Freeze mortality rate (%) of *D. octaedra* during exposure to frost for 3–47 days. Points represent average mortality at the individual time points (N=13–20). The line shows the linear regression of the entire data set.

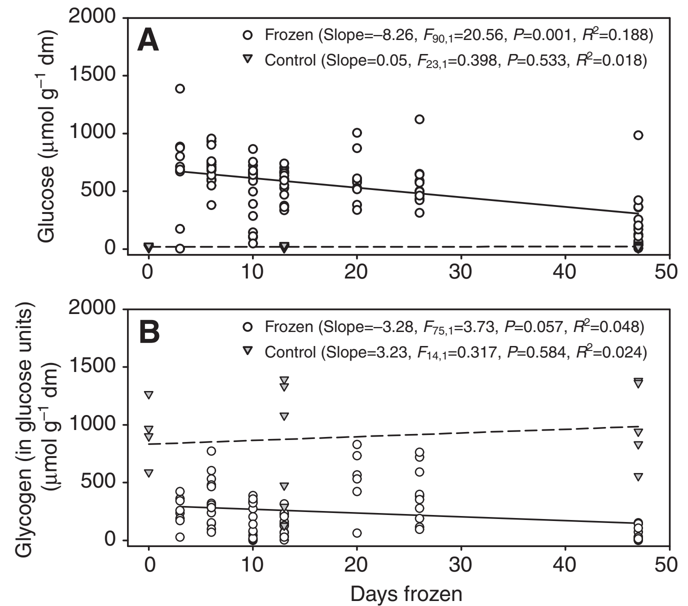

Fig. 3. Glucose (A) and glycogen (B) content in surviving *D. octaedra* during frost (solid line, white circles) and untreated control (dashed line and grey triangles). Dead worms were not included. Worms were frozen at –2°C for up to 47 days. Lines show the linear regression (slope and statistical significance are presented in the top right corner).

Temporal changes in metabolite levels during freezing

The metabolite concentrations reported in Table 1 are average values over the entire experimental period. However, in the surviving frozen worms, concentrations of the different metabolites change markedly over time whereas there were no significant changes in any of the metabolites measured in the untreated controls (Figs 3 and 4). In frozen worms that survived there was a highly significant decrease in glucose (Fig. 3A; $P<0.001$) and also a tendency for a reduction in glycogen content although this was not statistically significant (Fig. 3B; $P=0.057$). These changes were accompanied by increases in lactate (Fig. 4A; $P<0.001$) and alanine (Fig. 4B; $P<0.001$) but not succinate (Fig. 4C; $P=0.386$). On average the surviving worms metabolised more than half of their glucose reserves during the 47 days in a frozen state. Thus, average glucose concentrations of surviving earthworms was 124.1±24.3 µg mg^{-1} dm 3 days into the frost period and this was reduced to an average of only 49.1±13.4 µg mg^{-1} dm after 47 days of frost. The cumulative increase in alanine, lactate and succinate between day 3 and day 47 was from 4.0 to 10.1 µg mg^{-1} dm and this increase does, therefore, only account for 8.1% of the glucose decrease.

Temporal changes in metabolite levels in worms that died as a result of freezing were less clear (data not shown). Thus, dead frozen worms showed a significant temporal increase in alanine ($P=0.001$) while glucose ($P=0.203$), glycogen ($P=0.283$), lactate ($P=0.152$) and succinate ($P=0.727$) levels did not change significantly. However, it is possible that directional changes in metabolite levels are blurred by the fact that some worms had been dead for longer than others at the time of collection.

