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RESEARCH ARTICLE

Soil salinity increases survival of freezing in the enchytraeid *Enchytraeus albidus*

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SUMMARY

Enchytraeus albidus **is a freeze-tolerant enchytraeid found in diverse habitats, ranging from supralittoral to terrestrial and spanning temperate to arctic regions. Its freeze tolerance is well known but the effect of salinity in this strategy is still poorly understood. We therefore studied the combined effect of salinity (0, 15, 35, 50‰ NaCl) and sub-zero temperatures (−5, −14, −20°C) on the freeze tolerance of** ***E. albidus*** **collected from two distinct geographical regions (Greenland and Germany). A full factorial design was used to study survival, and physiological and biochemical end points. The effect of salinity on the reproduction of German** ***E. albidus*** **was also assessed. Exposure for 48 h to saline soils prior to cold exposure triggered an increase in osmolality and decrease in water content. Worms exposed to saline soils had an improved survival of freezing compared to worms frozen in non-saline soils, particularly at −20°C (survival more than doubled). Differential scanning calorimetry measurements showed that the fraction of water frozen at −5 and −14°C was lower in worms exposed to 35‰ NaCl than in control worms. The lowering of ice content by exposure to saline soils was probably the main explanation for the better freeze survival in saline-exposed worms. Glucose increased with decreasing temperature, but was lower in saline than in non-saline soils. Thus, glucose accumulation patterns did not explain differences in freeze survival. Overall, the physiological responses to freezing of** ***E. albidus*** **from Greenland and Germany were similar after exposure to saline soils. Soil salinity up to 30‰ improved reproduction by a factor of** ***ca.*** **10.**

Key words: ice content, freeze tolerance, osmolality, cryoprotectants, glucose.

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INTRODUCTION

Enchytraeids (Oligochaeta), commonly known as potworms, form a large and widely distributed group of saprophagous organisms, which inhabit the litter layer and the upper mineral soil of many terrestrial and supralittoral ecosystems (Didden, 1993; Giere, 2006). They play important functions, namely as soil structure promoters, in the decomposition of dead organic matter and nutrient mobilization (Petersen and Luxton, 1982; Didden, 1993). *Enchytraeus albidus* Henle 1837 can be found in organic matter-rich soils as well as in decaying seaweed at the supralittoral zone along the coast, e.g. in Germany and Greenland (Christensen and Dózsa-Farkas, 2006; Giere, 2006). This species shows a large physiological tolerance range to salinity (Kähler, 1970; Generlich and Giere, 1996) and freezing temperatures (Slotsbo et al., 2008) of its habitat.

Cold hardiness in enchytraeids originating from temperate or arctic environments has been reported in several studies (Sømme and Birkemoe, 1997; Block and Bauer, 2000; Bauer et al., 2001; Holmstrup and Sjursen, 2001; Holmstrup et al., 2002a; Pedersen and Holmstrup, 2003; Christensen and Dózsa-Farkas, 2006; Slotsbo et al., 2008). When ambient temperatures decrease below the melting point of the body fluids, enchytraeids, like any other ectothermic animal, face the risks associated with the freezing of body fluids (Zachariassen, 1985; Ramløv, 2000). As enchytraeids are small hygrophilic soil organisms with a high cuticular permeability for water, they are not likely to use supercooling as a cold tolerance strategy. Instead, two other cold tolerance strategies have been adopted by enchytraeids, namely cryoprotective dehydration (Sømme and Birkemoe, 1997; Pedersen and Holmstrup, 2003) and freeze tolerance after inoculative freezing of body fluids (Sømme and Birkemoe, 1997; Slotsbo et al., 2008). Cryoprotective dehydration and freeze tolerance are assisted by the accumulation of cryoprotectants such as glycerol, sorbitol and trehalose as well as free amino acids (Zachariassen, 1985; Storey, 1997; Holmstrup et al., 2002b). Enchytraeids accumulate glucose as a cryoprotectant, probably because it is the main blood sugar of oligochaetes (Holmstrup et al., 1999; Slotsbo et al., 2008), but glucose may also serve as an energy resource in the frozen organism (Calderon et al., 2009). The accumulation of cryoprotectants lowers the melting point, and with that the ice fraction at a given temperature, and dilutes the concentration of potentially toxic solutes (e.g. salts) in the unfrozen body fluids (Zachariassen, 1985; Ramløv, 2000).

With regard to the effects of salinity on enchytraeids, the published data has mainly been related to survival and osmoregulatory capacity. These studies show that *E. albidus* can rapidly adjust body fluid osmolality to changes in environmental salinity and remain hyperosmotic at salinities up to about 25‰; at higher salinities the worm osmoconforms (Kähler, 1970; Schöne, 1971; Generlich and Giere, 1996). A study by Schöne reported that reproduction can occur under full-strength seawater salinity but did not indicate an optimal salinity for fitness (Schöne, 1971).

Physiological mechanisms in cold or salinity tolerance of enchytraeids have been examined in several studies (Schöne, 1971; Block and Bauer, 2000; Bauer et al., 2001; Generlich and Giere, 1996; Sømme and Birkemoe, 1997; Pedersen and Holmstrup, 2003; Owojori et al., 2009; Slotsbo et al., 2008), but very little information is available on the combined effect of cold and salinity and the interactions between these two factors. One study has addressed this topic in a short-term experiment, indicating that acclimation to low temperature interacts with salinity in *E. albidus* (Kähler, 1970).

In the present study, we exposed *E. albidus* from two populations (Greenland and Germany) to a range of ecologically relevant salinities and low temperatures in a full-factorial experimental design. We assessed survival, glucose and glycogen levels, osmolality, supercooling point and estimated the ice content. Additionally, the effects of salinity on reproduction were assessed for the German population.

