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Cryoprotective and osmotic responses to cold acclimation and freezing in freeze-tolerant and freeze-intolerant earthworms

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Abstract In this paper we present the results of physiological responses to winter acclimation and tissue freezing in a freeze-tolerant Siberian earthworm, *Eisenia nordenskioeldi*, and two freeze-intolerant, temperate earthworm species, *Lumbricus rubellus* and *Aporrectodea caliginosa*. By analysing the physiological responses to freezing of both types we sought to identify some key factors promoting freeze tolerance in earthworms. Winter acclimation was followed by a significant increase in osmolality of body fluids in *E. nordenskioeldi*, from 197 mosmol kg^{-1} in 10 °C-acclimated animals to 365 mosmol kg^{-1} in animals acclimated to 0 °C. Cold acclimation did not cause any change in body fluid osmolality in the two freeze-intolerant species. As a response to ice formation in the body, the freeze-intolerant species produced copious amounts of slime and expulsion of coelomic fluids, and thereby lost 10–30% of their total water content. Contrary to this, the freeze-tolerant species did not lose water upon freezing. At temperatures down to −6.5 °C, the ice content in the freeze-tolerant *E. nordenskioeldi* was significantly lower than in *L. rubellus*. At lower temperatures there were no differences in ice content between the two species. Cold acclimated, but unfrozen, specimens of all three species had low levels of ammonia, urea, lactate, glycerol and glucose. As a response to ice formation, glucose levels significantly increased within the first 24 h of freezing. This was most pronounced in *E. nordenskioeldi* where a 153-fold increase of glucose was seen (94 mmol $\cdot l^{-1}$). In *L. rubellus* and *A. caliginosa* a 19-fold and 17-fold increase in glucose was seen. This is the first study on physiological mechanisms promoting freeze tolerance in *E. nordenskioeldi*, or any other oligochaete. Our results suggest that the cryoprotective system of this species more closely resembles that of freeze-tolerant anurans, which synthesize cryoprotectants only after tissues begin to freeze, than that of cold-hardy invertebrates which exhibit a preparatory accumulation of cryoprotectants during seasonal exposure to low temperature.

Key words Freeze tolerance · Earthworms · *Eisenia nordenskioeldi* · Cryoprotectants · Glucose

Abbreviations *DW* dry weight · *FW* fresh weight · *MP* melting point

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Introduction

Cold-hardy ectothermic animals have developed two different strategies for survival at temperatures below the melting point (MP) of the animal's body fluids (Zachariassen 1985; Lee 1991; Costanzo and Lee 1995). The freeze-avoiding species, which die if frozen, survive exposure to cold either by supercooling or by lowering their body fluid MP to ambient temperature. In animals with high integumental permeability, the equilibration of body fluid MP with ambient temperature may result from dehydration (Holmstrup and Zachariassen 1996). Another strategy is employed by freeze-tolerant species that are able to tolerate freezing of their extracellular body fluids. Most earthworms are freeze-intolerant organisms (Lee 1985; Holmstrup and Zachariassen 1996). At present only one species, the Siberian earthworm *Eisenia nordenskioeldi* Eisen, is known to be freeze-tolerant (Berman and Leirikh 1985; Holmstrup and Petersen 1997). The physiological mechanisms promoting freeze tolerance in *E. nordenskioeldi*, or any other oligochaete, have not previously been investigated. In the present paper we present results on physiological responses to winter acclimation and tissue freezing of the

freeze-tolerant earthworm *E. nordenskioeldi* and two species of freeze-intolerant earthworms. In this comparative study we have investigated four main responses: (1) changes in water status and body fluid osmolality during acclimation to winter temperatures, (2) amount of ice formed in the animals in relation to temperature, (3) behavioural responses to freezing, and (4) accumulation of cryprotectants as a response to acclimation and freezing.

E. nordenskioeldi is widely distributed in the Asiatic part of Russia, extending to the northern subarctic tundra regions of Siberia (Perel 1979). These areas have continuous permafrost which means that the species cannot migrate to deeper unfrozen layers and therefore is confined to soils that are entirely frozen for a large part of the year. Even though earthworms under dry conditions have moderate supercooling capacity, it is unlikely that *E. nordenskioeldi* in the subarctic can avoid freezing during winter due to inoculative freezing from surrounding ice in the soil (Holmstrup and Zachariassen 1996). Freeze tolerance is therefore necessary for its existence in these areas. The two freeze-intolerant species, *Lumbricus rubellus* Hoffmeister and *Aporrectodea caliginosa* (var. *tuberculata*) Savigny, are cosmopolitan species found in a variety of temperate habitats. The upper soil layers in temperate areas may frequently freeze during winters but earthworms of these regions avoid frost by migrating to deeper, unfrozen soil layers (Rundgren 1975). The earthworms we studied may occupy the same habitats in Eastern European regions (Perel 1979). They belong to the same family (Lumbricidae), are of similar size and have, in the main, the same morphology and anatomy. For these reasons they are judged appropriate models in a comparative study. By analysing the physiological responses to freezing of both types we sought to identify some key factors promoting freeze tolerance in earthworms.