Table 1. Concentration of glucose, glycogen, lactate, alanine and succinate in unfrozen control worms and in worms that either survived or died during frost

	Control (µg mg^{-1} dm)	Survived after frost (µg mg^{-1} dm)	Dead after frost (µg mg^{-1} dm)
Glucose	3.6±0.3^{a} (24)	100.3±4.9^{b} (91)	25.7±6.4^{a} (28)
Glycogen	162.2±19.3^{a} (15)	44.4±4.4^{b} (75)	12.3±5.5^{c} (10)
Lactate	1.0±0.2^{a} (16)	1.7±0.1^{b} (90)	1.3±0.2^{a,b} (30)
Alanine	1.3±0.2^{a} (12)	5.9±0.4^{c} (84)	3.6 ±0.5^{b} (27)
Succinate	0.5±0.1^{a} (9)	0.5±0.1^{a} (25)	0.5±0.1^{a} (10)

Values are means ± s.e.m. Different letters indicate significant differences (one-way ANOVA).
The numbers in parentheses are the numbers of worms analysed.

Calculations of metabolic rate

The working hypothesis of this study was that the metabolism of frozen worms is entirely anaerobic. Using the declining slope of glucose concentration in frozen worms (Fig. 3A) we calculated the ATP production rate to be 2.6 µmol ATP g^{-1} wet mass (wm) day^{-1} (Fig. 5A) assuming a dry/wet mass ratio of 0.16 (personal observations) and that 1 mol glucose results in the generation of 2 mol ATP. If 1 mol glucose results in the formation of 6 mol ATP as would be expected from the formation of propionate (see Discussion), we calculate the ATP consumption rate to be 7.9 µmol ATP g^{-1} wm day^{-1}. Finally if it is assumed that only 8% of metabolism is anaerobic (corresponding with the observed increase in lactate and alanine) and that 92% is aerobic (giving 36 mol ATP mol^{-1} glucose) we estimate a metabolic rate of frozen worms to be 44 µmol ATP g^{-1} wm day^{-1}. These values are all lower than the value for unfrozen worms at –2°C estimated by simple Q_{10} effects, by factors of 90, 30 or 5.3, respectively (see Fig. 5A).

To put our calculated ATP consumption rates into context we estimated ATP consumption rates from prior respiration measurements of *D. octaedra* performed at 10 and 5°C by Uvarov and Byzova (Byzova, 1965; Uvarov, 1998). In these previous studies, oxygen consumption rates of *D. octaedra* were determined to be an average of 83.5 µl O_2 g^{-1} wm h^{-1} at 10°C and 59 µl O_2 g^{-1} wm h^{-1} at 5°C. Assuming that 1 mol O_2 generates 6 mol ATP these oxygen consumptions can be re-calculated to 536 and 379 µmol ATP g^{-1} wm day^{-1} at 10 and 5°C, respectively. Since *D. octaedra* does not show a critical thermal minimum and remains active until freezing we used the Q_{10} from these respiration measurements (Q_{10}=2.43) to estimate the aerobic metabolism of a supercooled worm at –2°C to be 233 µmol ATP g^{-1} wm day^{-1} (Fig. 5A) which is a value considerably higher than the estimates calculated from the glucose loss.