MATERIALS AND METHODS

Test species

Enchytraeus albidus (Oligochaete: Enchytraeidae) from Germany were obtained from a commercial supplier (Büchner Zierfischfutter, Jena, Germany; coordinates: 51°51′N, 9°50′E). These worms were originally collected from garden compost, and cultured for several years in the laboratory in agricultural (loamy) soil at 20.0±1°C and fed weekly with rolled oats mixed with dried and crushed macroalgae (predominantly *Fucus* spp., collected near Aarhus, Denmark). Worms from Greenland (Kobbefjord, about 20 km from Nuuk; coordinates: 64°8′N, 51°23′W) were collected in 2010 from decaying seaweed near the sea shore and kept in the laboratory at 5°C in agricultural soil (as used for German worms) for about 1 year prior to experiments. Before the experiments began, the organisms were cold acclimated at 5°C for 6 weeks and then at 2°C for 1 week.

Test soil and salt spiking procedure

All experiments were conducted with the natural standard soil LUFA 2.2 (Speyer, Germany). In short, this soil has *ca*. 6% clay, 17% silt, 77% sand and 4.4% organic matter. The pH ($CaCl_2$) of LUFA soil is 5.5. This soil is within the optimum range of pH in natural soils where *E. albidus* are found (Jänsch et al., 2005).

Salt spiking was performed using NaCl (99.5% purity, Merck, Darmstadt, Germany), added as aqueous solution to the dry soil. Soil water content was 22 ml 100 g^{-1} dry soil, which is equivalent to 50% of the water-holding capacity. For the survival test we used the following NaCl concentrations: 0‰, 15‰, 35‰ and 50‰ NaCl. For the reproduction test the concentration range was 0‰, 2‰, 4‰, 6‰, 8‰, 10‰, 20‰, 30‰ and 40‰. The NaCl-spiked soil was transferred to 1 l cylindrical plastic vessels and allowed to equilibrate for 1 day before being used in tests.

Experimental setup and survival assays

Each replicate consisted of a test vial (3 cm height, 2 cm diameter) containing 5 g of test soil and 15 mg oatmeal. Five worms per replicate were used. The vials were covered with a perforated lid to allow ventilation. For survival assessment, five replicates were used; for physiological and biochemical measurements, three to six additional replicates were prepared.

Vials with worms were kept at 2°C for 48 h, after which the vials were transferred to −2.0±0.2°C (a subset of vials was kept at 2°C as non-frozen controls). Once at −2°C, an ice crystal was added after 6 h to induce freezing of the soil water. This procedure has been shown to ensure inoculative freezing of enchytraeids once the temperature becomes lower than the body fluid melting point (Slotsbo et al., 2008). After 24 h at −2°C, when the soil was frozen (verified by visual inspection), the vials were transferred to programmable cooling cabinets in which temperature was gradually lowered by 3°C day^{-1} (0.125°C h^{-1}) until it reached −5, −14 or −20°C depending on the required treatment/sampling. Worms were kept at their target temperature until 2 days after the coldest cabinet had reached its final temperature (−20°C). Hence, each group remained at sub-zero temperatures for 9 days. Mortality was assessed 1 day after thawing at 5°C. Only the worms that reacted normally to tactile stimuli and showed no freezing damage (deformations and rupture of the skin) were scored as surviving.

Water content, osmolality and supercooling point measurements

Fresh and dry mass, water content, osmolality and supercooling point were determined for worms that were acclimated to 2°C for 48 h, i.e. just before exposure to sub-zero temperature. The water content of individual enchytraeids was calculated from measurements of fresh mass and of dry mass after drying at 60°C for 24 h (N=6) using a ±1 μg accuracy scale (Sartorius AG, Goettingen, Germany). The body fluid osmolality of single individuals was measured by placing an enchytraeid in a sample holder, quickly crushing it with a pestle in order to expose the body fluids, and then placing it in a Wescor C-52 sample chamber connected to a Wescor HR 33 T Dew Point Microvoltmeter (Wescor, Logan, UT, USA) operated in the dew point mode (N=6). Soil water osmolality was also measured by quickly filling the bottom of the sample holder with soil particles and following the same measurement procedure as for enchytraeids (N=3). Melting point was calculated using the osmolal melting point depression constant (1.86°C $osmol^{-1}$ kg water). The supercooling point was measured using copper–constantan thermocouples as described elsewhere (Pedersen and Holmstrup, 2003). The worms were gently surface dried with filter paper and carefully attached to a thermocouple with adhesive tape. The cooling rate was ~1°C min^{-1}. Supercooling points of N=8–14 individuals from each population and treatment were determined.

Quantification of glycogen and glucose

In order to assess the glycogen reserves and glucose concentration after acclimation at +2°C in test soil for 48 h (but before exposure to sub-zero temperature; day 0), five samples of three representative worms were taken from both control soil and treated soils. Glucose concentration was also determined for frozen worms (−5, −14 and −20°C). The sampling of frozen worms was carried out during the frost exposure, on days 2, 3, 6 and 8. Another sampling was made at the end of the freezing exposure, on day 9, at all target temperatures (+2, −5, −14 and −20°C). Groups of 15 worms were quickly thawed with deionized water and cleaned of excess soil; three organisms were pooled per sample in Eppendorf tubes, snap-frozen in liquid nitrogen and thereafter kept at −80°C until analysis. Because it was not possible to discriminate between dead and alive worms after rapid thawing, glucose measurements were (for some treatments) based on a mixture of surviving and dead worms. Glucose and glycogen analysis was carried out as previously described (Overgaard et al., 2007) using spectrophotometrically based enzymatic test kits (Gluc-DH FS from DiaSys Diagnostic Systems, Holzheim, Germany).

Direct measurement of ice content

Ice content (fraction of water that was frozen) was measured using differential scanning calorimetry (DSC). Because of the

extensiveness and complexity of our experiment, we only measured ice content in a subset of the treatments: worms acclimated to two salinities (0‰ and 35‰ NaCl), and frozen at two target temperatures (−5 and −14°C).