Materials and methods

Animals and acclimation protocols

E. nordenskioeldi was sampled from the northern shore of Taimyr Lake, Taimyr Peninsula, Siberia (74°N, 105°E) and bred at 15 °C in a Danish loam soil with cow dung as food. The *E. nordenskioeldi* used in the experiments were adults and large juveniles (second generation individuals) and had a mean fresh weight (FW) of 405 mg (range: 110–700 mg). Adult and large juvenile individuals of *L. rubellus* were obtained from a local fish-bait dealer (Oxford, Ohio, USA) and had a mean FW of 480 mg (range: 280–960 mg). Adult and large juvenile individuals of *A. caliginosa* (var. *tuberculata*) were collected in December 1997, from an agricultural soil in the vicinity of Oxford, Ohio. The mean FW of *A. caliginosa* used in the experiments was 445 mg (range: 200–1300 mg).

The test animals were kept in the moist soil in which they had been bred or from which they had been collected. They were divided into three experimental groups representing summer active individuals (10 °C), autumn active animals (0 °C) and winter estivating animals (0 °C). The two former groups were acclimated for at least 10 days at the respective temperatures. Only *E. nordenskioeldi* entered estivation. This occurred after 20–30 days at 0 °C during which period the worm excavated a soil cell in which it then coiled and entered quiescence. The experimentation was done during winter months.

Osmometry and water content determination

Osmolality of body fluids was determined by a psychrometric vapour pressure depression technique using Wescor C-52 sample chambers connected to a Wescor HR 33 T Dew Point Microvoltmeter operated in the dew point mode (Wescor, Logan, Utah, USA). The accuracy of this technique exceeds 10 mosmol kg^{-1}. Body fluids were sampled by excising the anterior 3–5 segments of the worm and cutting the body wall dorso-longitudinally to expose the body cavity (coelom) of the anterior 15–20 segments. Body fluids were then sampled by blotting them with filter paper discs that were fitted to the sample holder of the C-52 sample chamber. For each animal, 1–3 samples were taken (depending on its size) and the mean value of these measurements was used to represent the animal's body fluid osmolality. During dissection care was taken not to puncture the gut. The term, "body fluids", therefore refers to a mixture of blood and coelomic fluids but not gut fluids.

Water content was measured by lightly removing surface moisture from the worm with filter paper, weighing the specimen to the nearest 0.1 mg, drying it at 60 °C for 24 h, and determining the dry weight (DW).

Determination of ice content

Ice content of frozen worms (*E. nordenskioeldi*: 0 °C, estivating; *L. rubellus* and *A. caliginosa*: 0 °C, active) was determined with a slightly modifidied calorimetric method previously described by Lee and Lewis (1985; and references therein). The calorimetry system consisted of a 300 cm^3 thermos in which was placed a 10-ml plastic cup containing approximately 7 ml water continuously stirred by a magnetic stirrer. The change in water temperature was measured using a thermistor (CELS, World Precision Instruments, New Haven, Conn., USA) interfaced to a MacLab/8 data acquisition system. With this system temperature changes could be accurately determined to the nearest 0.02 °C.

Animals were encaged between two 4-ml conical plastic cups gently squeezed together and cooled to −1.5 °C. Freezing was initiated by a brief spray of quick-freeze applied to the outside of the plastic cup. The freezing of animals was documented by a clear freezing exotherm measured by a 36-gauge thermocouple in contact with the encaged specimen. After nucleation the animals were cooled to the desired temperature (cooling rate approximately 1 °C h^{-1}). Because worms during cooling and freezing would often eject coelomic fluids through dorsal pores and secrete significant amounts of slime, it was necessary to correct for this to obtain the actual fresh mass of the frozen animal. This was done by weighing the container with the animal before cooling and subtracting the weight of the empty container (with ejected body fluids) after the frozen animal had been removed for ice content measurement. The water content and DW was determined for each specimen used in the calorimetric experiments and used in the calculation of ice content.

The frozen animals were quickly transferred from the cooling vessel to the calorimeter and the temperature change during warming and melting was recorded until the temperature of the calorimeter became constant. The amount of ice in the animals was calculated from the change in temperature of the water in the calorimeter as described by Lee and Lewis (1985). The calorimeter constant (F) was determined by melting known amounts of ice in the calorimeter cup; F values ranged from 1.02 to 1.07 (depending on mass of ice) but a fixed value of 1.05 was chosen for all calculations. The specific heat of dry earthworm tissues was experimentally determined to be 0.30 ± 0.04 cal g^{-1} $°C^{-1}$ (mean ± SD; n = 4).

Loss of body fluids due to freezing

Loss of ejected coelomic fluids and secretion of slime during freezing was determined for cold-acclimated worms (*E. nor-*

denskioeldi: 0 °C, estivating; *L. rubellus* and *A. caliginosa*: 0 °C, active). Initial FW was determined whereafter the worms were frozen at −1.5 °C for 10 h or 34 h following the freeze protocol described above. After thawing at 5°C (for approximately 10 min), the specimen was carefully blotted with filter paper and weighed, followed by drying and determination of DW. The water loss due to freezing was calculated as the weight of lost water in relation to the initial weight of body water. Since water loss could also be a reaction simply to encaging, the results were compared to water loss of animals kept under similar circumstances at 0 °C.