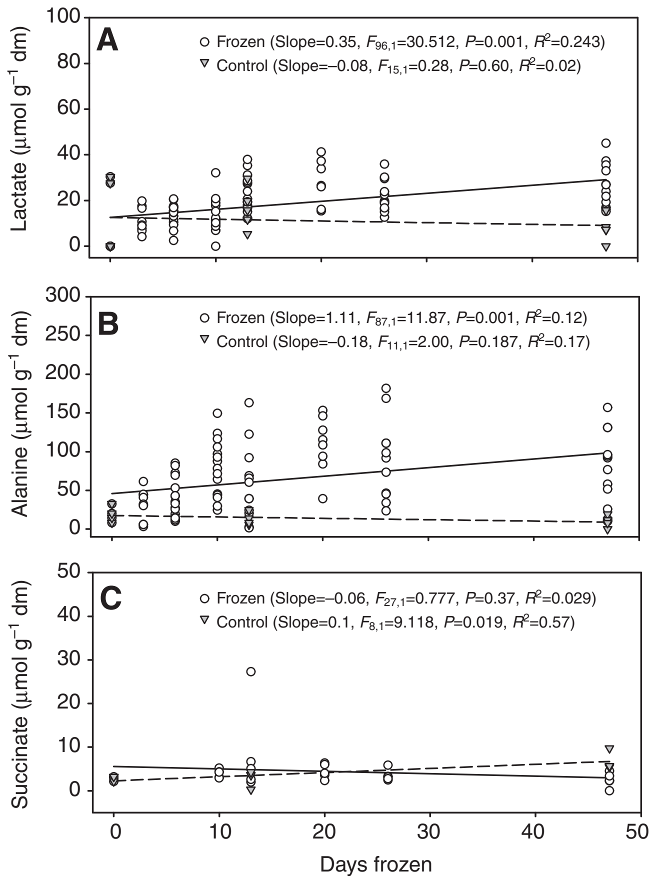

Fig. 4. Lactate (A), alanine (B) and succinate (C) in surviving *D. octaedra* during frost (solid line, white circles) and untreated control (dashed line and grey triangles). Dead worms were not included. Worms were frozen at –2°C for up to 47 days. Lines show the linear regression (slope and statistical significance are presented in the top right corner).

Calorimetry

In order to determine the metabolic rate of frozen worms we used calorimetry, which gives a direct measurement of the heat produced by metabolism. The calorimetric measurements were recalculated to ATP consumption rates using a conversion of 2500 kJ mol^{-1} glucose and 36 mol ATP mol^{-1} glucose (i.e. ~70 kJ/mol ATP). Measurements at 10, 6 and 2°C showed a decrease in metabolism with temperatures characterised by a Q_{10} of 3.76 (Fig. 5B). Using this Q_{10} we estimated a value for metabolism of a supercooled worm at –2°C to be 99 µmol ATP g^{-1} wm day^{-1}. The measured heat production of frozen worms at –2°C corresponded to 10.0±2.8 µmol ATP g^{-1} wm day^{-1} which is close to, but slightly higher, than the values calculated assuming exclusively anaerobic metabolism or mixed aerobic/anaerobic metabolism (compare Fig. 5A and 5B).

DISCUSSION

Is glucose an energy source or a cryoprotectant?

Freezing of the extracellular fluid in many animals will, in addition to cellular dehydration, impair the convective transport of extracellular body fluids impeding the exchange of respiratory gases, nutrients and waste products between the respiring cells and their surroundings. This is probably of particular relevance to animals such as amphibians, limpets and earthworms that rely on blood/haemolymph for cellular oxygen transport, whereas it is possible that tracheal oxygen and carbon dioxide exchange can sustain gas transport to some extent in frozen insects (Lundheim and Zachariassen, 1993; Irwin and Lee, 2002; Sinclair et al., 2004). In earthworm species such as *D. octaedra* and *E. nordenskioldi* it has previously been assumed that the rapid accumulation of cryoprotectants serves to limit the extent and speed of ice formation and hence reduce freeze-induced cell shrinking (Holmstrup and Zachariassen, 1996; Overgaard et al., 2007). However, it is also possible that glucose accumulation is important for distributing fermentable energy resources before long-term freezing events. Clearly this would help worms to maintain sufficient anaerobic ATP production rates during extended periods in a frozen state. This hypothesis is consistent with a number of observations. Generally, osmolyte/cryoprotectant systems have converged to include a fairly limited number of substances that are characterised by being compatible, even in high concentrations, with sustained cellular and protein function (Yancey et al., 1982). However, earthworms and amphibians are the only animal groups that utilize glucose as their primary cryoprotectant, although this osmolyte may have some potential disadvantages. Thus, insects, bacteria and plants often use osmolytes of lower molecular mass such as glycerol, betaine and TMAO (trimethylamine *N*-oxide), which increases the number of osmotically active particles, as compared to larger osmolytes such as glucose and trehalose (Yancey et al., 1982; Storey and Storey, 1996).