Before DSC analysis, the worms were quickly and gently cleaned with filter paper pre-moistened with distilled water and weighed to determine fresh mass (Mettler Toledo, Lisbon, Portugal; accuracy ±10 μg). Each worm was then placed in a hermetically sealed 50 μl aluminium test-pan and the combined mass of the worm and pan was determined (total fresh mass). Thermal analyses of whole worms were conducted using a DSC4000 calorimeter (Perkin Elmer, Waltham, MA, USA) as described previously (Koštál et al., 2012). For measurement of ice content at −5°C the following temperature program was used: (i) hold for 1 min at 10°C; (ii) cool to −20°C at a rate of 5°C min^{-1}; (iii) hold for 5 min at −20°C; (iv) heat to −5°C at a rate of 5°C min^{-1}; (v) hold at −5°C for 30 min; and (vi) heat to 5°C at a rate of 1°C min^{-1} (N=9). For measurement of ice content at −14°C, the same temperature program was followed, except in for steps iv–vi: (iv) heat to −14°C at a rate of 5°C min^{-1}; (v) hold at −14°C for 30 min; and (vi) heat to 5°C at a rate of 1°C min^{-1} (N=4).

After thermal analysis, the test-pans were perforated to allow the worms to be dried for 3 days at 60°C. Test-pans containing dried worms were weighed (total dry mass). The amount of frozen water was calculated from the area under the melt endotherm using the value of 334.5 J g^{-1} for the enthalpy of water. The amount of unfrozen water was determined by subtracting the mass of frozen water from the total water mass (calculated from the difference of total fresh mass and total dry mass). The relative amount of total osmotically active water (OAW) and total osmotically inactive water (OIW, or 'bound water') was determined as described elsewhere (Holmstrup and Westh, 1994) by cooling worms to −70°C at a rate of 10°C min^{-1}, holding them here for 30 min and then heating them to 30°C at a rate of 5°C min^{-1} (N=5 for each population).

Estimation of ice content

The relative ice content of frozen worms was estimated at each combination of sub-zero temperature (−5, −14, −20°C) and salinity (0‰, 15‰, 35‰ and 50‰), by using the values of melting point and water content (measured before freezing) and glucose concentrations (measured after freezing). These values were randomly paired before calculations, leading to five replicated estimates of ice fraction. The osmotic contribution of glucose was based on the assumption that all glucose is osmotically active, resulting in 1 mol l^{-1} glucose being equivalent to ~1 osmol kg^{-1}, and that 60% of the worm's water content could be regarded as OAW (see Results). The melting point depression of accumulated glucose was calculated using the osmolal melting point depression constant, and added to the measured melting point of unfrozen individuals. The ice fraction, F, at a given temperature was calculated according to the formula: F=1–(MP/T), where MP is the melting point and T is ambient temperature (Zachariassen and Husby, 1982).

Reproduction test

The test was performed according to the standardized guideline for the enchytraeid reproduction test used in ecotoxicological studies (ISO, 2004; OECD, 2004), with minor adjustments. Because of the large number of worms required for testing and in order to spare the natural population from Greenland, only worms from Germany were used.

In short, eight adult worms with well-developed clitellum were introduced into glass vessels (50 ml) containing 25 g of test soil (moistened to 50% of the water-holding capacity) plus food supply (50 mg of finely ground and autoclaved rolled oats, half of the amount supplied every week). Four replicates per salinity treatment and eight controls were used. The tests were run at 20±1°C with a 16 h:8 h light:dark photoperiod, for 6 weeks. Soil moisture content was checked each week and mass loss replenished with the appropriate amount of deionized water. At the end of the test, the juveniles were immobilized with 80% alcohol and counted under a dissection microscope.

Statistical analysis

Comparisons between treatments were tested using ANOVA. Dunnett's, Holm–Sidak and Tukey tests were used to assess significant differences after one-way or two-way ANOVA. All statistical analyses were performed using Sigmaplot for Windows Version 11.0 (Systat software Inc., Chicago, IL, USA).

RESULTS

Survival

Worms from both populations showed a significant improvement in survival in saline soils within the tested range (for salinity, Germany: $F_{3,64}$=73.90, P<0.001; Greenland: $F_{3,64}$=51.14, P<0.001) (Fig. 1). This effect was more pronounced in worms from Germany than in those from Greenland, where significant differences were already apparent at −5°C between 0‰ and all other salinities (Dunnett, P<0.05). At −20°C, 20% of German worms survived in non-saline soils (0‰) compared with 70%, 85% and 100% in soils with 15‰, 35‰ and 50‰ salinity, respectively. Comparison between the two populations in non-saline soil showed that the Greenland population had higher survival after freezing than the German population ($F_{1,24}$=6.13, P<0.05) for all freezing temperatures (−5, −14 and −20°C).

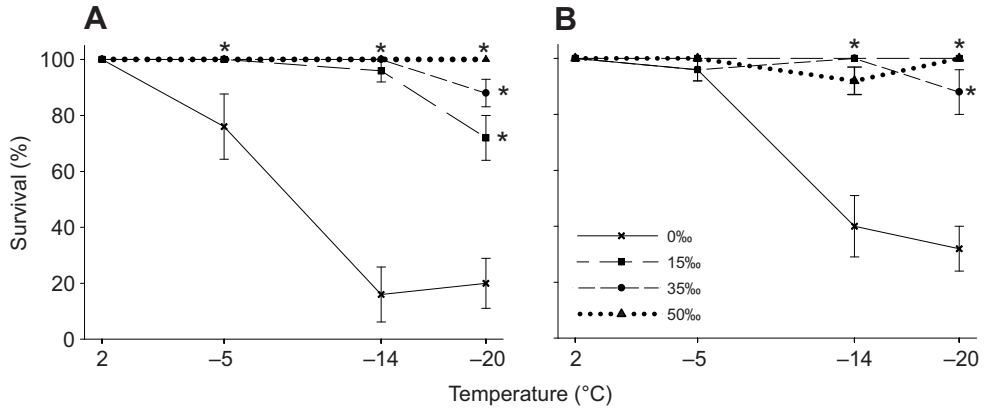

Fig. 1. Freeze survival of *Enchytraeus albidus* from Germany (A) and Greenland (B) when exposed to soil with a range of salinities (0‰, 15‰, 35‰ or 50‰ NaCl). Results are shown as means ± s.e.m. (N=5). Significant difference from 0‰ salinity is shown (Dunnett, *P<0.05).