Metabolite analyses

We analysed worms for changes in certain metabolites associated with acclimation to 10 °C and 0 °C, and with freezing at −1.5 °C for 24 h. Worms were gently blotted on filter paper to remove excess surface moisture and any soil particles, weighed to the nearest 1.0 mg, and homogenized in ice-cold $HClO_4$ (7%, v/v). Deproteinized extracts were prepared by centrifuging the homogenate (14,000 *g*, 5 min) and neutralizing the supernatant with KOH. Neutralized extracts were stored at −80 °C until assayed for metabolite concentrations.

Urea nitrogen and ammonia concentrations were measured using a urease/nitroprusside method (Sigma no. 640). Enzymatic assays were used to measure concentrations of glucose (Sigma no.510), glycerol (Sigma no. 337), and lactate (Sigma no. 735) in the extracts. Owing to pronounced variation in tissue water contents of worms in different treatment groups and among species, metabolite concentrations were expressed as μmol g^{-1} DW.

Results

Water content and body fluid osmolality

For *E. nordenskioeldi*, acclimation to cold caused a slight increase in water content, estivating individuals having a significantly higher water content than active animals at 10 °C (Table 1). The same trend was seen for *A. caliginosa* where a large difference in water content was recorded between worms acclimated at 10 °C and 0 °C. It was observed that active worms (10 °C) of these two species had guts filled with ingested soil, whereas animals acclimated at 0 °C had empty guts. The differences in water content were therefore, for the most part, due to filled/empty guts and probably not so much due to a true physiological shift in water content. *L. rubellus* differed in this respect, and no increase in water content was observed with acclimation to low temperature. This species was kept in a compost medium, and the worms did not have a normal feeding behaviour, their guts having only traces of soil within them.

The body fluid osmolality of *E. nordenskioeldi* increased during cold acclimation from 197 ± 27 mosmol kg^{-1} in 10 °C (active specimens) to 365 ± 65 mosmol kg^{-1} in 0 °C (estivating specimens) (Table 1). Even acclimation at 0°C for only 14 days, at which time the worms had not yet entered estivation, caused a significant increase in osmolality to 272 ± 24 mosmol kg^{-1}. In *A. caliginosa* and *L. rubellus* a slight, but not statistically significant, increase in osmolality was observed during cold acclimation. There was no difference in osmolality between warm-acclimated *E. nordenskioeldi* and *L. rubellus*, whereas the osmolality of *A. caliginosa* was significantly lower than in the other species (ANOVA/Tukey; $P < 0.05$). Among the animals acclimated at 0 °C, active *E. nordenskioeldi* had a significantly higher osmolality than both *L. rubellus* and *A. caliginosa* (ANOVA/Tukey; $P < 0.05$). Again, *A. caliginosa* had the lowest osmolality of the three species (Anova/Tukey; $P < 0.05$).

Behavioural responses to cold acclimation and freezing

Acclimation at 0 °C caused *E. nordenskioeldi* to enter estivation within 20–30 days, whereas the other species did not become quiescent, even after acclimation at this temperature for more than 30 days. Estivation in *E. nordenskioeldi* comprised excavation of a small soil cell and a coiling of the worm. In containers having more than one specimen, several individuals were sometimes observed to estivate in the same soil cell, tightly coiled together. However, handling of the worms in connection with the experiments immediately caused them to resume activity. Cold-acclimated *L. rubellus* and *A. caliginosa* stopped feeding and burrowing but other responses were not observed.

When their body fluids froze, a typical response of the earthworms was the (sometimes violent) secretion of mucous and apparent expulsion of coelomic fluids through the dorsal pores. However, the three species responded very differently to freezing. *A. caliginosa* lost about 30% of its body water when frozen at −1.5 °C under the experimental conditions (Table 2). A control

Table 1 Mean (± SD) water content and body fluid osmolality of active and estivating earthworms. Number of observations is indicated in parentheses. Within columns, mean values identified by different letters were statistically distinguishable (ANOVA/Tukey; $P < 0.05$)

State/treatment	Water content (g g^{-1} dry mass)			Body fluid osmolality (mOsm)		
	Eisenia nordenskioeldi	*Lumbricus rubellus*	*Aporrectodea caliginosa*	*E. nordenskioeldi*	*L. rubellus*	*A. caliginosa*
Active/10 °C	2.58 ± 0.27 (5)[a]	4.90 ± 0.37 (6)[a]	2.23 ± 0.29 (6)[a]	197 ± 27 (6)[a]	191 ± 31 (6)[a]	137 ± 19 (6)[a]
Active/0 °C	3.28 ± 0.75 (5)[a,b]	4.37 ± 0.20 (4)[b]	4.06 ± 0.61 (7)[b]	272 ± 24 (5)[b]	212 ± 22 (8)[a]	167 ± 31 (8)[a]
Estivating/0 °C	3.64 ± 0.41 (9)[b]	–	–	365 ± 65 (11)[c]	–	–
Frozen/−1.5 °C	–	–	–	448 ± 37 (6)[d]	–	–