The reasons for using glucose could relate to the natural history of freeze-tolerance in earthworms and amphibians. Both these animal groups overwinter in or near the soil surface and they cannot be expected to supercool to any considerable extent because their integuments are permeable to inoculative freezing from the external environment. Another general difference from more exposed cold hardy animals is that freeze tolerant amphibians and earthworms will probably experience relatively stable subzero temperatures that will rarely drop below –10°C and consequently they will be expected to experience few but long periods of freezing at relatively high subzero temperatures constraining these animals to anaerobic ATP production over long periods (see Fig. 1 for an example of soil temperature profiles from one of the most northerly distributions of *D. octaedra*). Clearly these animals cannot fully exploit the thermal reduction in metabolic rate that more cold exposed animals can, nor can they rely on occasional thaw periods because of the thermal inertia of the frozen soil in which they reside. The results of our study strongly support the hypothesis that *D. octaedra* relies on anaerobic metabolism of the accumulated glucose, as we found a highly significant gradual decline in glucose levels during the 47 days that worms were frozen (Fig. 3). When the slope of decreasing glucose concentration was recalculated to ATP consumption (assuming that all metabolism was anaerobic), the estimated metabolic rate was very similar to that measured empirically by calorimetry (Fig. 5).

The finding that glucose may be important as a metabolic fuel does not diminish its role as a potentially important cryoprotectant/osmolyte. However, glucose accumulation may have evolved primarily to support metabolic needs during long-term freezing since the present study demonstrates that animals that died during the long-term freeze test were characterised by fourfold lower glucose and glycogen levels than those surviving (Table 1). This suggests that dead individuals succumbed because they had exhausted their energy reserves during freezing. Mortality could obviously also be linked to the lower levels of osmolytes/ cryoprotectants in these individuals, or alternatively low glucose