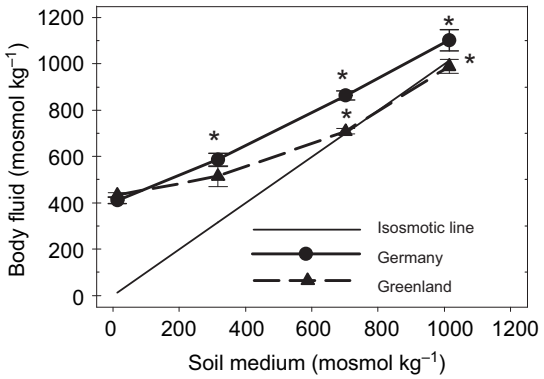

Fig. 2. Osmolality of body fluids of *E. albidus* from Germany and Greenland after 48 h exposure to different salinities. Results are shown as means ± s.e.m. (*N*=6). Significant difference from 0‰ salinity is shown (Dunnett, **P*<0.05).

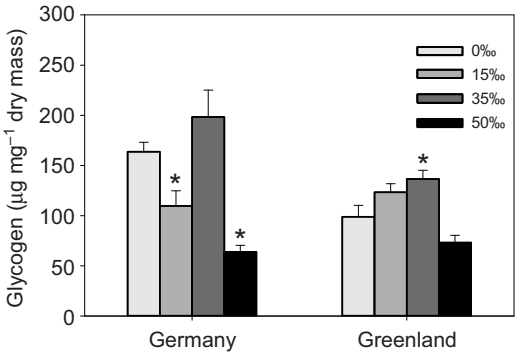

Fig. 3. Glycogen content of *E. albidus* from Germany and Greenland after 48 h exposure to different salinities. Results are shown as means ± s.e.m. (*N*=5). Significant difference from 0‰ salinity is shown (Dunnett, **P*<0.05).

Osmolality, melting point, supercooling point, water content and dry mass

Both populations showed an increase in the osmolality of body fluids with exposure to increasing salinity in soil (Fig. 2). Despite similar osmoregulatory responses, there was a significant interaction between population and soil salinity ($F_{3,40}$=10.78, P<0.001). At lower salinities (0‰ and 15‰) both populations were hyperosmotic in relation to the soil medium. At higher salinities (35‰ and 50‰) worms from Greenland became osmoconforming, whereas the worms from Germany remained slightly hyperosmotic to the soil medium.

The corresponding melting points are shown in Table 1, along with supercooling point, water content and dry mass measurements. Water content and supercooling point decreased with the salinity increase in both populations; however, this decrease was not of statistical significance (Table 1). Water content differed significantly between populations in non-saline soils, being lowest in worms from Greenland. The worms from Greenland were also significantly larger than the worms from Germany, as shown by their higher dry mass (Table 1).

Glycogen and glucose

Salinity (without freezing) had an effect on glycogen reserves (Fig. 3), causing a significant decrease at 15‰ and 50‰ in worms from Germany. In the worms from Greenland, no significant decrease was observed; instead, a small but significant increase in glycogen was observed at 35‰.

Temperature and salinity had an effect on glucose accumulation (Fig. 4). At the end of freeze exposure (day 9), worms from Germany had the highest levels of glucose at the lowest sub-zero temperatures (20- to 30-fold increase compared with +2°C, independently of soil salinity), revealing a significant positive effect of sub-zero temperature ($F_{3,64}$=484.51, P<0.001), a negative effect of salinity ($F_{3,64}$=38.07, P<0.001) and also an interaction between temperature and salinity ($F_{9,64}$=10.80, P<0.001). At −5°C we observed an increase in glucose with an increase in the time of exposure (from 3 to 9 days) ($F_{1,32}$=49.02, P<0.001). At −14°C the interaction between time of exposure and salinity was significant ($F_{3,32}$=3.03, P<0.05).

Similar to worms from Germany, worms from Greenland also showed a negative effect of salinity ($F_{3,64}$=8.77, P<0.001), a positive effect of sub-zero temperature ($F_{3,64}$=419.41, P<0.001) and a significant interaction between temperature and salinity on glucose accumulation ($F_{9,64}$=5.15, P<0.001). Time of exposure generally increased the glucose levels in worms at 2°C ($F_{1,32}$=5.55, P<0.05), −5°C ($F_{1,32}$=74.19, P<0.001) and −14°C ($F_{1,32}$=9.01, P<0.05). There was no significant interaction between time of exposure and salinity.

