The Journal of Experimental Biology 211, 1903-1910
Published by The Company of Biologists 2008
doi:10.1242/jeb.017558

Slow desiccation improves dehydration tolerance and accumulation of compatible osmolytes in earthworm cocoons (*Dendrobaena octaedra* Savigny)

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Accepted 2 April 2008

SUMMARY

The earthworm, *Dendrobaena octaedra*, is a common species in temperate and subarctic regions of the northern hemisphere. The egg capsules ('cocoons') of *D. octaedra* are deposited in the upper soil layers where they may be exposed to desiccation. Many previous studies on desiccation tolerance in soil invertebrates have examined acute exposure to harsh desiccating conditions, however, these animals are often more likely to be exposed to a gradually increasing drought stress. In the present study we slowly desiccated *D. octaedra* cocoons to simulate ecologically realistic drought conditions and the results clearly demonstrate that gradually dehydrated cocoons show an increased tolerance of extreme drought compared with acutely dehydrated cocoons. NMR spectroscopic analysis of compatible osmolytes revealed the presence of sorbitol, glucose, betaine, alanine and mannitol in dehydrated embryos. The superior drought survival of gradually desiccated embryos could partly be attributed to a higher accumulation of osmolytes (especially sorbitol). Thus, gradually and acutely desiccated embryos accumulated ~2 mol l^{-1} and 1 mol l^{-1} total osmolytes, respectively. However, in addition to osmolyte accumulation, the gradually desiccated cocoons also tolerated a higher degree of water loss, demonstrating that gradually dehydrated *D. octaedra* cocoons are able to survive loss of ~95% of the original water content. Although *D. octaedra* embryos can probably not be categorized as a truly anhydrobiotic organism we propose that they belong in a transition zone between the desiccation sensitive and the truly anhydrobiotic organisms. Clearly, these earthworm embryos share many physiological traits with anhydrobiotic organisms.

Key words: anhydrobiosis, betaine, dehydration, sorbitol, water loss.

INTRODUCTION

Earthworms have no means of reducing evaporative water loss across their skin, and their activity is largely restricted to periods where free water is available in the soil (Holmstrup, 2001). If soils become too dry, earthworms often move to deeper soil layers where moisture conditions are more favourable because even small decreases in soil water potential may cause a quick loss of body water. Earthworm egg capsules ('cocoons') are, however, mostly situated in the upper soil layers (Gerard, 1967) and must therefore depend on tolerance of desiccation during dry periods. This is in particular the case for surface dwelling species that place their cocoons in leaf litter on the soil surface (Rundgren, 1975).

The moss worm, *Dendrobaena octaedra*, lives in the litter layer of the forest floor and under moss or lichens. It is a widely distributed species and is found in most of the European forest zone and the European tundra, Eastern Siberia, North America and Greenland (Stöp-Bowitz, 1969). The cocoons of *D. octaedra* are often exposed to desiccating conditions during dry periods because of their surface habitat choice. Previous studies have shown that cocoons of *D. octaedra* are very tolerant to desiccation in comparison with other earthworm species (Holmstrup and Westh, 1995). Thus, about 50% of cocoons from a Danish population tolerate exposure to 93% relative humidity (RH) for 14 days (Holmstrup and Westh, 1995). The permanent wilting point of plants is by convention set at 98.9% RH so a RH of 93% represents a severe level of desiccation. Because of their location in the topsoil, however, it is likely that cocoons may need to tolerate even lower humidities. The embryos within these cocoons have therefore evolved physiological adaptations to meet this desiccation stress and they are able to tolerate the loss of practically all osmotically active water (approximately 85% water loss) (Holmstrup and Westh, 1995). Dehydration can be harmful to the embryos because cellular shrinkage is potentially damaging, with proteins becoming irreversibly denatured in dehydrated cells, and because cellular membranes may lose their normal conformation in a liquid crystalline state (Crowe et al., 1992). However, it is well known that protective substances such as sugars and polyhydric alcohols, or other compatible osmolytes, may ameliorate these effects (Crowe, 2002; Yancey, 2005). In addition to protein-protective effects, compatible osmolytes may also limit the water loss of the embryo during desiccation by their osmotic effects even though examples of this are rare in the literature (Bayley and Holmstrup, 1999).

It is clear that the survival of dehydration by invertebrates depends not only on degree of water loss, but also on the rate of dehydration. Previous reports have shown that pre-acclimation to a relatively mild desiccation stress can improve severe desiccation tolerance in other soil invertebrates such as nematodes, Collembola and midge larvae (Hayward et al., 2007; Sjursen et al., 2001; Womersley and Ching, 1989). In natural environments, soil organisms will be subjected to gradually increasing desiccation stress because drying of soils is a

buffered process occurring over periods of days or weeks rather than hours. Studies that explore the water balance, production of compatible osmolytes and survival of soil organisms within these time and moisture scales have therefore greater ecological relevance than studies using acute exposure regimes. However, such studies are rare and have not yet been performed on earthworm cocoons. The aim of the present study was therefore to simulate naturally occurring desiccation rates and investigate how rate affects the responses of *D. octaedra* in terms of desiccation tolerance, survival and accumulation of compatible osmolytes.