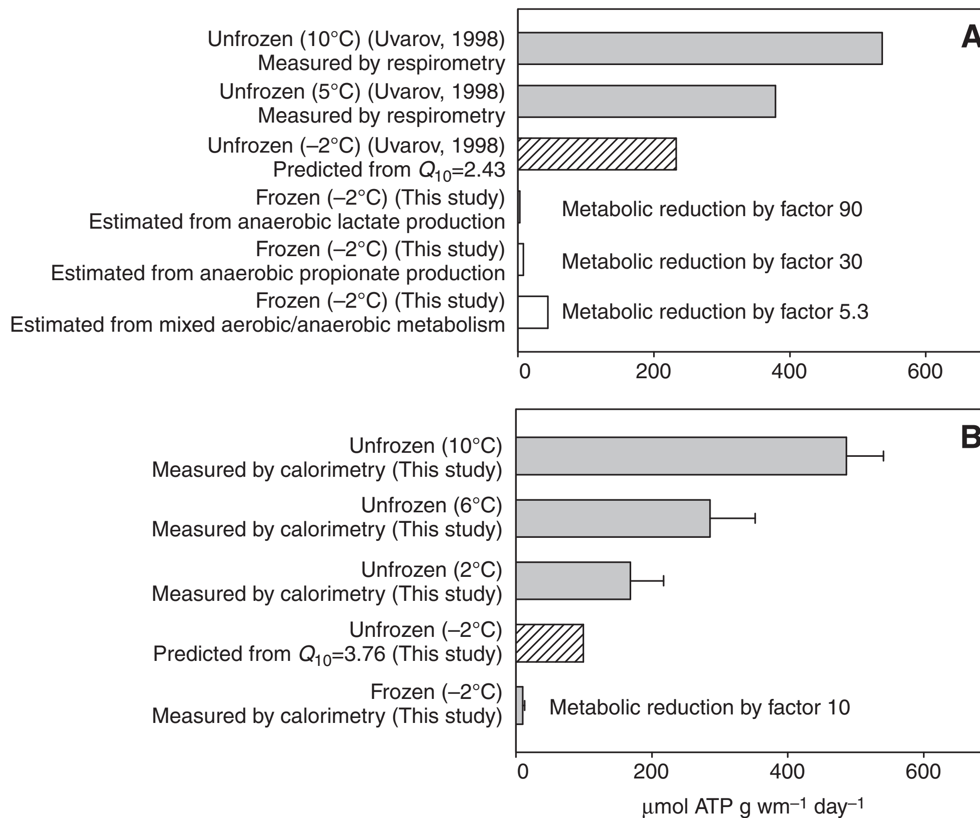

Fig. 5. Metabolic rate of frozen and unfrozen *D. octaedra* estimated from respirometry and production of anaerobic metabolites or from heat production. (A) ATP consumption rates at 10 and 5°C were calculated from previously published measurements of oxygen consumption rates (Uvarov, 1998) using the assumption that each mole of O_2 generates 6 ATP. The hatched bar depicts ATP consumption rate of unfrozen and aerobic worms at –2°C as estimated using a Q_{10} of 2.43 (from Uvarov's data). ATP consumption rate of frozen *D. octaedra* at –2°C are calculated from the temporal decrease in glucose shown in Fig. 3 using different assumptions regarding the proportion of anaerobic/aerobic metabolism and different assumptions of ATP yield per glucose molecule (see text for further explanation). (B) Metabolic rate of frozen and unfrozen *D. octaedra* measured directly by calorimetry. The hatched bar depicts ATP consumption rate of unfrozen and aerobic worms at –2°C as estimated using a Q_{10} of 3.76 (from calorimetric data).

levels could be a result of post-mortem microbial respiration. However, previous studies using short-term freeze tests did not show mortality linked to individual glucose levels of *D. octaedra* (Overgaard et al., 2007), and glucose levels are always highly variable between individuals of *D. octaedra*, indicating that the low levels found in dead worms are not an artefact of microbial respiration. Further support for this notion comes from the vague interdependence of glucose accumulation and freeze survival in *D. octaedra* populations from the northern hemisphere (Holmstrup et al., 2007) and among different freeze-tolerant earthworm species (Holmstrup and Overgaard, 2007). Similarly, there was no clear relationship between the ability to survive freezing and glucose accumulation when eight species of hatchling freshwater turtles were compared with respect to freeze tolerance (Costanzo et al., 2006). Indeed, freeze-tolerant reptiles generally accumulate much lower levels of putative cryoprotectants than freeze-tolerant anurans, insects or earthworms, but freeze-tolerant reptiles are also only able to survive fairly short episodes of freezing temperatures (Storey, 1990; Storey, 2006). It seems possible, therefore, that the level of glucose accumulation in general may be more important for the ability to survive freezing over long periods than for the actual absence/presence of freeze tolerance in freeze-tolerant animals.

Anaerobic metabolism in frozen *D. octaedra*

In general, earthworms survive without oxygen for several hours or even days at normal summer temperatures (Gruner and Zebe, 1978). Under these conditions, energy will be generated by anaerobic metabolism, although with poor efficiency, where the end product can include a variety of compounds such as succinate, lactate, alanine, valeric acid, propionate or acetate (Hochachka et al., 1973; Gruner and Zebe, 1978; Pörtner et al., 1984; Loomis et al., 1989; Bundy et al., 2003).

It is often found that anoxia tolerance is an important physiological trait favouring freeze tolerance and the working hypothesis of this study was that metabolism of frozen *D. octaedra* is completely anaerobic. However the evidence for facultative anaerobic metabolism in this study is confusing. Thus, being frozen for 47 days at –2°C resulted in a small accumulation of the anaerobic metabolic waste products, alanine and lactate, in *D. octaedra* (Fig. 4). This increase only accounted for 8% of the decrease in glucose levels and additional screenings of the entire metabolome by use of ^{1}H-NMR spectroscopy [as described by Malmendal et al. (Malmendal et al., 2006)] showed no other obvious candidates for anaerobic waste products in the extracts of frozen worms (data not shown). Although this may suggest that worms were aerobic we found that the metabolic rate measured by calorimetry was lower than that calculated from the glucose loss assuming a primarily aerobic metabolism. Metabolic rates were only slightly higher than the values calculated assuming that metabolism was exclusively anaerobic (Fig. 5A) and the most probable explanation for this discrepancy must be that the metabolism of frozen worms was a mixture of aerobic and anaerobic metabolism. The anaerobic contribution to total metabolism may be underestimated if only lactate and alanine are taken into account as it is possible that additional waste products are lost from the earthworm tissues, either in the frozen state during the experiment or during the sample preparation. A number of facultatively anaerobic invertebrates including several annelid species have the biochemical capacity to metabolise succinate further to propionate during anoxia (Surholt, 1977; Gruner and Zebe, 1978; Pörtner et al., 1984). Production of propionate as the primary anaerobic waste product has previously been demonstrated in different earthworm species such as *Lumbricus terrestris* and *L. rubellus* (Gruner and Zebe, 1978) and it is possible that anaerobic *D. octaedra* produce propionate under frozen