Comparing the two populations, worms from Greenland tended to accumulate more glucose than worms from Germany, especially at −5 and −14°C in soils with 0‰ ($F_{1,32}$=39.50, P<0.001), 35‰

Table 1. Body fluid melting point, supercooling point, water content and dry mass of *Enchytraeus albidus* from Germany and Greenland sampled after 48 h at 2°C

		Salinity (NaCl ‰)			
Physiological parameter	Population	0	15	35	50
Melting point (ºC)	Germany	–0.76±0.03^{a}	–1.09±0.05^{a,*}	–1.61±0.04^{a,*}	–2.05±0.09^{a,*}
	Greenland	–0.81±0.02^{a}	–0.96±0.08^{a}	–1.31±0.02^{b,*}	–1.84±0.06^{b,*}
	Soil	–0.02±0.01^{b}	–0.59±0.01^{b,*}	–1.30±0.01^{b,*}	–1.89±0.01^{b,*}
Supercooling point (ºC)	Germany	–3.50±0.47^{a}	–4.50±0.50^{a}	–5.55±0.66^{a}	–5.52±0.55^{a}
	Greenland	–4.18±0.42^{a}	–5.24±0.66^{a}	–5.17±0.80^{a}	–5.91±0.71^{a}
Water content (mg mg^{-1} dry mass)	Germany	4.40±0.66^{a}	3.52±0.10^{a}	3.25±0.13^{a}	2.84±0.05^{a}
	Greenland	2.86±1.04^{b}	2.68±0.28^{a}	2.22±0.11^{a}	2.13±0.14^{a}
Dry mass (mg)	Germany	0.80±0.08^{a}	0.75±0.08^{a}	0.42±0.05^{a,*}	0.69±0.07^{a}
	Greenland	2.10±0.24^{b}	1.87±0.20^{b}	1.49±0.19^{b}	2.55±0.28^{b}

Results are shown as means ± s.e.m. (*N*=6–8).
Significant differences between 0‰ and other salinities are shown (Dunnett, **P*<0.05).
Significant differences between populations are indicated by different superscript letters (*t*-test, *P*<0.05).

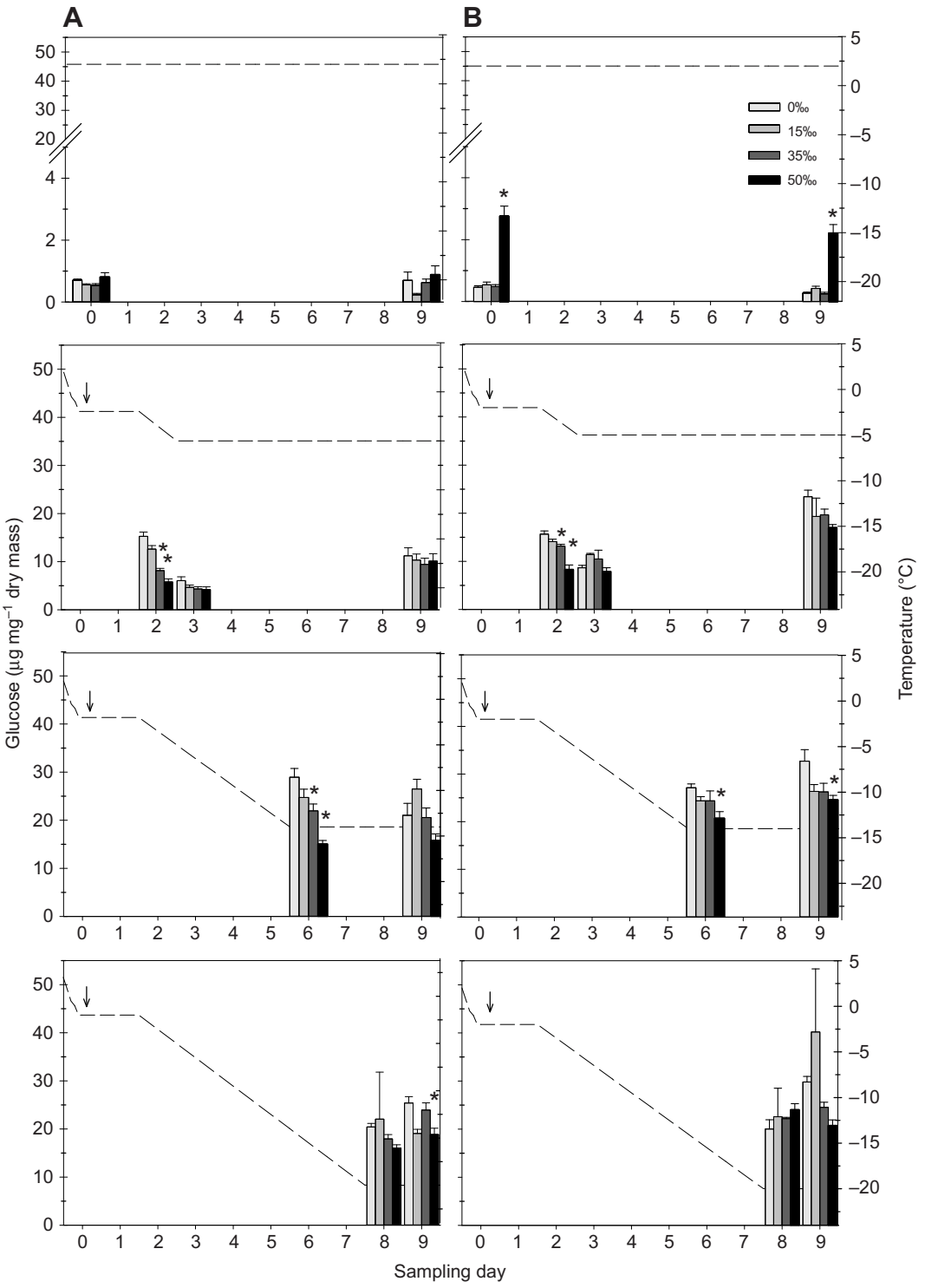

Fig. 4. Glucose content of *E. albidus* from Germany (A) and Greenland (B) measured at different sampling times, when exposed to soil with a range of salinities (0, 15, 35 or 50‰ NaCl) under different temperature regimes (2, −5, −14 and −20°C). The arrow marks the point in time when ice crystals were added to the soil surface to induce freezing. The dashed line indicates the target temperature. Results are shown as means ± s.e.m. (*N*=5). Significant difference from 0‰ salinity is shown (Dunnett, **P*<0.05).