Table 2 Mean (±SD) amount of body water (percent of initial total water) lost during freezing at −1.5 °C of earthworms acclimated at 0 °C. Number of observations is indicated in parentheses. Within columns, mean values identified by different letters were statistically distinguishable (ANOVA/Tukey; $P < 0.05$)

Treatment	Water loss (percent of initial body water)		
	E. nordenskioeldi	*L. rubellus*	*A. caliginosa*
0 °C/10 h	4.1 ± 1.1 (4)[a]	6.2 ± 1.0 (4)[a,b]	14.2 ± 4.6 (4)[a]
0 °C/34 h	2.7 ± 2.3 (4)[a]	4.4 ± 0.9 (4)[a]	12.6 ± 1.4 (4)[a]
Freezing at −1.5 °C/10 h	3.3 ± 1.0 (4)[a]	9.8 ± 4.3 (4)[b]	31.0 ± 5.6 (4)[b]
Freezing at −1.5 °C/34 h	2.7 ± 0.8 (4)[a]	11.0 ± 2.8 (4)[b]	28.3 ± 4.5 (4)[b]

group of this species kept at 0 °C in the same vials as used in freeze experiments lost about 14% of the initial body water, significantly less than when worms were frozen. *L. rubellus* frozen individuals also lost significantly more water than unfrozen control animals encaged similarly (Table 2). In contrast, estivating *E. nordenskioeldi* showed no increase in water loss due to freezing over that due to confinement, and the water loss (3%) of frozen animals was much lower than in the other two species (ANOVA/Tukey; $P < 0.05$).

Viability of the animals used in the ice content experiments and similar supplementary experiments was checked after 24 h of recovery at 5 °C. *E. nordenskioeldi* in general had a high survival rate down to −10 °C (Table 3). At −14.5 °C all animals eventually died, but a few reacted to tactile stimuli after thawing, even though severe tissue damage was observed (swellings, oedema, epidermal ruptures). It should be noted that viability measurements were based on animals that were rapidly thawed at 20 °C in the calorimeter which may underestimate true survival in nature. Survival after freezing was never observed in *L. rubellus* or *A. caliginosa*, not even after freezing at −1.5 °C and subsequent slow thawing at 5 °C.

Ice content

Ice content of individuals frozen at various temperatures was determined for *E. nordenskioeldi* and *L. rubellus* only. In *A. caliginosa* the excretion of mucus and expulsion of body fluids was so copious that this species, for practical reasons, was not suitable for such measurements.

The ice contents at −1.5 °C of *E. nordenskioeldi* and *L. rubellus* were approximately 30% and 45% of the total water content, respectively (Fig. 1). When the data were grouped with respect to period of freezing (<15 h; between 15 h and 25 h; >25 h) a statistical analysis showed that there was no change in ice content during the experimental period (ANOVA/Tukey; $P > 0.05$) for either of the species. The equilibrium ice content at −1.5 °C was therefore apparently reached within 10 h after nucleation.

The influence of temperature on ice content is shown in Fig. 2. At −1.5 °C, −2.5 °C and −4.1 °C the ice content of *E. nordenskioeldi* was significantly lower than that of *L. rubellus* (ANOVA/Tukey; $P < 0.05$). At the lower temperatures (−6.5 °C and −10.0 °C) there was no difference in ice content between the two species. The ice content at −6.5 °C was about 65% of the total water content for both species and lower temperatures did not cause any measurable increase of the ice content.

Table 3 Percent viability of earthworms frozen at various temperatures for periods between 10 h and 40 h. Number of observations is indicated in parentheses

Temperature (°C)	Viability (%)		
	E. nordenskioeldi	*L. rubellus*	*A. caliginosa*
−1.5	92 (12)	0 (14)	0 (14)
−2.5	25 (4)	0 (4)	–
−4.1	100 (6)	0 (6)	–
−6.5	63 (8)	0 (4)	–
−10.0	75 (8)	0 (4)	–
−14.5	0 (11)*	–	–

* Three specimens reacted to tactile stimuli after thawing but ultimately succumbed

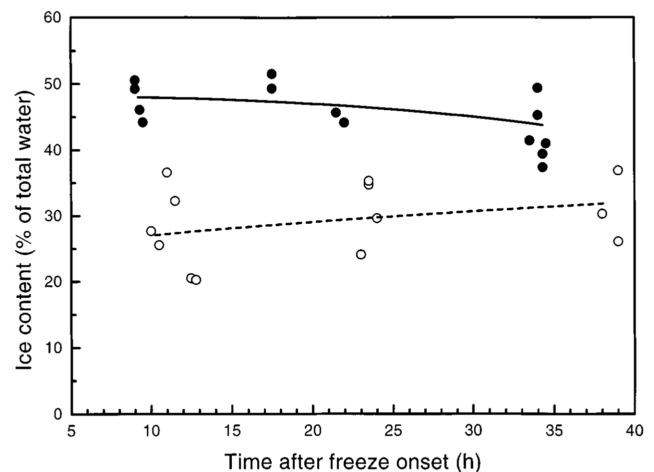

Fig. 1 The ice content of individual *Eisenia nordenskioeldi* (*open circles*) and *Lumbricus rubellus* (*closed circles*) frozen at −1.5 °C for different periods of time