conditions. The use of this metabolic pathway could favour long-term anaerobic survival since the ATP yield per mole of glucose is higher (Pörtner et al., 1984) and it is possible that we missed this metabolite in our NMR measurements since propionate is a highly volatile fatty acid. It has been shown that close to 90% of this anaerobic waste product was lost to the surrounding water when the marine annelid *Arenicola marina* was maintained anoxic for 48 h (Surholt, 1977).

In this study the levels of anaerobic waste products were similar in surviving and dead worms, and mortality was, therefore, not related to the accumulation of anaerobic waste products (Table 1). In fact, previous studies have shown that the accumulation of some waste products such as alanine may have a cryoprotective role (Loomis et al., 1989; Kukal et al., 1991). However, further study of the biochemical pattern of anaerobic metabolism in *D. octaedra* and in particular on the putative origin and fate of the metabolic end products and their associated protons are needed to clarify the potential effects of continued anaerobic metabolism. Thus, in vertebrates it seems clear that the ability to tolerate long-term anoxia is tightly linked to the handling of the ensuing acid load (Lutz and Nilsson, 1997; Jackson, 2002).

Metabolic depression and duration of freeze-tolerance in *D. octaedra*

The calorimetrically based estimates of ATP consumption rates generally corresponded well with or were slightly lower than those calculated under oxygenated and unfrozen conditions (Uvarov, 1998) (compare Fig. 5A and 5B). The differences that there were may be partially attributed to differences between the populations studied by Uvarov (Uvarov, 1998) and us, and/or experimental setup. Furthermore, the calculated estimates are obviously very sensitive to the assumptions chosen to calculate ATP turnover rate.

A major objective of measuring metabolic rate was to assess if a metabolic depression occurred during freezing in *D. octaedra* and we conclusively found this to be the case. Thus, regardless of the assumptions of the mode of metabolism it seems clear that the worms undergo a marked metabolic depression when frozen (see Fig. 5A). Furthermore, our results generally support the hypothesis that metabolism of frozen worms is anaerobic although it is possible that there is a slight contribution of aerobic metabolism. Thus, to arrive at the same value as that measured calorimetrically it must be assumed that 84–94% of the glucose is metabolised anaerobically and only 6–16% aerobically, depending on the assumptions regarding the anaerobic metabolic pathway used. This assumption is in accordance with observations from the freeze-tolerant lizard *Lacerta vivipara* where oxidative metabolism persists, albeit at a lowered rate, even after ice equilibrium has been reached (Voituron et al., 2002b). Furthermore, different insect species also maintain some degree of oxidative metabolism during extracellular freezing (Irwin and Lee, 2002; Sinclair et al., 2004) and our results could suggest that frozen *D. octaedra* may also be able to maintain a slight exchange of respiratory gasses and waste products. However, this must be dependent on the proportion of frozen body water, which at –2°C can be estimated to be vary between 40 and 80% depending on the magnitude of the glucose accumulation (Overgaard et al., 2007). Moreover, it is possible that free-ranging earthworms become trapped in frozen soil which would rapidly bring the oxygen supply to a minimum as has been reported for other freeze-tolerant invertebrates (Scholander et al., 1953; Conradi-Larsen and Sømme, 1973; Sømme and Conradi-Larsen, 1977).