($F_{1,32}$=19.21, P<0.001) and 50‰ salinity ($F_{1,32}$=40.09, P<0.001). At these salinities, the effects varied with temperature depending on the population source (0‰ $F_{1,3}$=7.51, P<0.001; 35‰ $F_{1,3}$=7.13, P<0.001; and 50‰ $F_{1,3}$=6.18, P<0.05). There was an interaction between population and salinity at +2°C ($F_{3,32}$=10.34, P<0.001) and −14°C ($F_{3,32}$=3.67, P<0.05).

Ice content determined by DSC

The total fraction of bound water (OIW, unfreezable water) was determined by DSC to be 38.6±2.3% (mean ± s.e.m.) for the worms from Germany (*N*=5) and 41.2±0.9% for the Greenland population (*N*=4). As these values were not significantly different, we used a mean value of 40% for OIW and 60% for OAW (freezable water) for both populations.

At the lower target temperature of −14°C, the ice content measured by DSC was significantly higher than that at −5°C ($F_{1,44}$=9.91, P<0.001), particularly in worms exposed to saline soils (by ~30%; Fig. 5). At each target temperature, the ice content was significantly lower in worms exposed to 35‰ salinity than in control worms (Fig. 5). Exposure to 35‰ salinity reduced the ice fraction

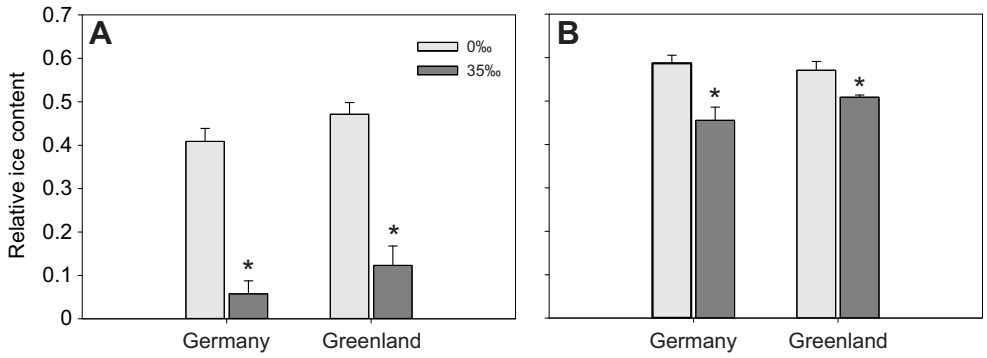

Fig. 5. Relative ice content of *E. albidus* from Germany and Greenland determined by differential scanning calorimetry when cooled to target temperatures of (A) −5°C and (B) −14°C, after 48 h exposure to salinities of 0 and 35‰ NaCl. Results are shown as mean values ± s.e.m. (*N*=10 and 5 for target temperatures of −5 and −14°C, respectively) (Dunnett, **P*<0.05).

of worms by 35% at −5°C in both populations (Fig. 5A). At −14°C, salinity reduced the ice fraction by 13% and 6%, in worms from Germany and Greenland, respectively (Fig. 5B). There was a significant interaction between temperature and salinity ($F_{1,44}$=22.70, P<0.001); however, no differences between populations were revealed.

Estimated ice content

At each target temperature the calculated relative ice content decreased with increasing salinity of the soil (Fig. 6). Comparing 0‰ and 50‰ salinity, the estimated ice content of worms from Germany was reduced by 32%, 8% and 7% at −5, −14 and −20°C, respectively. A similar pattern was found for worms from Greenland, with a reduction of 24%, 7% and 5% at −5, −14 and −20°C, respectively. The relative ice content increased with the decrease of temperature in both populations ($F_{2,48}$=892.26 in Germany, $F_{2,48}$=675.17 in Greenland; P<0.001). There was a significant interaction between salinity and temperature ($F_{6,48}$=41.58 in Germany, $F_{6,48}$=17.62 in Greenland; P<0.001). Differences between populations were observed at the target temperature of −5°C ($F_{1,32}$=403.13, P<0.001), −14°C ($F_{1,32}$=433.80, P<0.001) and −20°C ($F_{1,32}$=51.60, P<0.001).

The estimated ice content was in fairly good agreement with the DSC-determined values, except at relatively high sub-zero temperatures (−5°C), where the estimated ice content was much higher than the DSC-determined one. Relating the survival data to the estimated ice content showed that lethal effects occurred when the ice fraction reached a threshold between 0.56 and 0.57 (Fig. 7).

Reproduction

The reproduction test was valid according to the guidelines (ISO, 2004; OECD, 2004). The validity criteria of adult survival of 80% and a minimum of 25 juveniles per 10 adults (coefficient of variance <50%) were fulfilled. Reproduction was positively influenced by salinity from 2‰ to 40‰ salinity with a significant increase (Fig. 8). The results indicate a typical normal distribution curve for salinity, where the optimum seems to range between 10‰ and 30‰. To provide the estimate of the effect of salt concentration on reproduction, because the data describe an optimum distribution curve instead of a linear dose–response, calculations were done using 20‰ salinity as the optimum point in a two-parameter logistic curve fit. The 90% upper limit of NaCl was predicted to be 72.9‰ (39<CI<106).

DISCUSSION

Effect of salinity on freeze-tolerance

The most important finding of our study is that pre-acclimation to even modest salinities of soil water improved survival during freezing at low temperature considerably. For example, worms from Germany kept in 15‰ NaCl survived (>90%) freezing at −14°C whereas less than 20% of the worms kept in soil of 0‰ NaCl survived this temperature. Thus, *E. albidus* resembles many intertidal invertebrates in which changes in salinity can modify cold survival considerably (Aarset, 1982; Murphy, 1983; Elnitsky et al., 2009).