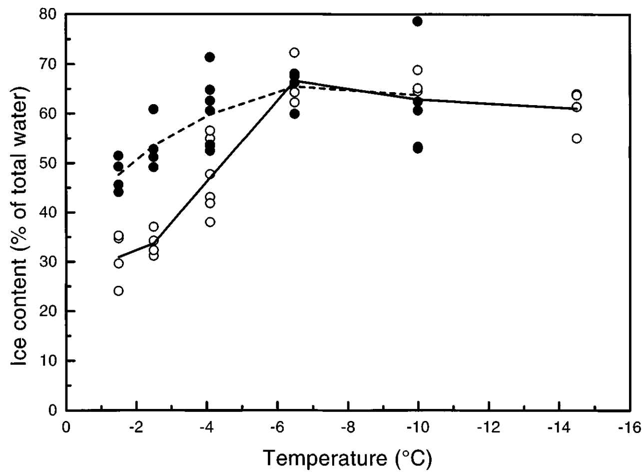

Fig. 2 The ice content of individual *E. nordenskioeldi* (*open circles*) and *L. rubellus* (*closed circles*) in relation to temperature. The *unbroken line* (*E. nordenskioeldi*) and *dashed line* (*L. rubellus*) indicate the mean ice content. The individuals were held at the respective temperature for 24 h before the ice content was determined

Metabolite concentrations

Ammonia and urea concentrations were compared among worms acclimated to 10 °C, acclimated to 0 °C, and frozen at −1.5 °C for 24 h following acclimation to 0 °C (Table 4). Acclimation temperature had no effect on concentrations of either ammonia or urea in *E. nordenskioeldi* and *A. caliginosa*. Warm-acclimated *L. rubellus* had ammonia concentrations that were approximately two-fold greater than those of cold-acclimated conspecifics, and five- to eight-fold greater than ammonia levels in warm-acclimated *E. nordenskioeldi* and *A. caliginosa*. Comparisons between active and estivating *E. nordenskioeldi* acclimated to 0 °C revealed that, whereas no differences occurred in ammonia concentrations, estivating specimens accumulated a small quantity of urea. Freezing exposure did not alter concentrations of ammonia or urea in any species (Table 4). Urea levels were low or imperceptible in all species, although concentrations of 2–3 μmol g^{-1} DW were measured in *L. rubellus*. Thus, ammonia was the principal excretory metabolite in these species.

Except for *A. caliginosa*, which contained slightly more glycerol when exposed to cold, acclimation to 10 °C or 0 °C did not influence glucose or glycerol concentrations in any species (Fig. 3). Worms generally maintained higher levels of glycerol (6–15 μmol g^{-1} DW) than glucose (1–3 μmol g^{-1} DW). Active *E. nordenskioeldi* acclimated to 0 °C had concentrations of glucose (2.6 ± 0.2 μmol g^{-1} DW; $n = 5$) and glycerol (9.3 ± 0.9 μmol·g^{-1} DW; $n = 5$) that were indistinguishable (ANOVA/Tukey; $P > 0.05$) from those of estivating *E. nordenskioeldi* (Fig. 3). Freezing exposure (−1.5 °C for 24 h) caused an increase in glucose concentration in all three species; however, the 153-fold increase in *E. nordenskioeldi* (to 413 ± 77 μmol g^{-1} DW) was markedly greater than that in *L. rubellus* (19 fold) and *A. caliginosa* (17 fold). Glycerol also accumulated in