Animals across a wide range of phyla lower their metabolic turnover when stressed by various environmental factors and annelids have also previously been shown to undergo metabolic depression when aestivating or when exposed to low temperature and anoxia (Guppy and Withers, 1999). Considering that frozen *D. octaedra* undergo severe cellular hypoxia and dehydration when frozen it is not unexpected that these worms also undergo metabolic depression under these conditions. With regard to metabolic depression it is generally assumed that freeze-tolerant animals have lower costs for maintenance of metabolic functions than animals using a supercooling strategy (Voituron et al., 2002a), however, to our knowledge no previous study has estimated the long-term metabolic rate in frozen annelids and records are generally scarce regarding long-term studies of freeze-tolerant animals. In the present study, the most reliable estimate of metabolic rate is that measured by calorimetry since this measures the heat of metabolism directly without bias regarding assumptions of the metabolic pathway. Using this method we found an estimated 90% reduction of metabolism in frozen worms, which is well within the 'normal' rate of metabolic depression (Guppy and Withers, 1999) but slightly larger than the 50 to 70% depression found in the frozen insects *Eurosta solidaginis* and *Upis ceramboides,* respectively (Lundheim and Zachariassen, 1993; Irwin and Lee, 2002). A large decrease in metabolic rate was also found in the sub-Antarctic caterpillar *Pringleophaga marioni* (Sinclair et al., 2004), however, in this species the metabolic depression was related to the critical thermal minimum rather than the process of freezing and it was suggested that metabolic depression is linked with reduced Na^+/K^+ pump activity and failure to maintain ion gradients.

In *D. octaedra* metabolic depression is probably a result of several different mechanisms including reduced activity and the presumed reduction in energy used on growth, reproduction, digestion and excretion. Metabolic depression is also generally associated with a number of biochemical changes including decreased pH, protein phosphorylation and prioritization of some cellular function (Na^+/K^+ pump) over others (protein synthesis) (Hand and Hardewig, 1996; Guppy and Withers, 1999; Cowan and Storey, 2003). Although frozen *D. octaedra* may conform to these general trends, a preliminary study found that extracellular $[K^+]$ increased markedly following frost exposure (Marie Rohde, personal communication) suggesting a possible reduction in Na^+/K^+ pump activity. Other freeze-tolerant animals, such as the larvae of *E. solidaginis,* have also been shown to suppress Na^+/K^+ pump activity during winter acclimation (McMullen and Storey, 2008), and it was also hypothesised that Na^+/K^+ pump activity becomes insufficient to maintain ion balance in the freeze-tolerant caterpillar *P. marioni* (Sinclair et al., 2004). It is, therefore, possible that a reduction in Na^+/K^+ pump activity also contributes to the metabolic depression of *D. octaedra*.

Most worms survived the 47 day freeze exposure used in this experiment and given the estimated metabolism of 8.3 μmol glucose g^{-1} dm day^{-1} (Fig. 3) the 'average' worm would be able to survive for 83 days before glucose supplies were exhausted. This may be sufficient to survive winter in the southerly distribution ranges of *D. octaedra.* However, as illustrated in Fig. 1, winter may be considerably longer for northerly distributed populations. *D. octaedra* exhibit a large variation between individuals in the amount of glucose accumulated and also large variation in the magnitude of the glycogen stores of autumn-collected worms (Rasmussen and Holmstrup, 2002; Holmstrup et al., 2007; Overgaard et al., 2007). Such variance may naturally affect the projected survival time as some individuals may have glucose levels of ~1100 μmol glucose g^{-1} dm, and glycogen levels prior to frost have in a few cases been measured at levels higher than

1500 μmol glucosyl units g^{-1} dm. Populations with large glycogen reserves, such as those found in Greenland, Finland, Poland and Canada, may also be more tolerant to long-term freezing than populations from Denmark and Sweden, which have smaller glycogen stores (Holmstrup et al., 2007). The projected survival time will also depend on external conditions such as temperature where a low soil temperature will decrease the use of glucose reserves, and therefore possibly extend the duration of freeze survival.

In conclusion, the main result of our study indicate a novel role of glucose in survival of long-term freezing, namely that glucose usage as fuel for metabolism is perhaps the most important function. Our results therefore could be the opening of a new view on the roles of so-called cryoprotectants for winter survival.

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