The survival of freezing is probably related to the amount of extracellular ice formed. Extracellular ice formation causes cell dehydration and shrinking, which at very low temperatures may reach a lower critical volume that affects membrane integrity, leading to irreversible cell collapse (Meryman, 1971; Zachariassen, 1985). It is also likely that extensive ice formation causes mechanical damage to tissues in the frozen organism. Thus, keeping ice formation to a minimum is crucial for cold tolerance and cellular homeostasis. When relating estimated ice content to survival rate it appears that lethal effects emerge when the ice fraction crosses a threshold between 0.56 and 0.57. In our case, worms that were

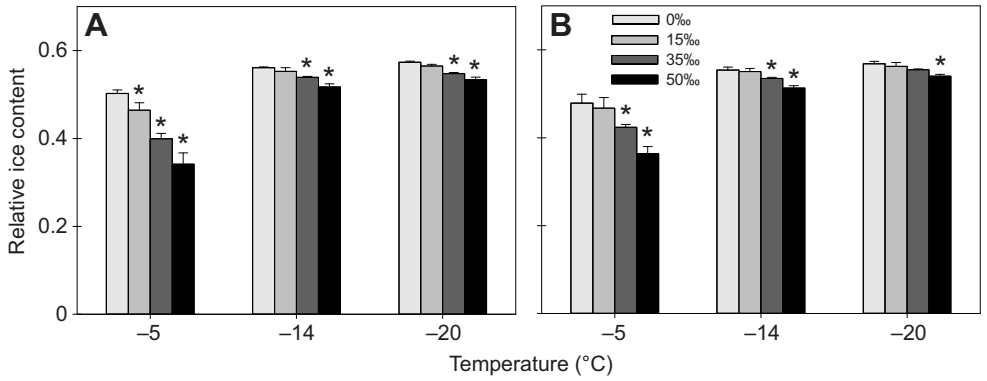

Fig. 6. Estimated relative ice fraction of *E. albidus* from Germany (A) and Greenland (B) when exposed to combinations of sub-zero temperature and salinity. Results are shown as mean values ± s.e.m. (*N*=5) (Dunnett, **P*<0.05).

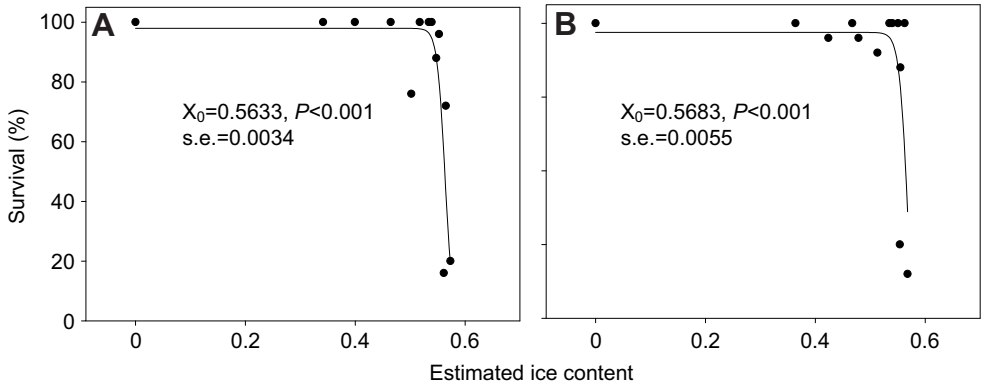

Fig. 7. Relationship between the relative ice content and survival of freezing in *E. albidus* from Germany (A) and Greenland (B). Each point represents the mean survival and the corresponding estimated mean ice fraction. A sigmoidal regression curve was fitted to the data. X_0 denotes the ice fraction causing 50% mortality. Standard error of the X_0 estimate is shown.

pre-acclimated in saline soils and frozen had a significantly lower ice content compared with those in the 0‰ soil (not salt acclimated), which can at least partly explain their much higher survival at sub-zero temperatures. The decrease in ice content with increasing salinity was observed in both estimated and DSC-measured ice content, despite lower values revealed in the latter at −5°C. However, at −14°C the estimated ice content matched the DSC-determined ice content quite well. The ice content measured by DSC must be carefully interpreted as inoculation at high temperatures by ice crystals was not possible and cooling rates were much more rapid than those used in the survival experiment. This probably prevented the accumulation of cryoprotectants during freezing. However, the estimated ice content also depends on assumptions that may not be accurate.

The main contribution to the lower ice fraction observed in worms exposed to saline soils was the decrease in melting point (by ~1.03–1.29°C in 50‰ saline soil) and to some degree from a decrease in water content. This decrease in melting point was probably due to the passive influx of Na^+ and Cl^- ions across the body wall, as demonstrated in other annelids, such as *Lumbricus* sp. (Ramsay, 1949; Dietz and Alvarado, 1970; Dietz, 1974; Prusch and Otter, 1977) and *Nereis* sp. (Smith, 1976). According to these previous results, the uptake of Na^+ and Cl^- increases with salinity, although the velocity of this process tends to diminish with time of exposure. At lower temperatures such as −14 and −20°C, the melting point-driven effect on ice fraction becomes less prominent; however, even small reductions in ice fraction at these temperatures may improve survival if the lethal threshold (~0.6) is not reached. At a relatively high sub-zero temperature (−5°C), the reduction in ice fraction caused by salinity is considerable, meaning that worms exposed to salinity would be subjected to a slower freezing of body fluids during cooling to very low temperatures (−14 or −20°C) than worms frozen in control soil. This reduced ice content in the early phase of the freezing process could be important for physiological mechanisms to prevent severe cellular dehydration, and would promote the stabilization of proteins and cell membranes by cryoprotectants (Zachariassen, 1985; Crowe et al., 1987) as discussed below.