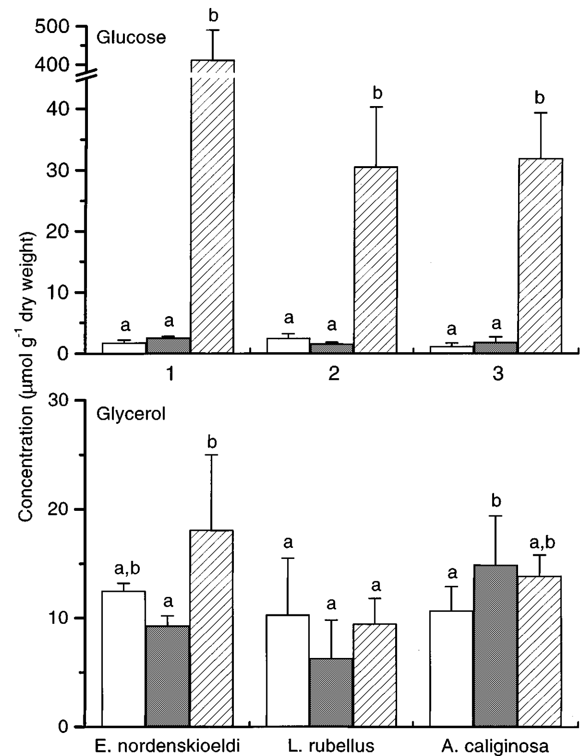

Fig. 3 Mean (±SD) concentrations of glucose and glycerol in *E. nordenskioeldi*, *L. rubellus*, and *A. caliginosa* acclimated to 10 °C (*white columns*) or 0 °C (*black columns*), or frozen at −1.5 °C for 24 h (*hatched columns*). Worms used in freezing trials were acclimated to 0 °C; specimens of *E. nordenskioeldi* were estivating. Sizes of the samples are as given in Table 4. Within a species, mean values identified by different letters were statistically distinguishable (ANOVA/Tukey; $P < 0.05$)

Table 4 Mean (±SD) concentrations of ammonia and urea in earthworms exposed to various thermal regimens. Number of observations is indicated in parentheses

State/treatment	Ammonia (μmol g^{-1} dry mass)			Urea (μmol g^{-1} dry mass)		
	E. nordenskioeldi	*L. rubellus*	*A. caliginosa*	*E. nordenskioeldi*	*L. rubellus*	*A. caliginosa*
Active/10 °C	9.2 ± 5.3 (6)[a]	47.9 ± 21.3 (8)[a]	5.8 ± 1.6 (8)[a]	0.8 ± 0.5 (6)[a]	3.4 ± 1.8 (8)[a]	0.2 ± 0.2 (8)[a]
Active/0 °C	15.3 ± 2.7 (5)[a]	21.2 ± 14.4 (8)[b]	7.8 ± 4.9 (11)[a]	0.0 ± 0.0 (5)[a]	2.7 ± 2.1 (8)[a]	0.3 ± 0.3 (11)[a]
Estivating/0 °C	13.3 ± 4.6 (8)[a]	–	–	3.0 ± 1.4 (8)[b]	–	–
Frozen/−1.5 °C	9.8 ± 5.1 (8)[a]	17.6 ± 4.6 (5)[b]	6.5 ± 1.5 (5)[a]	0.5 ± 0.4 (8)[a]	2.3 ± 1.5 (5)[a]	0.2 ± 0.2 (5)[a]

E. nordenskioeldi during freezing, although to much lower levels than glucose. No change in glycerol concentration accompanied freezing in either *L. rubellus* or *A. caliginosa* (Fig. 3).

Lactate concentrations in worms acclimated to 0 °C or 10 °C were 5–10 μmol g^{-1} DW (Fig. 4). Cold-acclimated *L. rubellus* had lactate concentrations 50% lower than those of warm-acclimated conspecifics; however, acclimation temperature generally had no effect on lactate level. Estivating *E. nordenskioeldi* had lactate levels that were indistinguishable (ANOVA/Tukey; $P > 0.05$) from those of active conspecifics acclimated to 0 °C (6.3 ± 0.9 μmol g^{-1} DW; $n = 5$), and from those of estivating conspecifics exposed to freezing (Fig. 4). In contrast, freezing caused a marked increase in lactate concentration of *L. rubellus* (3.5 fold) and *A. caliginosa* (1.7 fold).

Discussion

Cold acclimation of *E. nordenskioeldi* was associated with a marked increase in osmolality of the body fluids in both active and estivating specimens. Development of an estival state in cold-acclimated worms was associated with an additional osmotic increase of about 93 mosmol kg^{-1}, but was without change in hydration state (Table 1). Thus, preparation for overwintering in *E. nordenskioeldi* apparently involves an accumulation of osmotically active solutes, which in many cold-hardy ectothermic animals reflects changes in the concentrations of low-molecular weight carbohydrates, principal inorganic ions, amino acids, or excretory metabolites (Storey and Storey 1988; Loomis 1991). Osmolality of body fluids of temperate earthworms are reported to be in the range 120–180 mosmol kg^{-1} (Ramsay 1949; Holmstrup and Zachariassen 1996). In this respect *E. nordenskioeldi* differs markedly from previously studied species. We did not identify the additional osmolytes in cold-acclimated *E. nordenskioeldi*, although our results suggest that they were neither glucose nor glycerol.

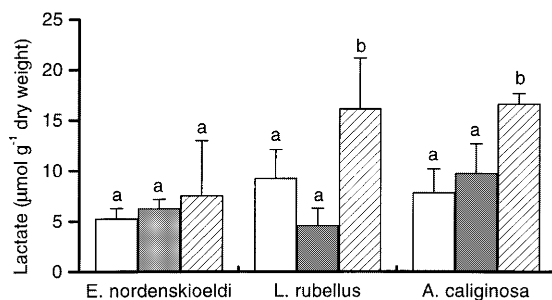

Fig. 4 Mean (±SD) concentrations of lactate in *E. nordenskioeldi*, *L. rubellus*, and *A. caliginosa* acclimated to 10 °C (*white columns*) or 0 °C (*black columns*), or frozen at −1.5 °C for 24 h (*hatched columns*). Worms used in freezing trials were acclimated to 0 °C; specimens of *E. nordenskioeldi* were estivating. Sizes of the samples are as given in Table 4. Within a species, mean values identified by different letters were statistically distinguishable (ANOVA/Tukey; $P < 0.05$)

Estivating *E. nordenskioeldi* accumulated urea, which was not detected in the tissues of active worms kept at the same temperature (Table 4), although urea concentrations in body fluids were relatively low (≤1.4 mmol·l^{-1}) and thus contributed little to the total osmotic pressure. The observed osmotic increase in estivating *E. nordenskioeldi* may enable this species to better resist desiccation during estivation under low environmental water potentials. On the other hand, in autumn where soils are usually moist, such a high osmotic pressure should cause an increased influx of water through the integument, requiring increased energy consumption to avoid salt losses in excreted urine (Holmstrup and Zachariassen 1996). In the Subarctic, however, the animal will be frozen for much of the winter, therefore water influx for the hyperosmotic worm is only a problem during the estivating stage until the animal freezes. No change in osmolality occurred during cold acclimation in *L. rubellus* or *A. caliginosa*, and the osmolality of these species remained in the range that are normally found for earthworms. In our laboratory study cold acclimation did not induce estivation in *A. caliginosa*. However, in the field this species may enter winter estivation, whereas *L. rubellus* does not (Nordstrøm 1975).

Seasonal changes in body water content and levels of some metabolites were studied in field-acclimatized *E. nordenskioeldi*, in Siberia, by Berman and Leirikh (1985). By sampling worms in late August, early October, and early March, these authors found that glycogen reserves were highest at the onset of winter (205 μmol g^{-1} wet mass) and were reduced by approximately 95% within 5 months. They also found, as we did, that the concentration of glycerol was four-fold higher than that of glucose in cold-acclimated, but unfrozen, worms (October sample), suggesting that glycerol is the predominant blood sugar. However, the levels of glycerol (13.4 mmol·l^{-1}) and glucose (3.4 mmol·l^{-1}) they measured in body fluids of field-acclimatized *E. nordenskioeldi* were about 5.5-fold higher than those measured in our study (2.5 mmol·l^{-1} and 0.6 mmol·l^{-1}, respectively).

Accumulation of glucose and, to a lesser extent, glycerol in *E. nordenskioeldi* was triggered by the freezing of its tissues rather than acclimation to low temperature, per se (Fig. 3). Thus, the cryoprotective system of this species more closely resembles that of freeze-tolerant vertebrates, which synthesize cryoprotectant only after tissues begin to freeze (Schmid 1982; Storey and Storey 1985a, b), than that of the cold-hardy invertebrates (Storey and Storey 1988; Lee 1991) and fishes (e.g., Umminger 1969; Raymond and Driedzic 1997) which exhibit preparatory accumulation of low-molecular-weight carbohydrates during seasonal exposure to low temperature. Deferring cryoprotectant synthesis to the moment of tissue freezing obviates the energetic expense of mobilizing cryoprotectant, and the potential for loss of energy-rich compounds from the body, that would be unnecessary so long as body

temperatures remain above freezing. This system also eliminates the osmoregulatory burden of maintaining body fluids hypertonic to the environment, an especially important benefit to annelids, amphibians, and other animals that have highly permeable integuments (Holmstrup and Zachariassen 1996).

Our laboratory evidence that freezing triggers mobilization of glucose and glycerol in *E. nordenskioeldi* is bolstered by the findings of Berman and Leirikh (1985), who noted that concentrations of these metabolites in free-ranging worms were elevated (9.8- and 3.6-fold, respectively) in late winter (i.e., after frost exposure) relative to autumn. Synthesis of these carbohydrates were likely to be supported by catabolism of glycogen reserves, which were much reduced in the winter sample. In some freeze-tolerant animals, freezing-induced stimulation of the key enzymes regulating glycogenolysis results in the rapid production of glucose and/or glycerol (Storey and Storey 1985a, b). Our finding that freezing exposure of *E. nordenskioeldi* raised the concentration of glucose to a greater extent than that of glycerol concurs with observations of Berman and Leirikh (1985), although concentrations of these metabolites in body fluids were much higher in our frozen specimens, averaging 94 $mmol \cdot l^{-1}$ for glucose (range 67–131 $mmol \cdot l^{-1}$) and 4 $mmol \cdot l^{-1}$ for glycerol (range 2–7 $mmol \cdot l^{-1}$). Accumulation of glucose and glycerol during freezing wholly accounts for the concomitant increase in osmolality (Table 1), indicating that no other major cryoprotectants were mobilized. Glucose, rather than glycerol, appears to be the primary cryoprotectant in *E. nordenskioeldi* even though glycerol is maintained at higher basal levels in unfrozen animals.