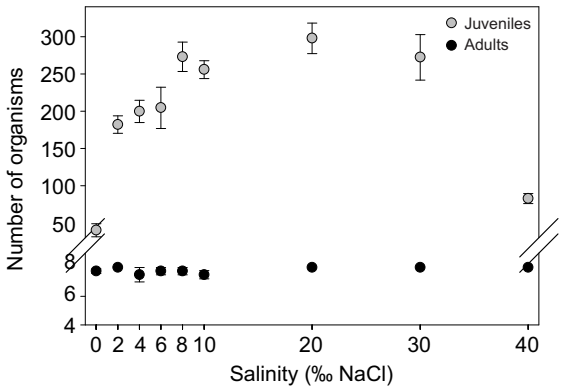

Fig. 8. Results from a reproduction test performed with *E. albidus* from Germany when exposed to a range of salinities in LUFA 2.2 soil, showing the number of surviving adults and juveniles. Results are shown as means ± s.e.m. (*N*=4).

The results of the freeze tolerance experiment showed that *E. albidus* from Germany and Greenland are highly tolerant to saline soils. No mortality due to salinity was observed, even at 50‰. Moreover, our results show that up to 30‰ salinity is beneficial for *E. albidus* reproduction, which is perhaps not so surprising considering that this species is commonly found in decaying seaweed on the sea shore. Taken together, our study shows that non-saline soils are in fact sub-optimal for *E. albidus*.

Role of glycogen and glucose

Previous studies on enchytraeids and other annelids have pointed to a positive correlation between glycogen reserves and the accumulation of glucose with the ability to survive extreme freeze events (Pedersen and Holmstrup, 2003; Holmstrup and Overgaard, 2007; Slotsbo et al., 2008). Glycogen is the principal source for mobilization of carbohydrate cryoprotectants in terrestrial oligochaetes (Holmstrup et al., 2007; Overgaard et al., 2007), and glucose acts as their main cryoprotectant, probably because it is the primary blood sugar of these animals (Prentø, 1987).

Previous work (Slotsbo et al., 2008) has shown that *E. albidus* from Greenland accumulated glucose to between 50 and 110 $\mu g\,mg^{-1}$ dry mass upon freezing at −2 and −14°C, respectively, equivalent to concentrations in OAW of ~120–250 $mmol\,l^{-1}$. As glucose is a reducing sugar and may cause damaging glycation of proteins at 10-fold lower concentrations in vertebrates (MacDonald et al., 2009), it is surprising that enchytraeids can tolerate such high glucose concentrations. The glucose concentration accumulated at −2°C was enough to reduce the ice fraction by ~20% at this temperature, which will help to ensure controlled tissue dehydration and glucose transportation across tissues. In our investigation, we also observed an increase in glucose concentration with the decrease in temperature. However, considering the relatively low glucose concentrations of worm tissue (20–30 $\mu g\,mg^{-1}$ dry mass), the contribution of glucose to lowering the ice fraction was negligible (<1%). Thus, in this case, the colligative effects of glucose were not of importance for freeze survival. This was also underlined by the fact that the worms exposed to saline soils (e.g. 50‰) had lower glucose concentrations

than the worms exposed to non-saline soils, but still had a higher freeze survival. In addition, as we were not able to discriminate between live and dead specimens at the time of sampling, the glucose content of worms exposed to non-saline soils at −14 and −20°C could be underestimated and thus corroborate the observed negative effect of salinity on glucose accumulation. These observations suggest that glucose synthesis is somehow triggered and regulated (*via* feedback mechanisms) by changes in body fluid osmolality. Even though glucose was the only cryoprotectant measured in the present study, the possibility of other carbohydrate cryoprotectants being of importance seems unlikely. Slotsbo and colleagues explored different types of low molecular weight cryoprotectants that were produced by *E. albidus*, and glucose was clearly the dominant cryoprotectant (Slotsbo et al., 2008).

Despite the low contribution of glucose to osmolality and reduced ice fraction, its importance in other processes such as stabilization of membranes and proteins is crucial (Anchordoguy et al., 1987; Crowe et al., 1987). It is believed that such sugars maintain the phospholipid membrane in a fluid phase in the absence of water, preventing damage from low temperatures or dehydration. They are also effective at preserving the structure and function of labile proteins that are important for maintaining vital functions (Crowe et al., 1987). Lastly, glucose may also alleviate the potentially toxic effect of high Na^+ and Cl^- concentrations during freezing. It should be noted that these and glucose will increase to at least 10-fold higher concentrations in the unfrozen parts of the body fluids bathing the cells when worms are frozen at very low temperatures. Worms from Greenland had the highest glucose levels, and this may possibly explain their better freeze survival.

Glucose also increased as a response to osmotic stress caused by salinity without freezing, as has also been shown in other invertebrates (Elnitsky et al., 2009). However, the glucose concentrations were low under these conditions compared with glucose levels in frozen worms. Furthermore, the decrease in glycogen levels due to salinity exposure was not matched by similar glucose concentrations, which points to the conversion of glycogen to other low molecular weight carbohydrates in addition to glucose.

Conclusions

Despite the similar overall physiological and biochemical response mechanisms of the two populations, our study indicates significant differences in their survival, absolute values of physiological parameters and the dynamics of these responses. Taken together, these differences led to an overall superior survival at sub-zero temperatures in the worms from Greenland. It seems reasonable to assume that genetically based adaptations to extreme environmental conditions of worms from Greenland are the reason to these population differences.

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AUTHOR CONTRIBUTIONS

A.L.P.S., M.H. and M.A. designed the overall experiment, and A.L.P.S. carried out the main experiment and measurements. A.L.P.S., M.H. and V.K. designed the experiments measuring ice content. A.L.P.S. and V.K. performed the ice content measurements. A.L.P.S. and M.A. carried out data analysis. All authors wrote the paper.

COMPETING INTERESTS

No competing interests declared.

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