Although our data unequivocally attest that glucose and glycerol are mobilized during freezing, we must infer the cryoprotective role of these compounds in *E. nordenskioeldi* from studies with other organisms. This is a reasonable inference given the demonstrated efficacy of both glucose and glycerol in protecting against freezing injury at the whole-animal level of organization (Costanzo et al. 1991, 1993; Layne 1999). Berman and Leirikh (1985) stated that the concentrations of these agents (about 82 $mmol \cdot l^{-1}$, combined) in winter *E. nordenskioeldi* would be insufficient to ensure ‘‘resistance to freezing’’, apparently in reference to the colligative reduction in tissue freezing point. However, cryoprotectants may function in a non-colligative manner to preserve the integrity of membranes and macromolecules in frozen tissues (Mazur 1984; Karow 1991). Glucose is an unusual cryoprotectant among invertebrates (Lee 1991; Loomis 1991), although this compound promotes supercooling in certain fishes (Umminger 1969) and freeze tolerance in some anurans (Schmid 1982; Storey and Storey 1985b; Costanzo et al. 1993) and reptiles (Churchill and Storey 1992a, b; Costanzo et al. 1995). Blood glucose in fully frozen wood frogs (*Rana sylvatica*) may approach 250 $mmol \cdot l^{-1}$ (Storey and Storey 1985b), although other animals that tolerate freezing produce much lower concentrations of glucose (e.g., Churchill and Storey 1992a). In our study, glucose levels averaging 413 $\mu mol\ g^{-1}$ DW (94 $mmol \cdot l^{-1}$ body fluid) apparently provided protection against freezing damage in some specimens of *E. nordenskioeldi* exposed to temperatures as low as −10 °C (Table 4). Whether higher concentrations of glucose and glycerol may be achieved during longer periods of freezing or during slower freezing remains to be determined. It should be stressed that the experiments undertaken in the present study were not designed to assess freeze tolerance in terms of survival. Previous studies show that *E. nordenskioeldi* is able to survive freezing at temperatures down to −30 °C for months when the worms are exposed in soil at natural cooling and thawing rates (Berman and Leirikh 1985; Holmstrup and Petersen 1997).

Freezing caused a marked increase in the concentration of glucose (but not glycerol) in freeze-intolerant *L. rubellus* and *A. caliginosa*, although the magnitude of the increase was substantially lower than that in *E. nordenskioeldi*. Such a pattern suggests that glycaemia is a fundamental response to stress that has been enhanced in *E. nordenskioeldi* to promote freeze tolerance. This hypothesis, which has been offered to explain similar findings among freeze-tolerant and intolerant anurans (Costanzo et al. 1993) and reptiles (Churchill and Storey 1992b), provides an intriguing mechanism by which cryoprotectant systems of freeze-tolerant animals may evolve.

Another clue to the cryoprotective system used by *E. nordenskioeldi* is implicit in the observations of Berman and Leirikh (1985), who noted that much of the ice forming within the body was located within the coelom. This arrangement is reminiscent of the case with *Rana sylvatica*, in which about 25% of the ice forming during moderate freezing exposure becomes sequestered within the coelom and lymphatic spaces (Lee and Costanzo 1998). Water that ultimately freezes in these compartments originates in various tissues and organs, which may dehydrate by > 50% during freezing. Extraorgan ice sequestration may reduce cryoinjury associated with physical disruption of tissues by ice and may concentrate cryoprotectant in the remaining tissue fluids (Lee and Costanzo 1998). Our results clearly demonstrate that the high osmolality of body fluids of cold-acclimated and frozen *E. nordenskioeldi* (Table 1) resulted in a much lower ice content than observed for *L. rubellus*, at least at high subzero temperatures (Figs. 1, 2). The ability to keep the ice content low during the first winter freeze may allow *E. nordenskioeldi* to synthesize and distribute cryoprotectants before the ice content reaches its maximum (about 65% of total water) at temperatures around −6 °C (Fig. 2). This reaction much resembles the situation in freezing wood frogs (*R. sylvatica*) where glucose is synthezised in the liver and distributed to other tissues and organs before the ice content reaches its maximum (Costanzo et al. 1992). Similarly, it may be speculated that, in *E. nordenskioeldi*, glucose is synthesized in the chloragogen tissue. This tissue is always closely associ-

ated with blood vessels and capillaries, contains large glycogen reserves, and is thought to have a liver-like function (Laverack 1963).

We detected no differences in lactate concentrations between warm- and cold-acclimated *E. nordenskioeldi*, suggesting that this metabolite plays no role in the development of the estival state or cold hardening in this species. During winter, some intertidal invertebrates accumulate lactate which may confer some degree of cold hardening, as this compound has cryoprotective properties (Loomis 1991). We also detected no significant change in lactate levels in the tissues of *E. nordenskioeldi* exposed to freezing. Because little oxygen is available to cells of frozen tissues, energy production in frozen animals is based on stored phosphagens and anaerobic glycolysis. Consequently, the fermentation products lactate and alanine gradually accumulate during freezing exposure, potentially leading to metabolic perturbations (Storey and Storey 1988). Our finding that lactate levels in *E. nordenskioeldi* had not yet increased after 24 h of freezing at −1.5 °C suggests that the low ice content at this temperature did not cause anoxic conditions. In contrast, freeze-intolerant *L. rubellus* and *A. caliginosa* showed marked increases in lactate concentration during an identical exposure, probably due to the high ice content.

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