MATERIALS AND METHODS

Production of cocoons

Approximately 300 adult *Dendrobaena octaedra* Savigny were collected in a mixed forest near Silkeborg, Denmark, between August and October 2005. The earthworm cultures were maintained as described previously (Holmstrup et al., 1991). Briefly, the earthworms were kept at 15°C in 1 l plastic pots (10 worms in each) with soil and moist cow dung as substrate. Cocoons were harvested approximately every third week by washing and sieving, after which they were placed on moist filter paper in Petri dishes. The cocoons were then incubated at 20°C until they had developed to an early differentiated stage characterized by the embryos having a worm-like shape but with no visible internal structures or blood pigment (Holmstrup, 1994). Once cocoons had developed to this stage they were placed at 1°C to prevent them from developing further until the experiments were carried out.

Desiccation experiments

Two experiments were designed to compare the effects of gradually increasing desiccating conditions with the effects of acute exposure to harsh desiccation. The first experiment examined water content, osmolyte accumulation and survival of *D. octaedra* cocoons during a 10 day gradual lowering of RH from 100% to a final RH of 91%. These results were compared with those of cocoons that were acutely exposed to 91% RH (Fig. 1). For desiccation, fully hydrated cocoons were gently blotted with filter paper to remove surface water and placed in open-top plastic sample vials (3 cm high, 1.6 cm diameter) in the centre of a 160 ml plastic cup (4.2 cm high, 7 cm diameter) containing 25 ml aqueous NaCl solution, sealed with a tightly fitting plastic lid. The air in this small closed system rapidly equilibrates with the salt solution (following Raoult's law). The RH is defined by the molar fraction of water: 55.56/(Osm+55.56)×100%, where Osm is the osmolality of the NaCl solution. By varying the NaCl concentration between 0 and 158 g l^{-1} and by keeping the chambers at 20±0.1°C, humidity levels of between 100 and 91±0.1% RH were created (Holmstrup et al., 2001). The gradually desiccated cocoons were exposed to a series of decreasing humidities by replacing the salt solutions of the desiccation chambers daily over a 10 day period (Fig. 1A). After this period the cocoons were left at the final humidity (RH 91%) for 4 days (short exposure) or 14 days (long exposure), respectively, at which time five replicates of ten cocoons were sampled for survival assays. For the gradually desiccated cocoons, samples for measurements of water content were taken daily for the first 14

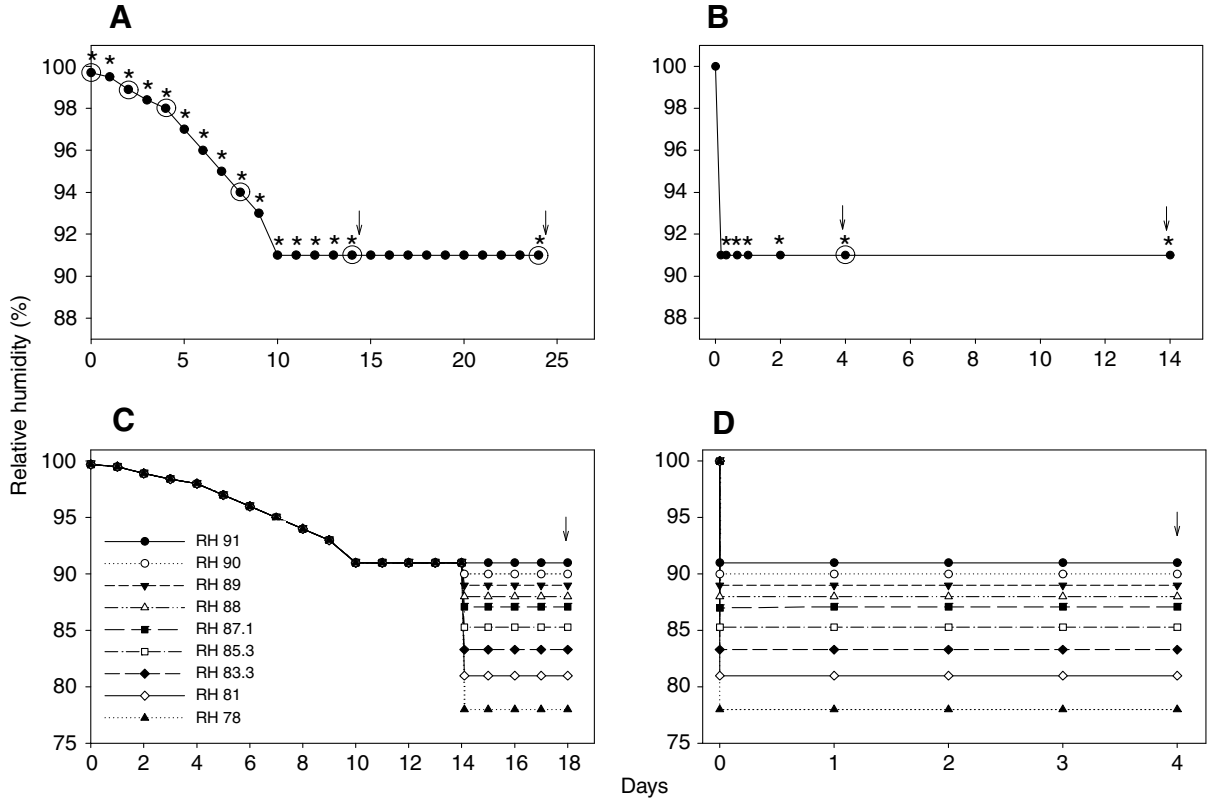

Fig. 1. Upper panels: experimental protocol for gradually (A) and acutely (B) desiccated cocoons of *D. octaedra*. Arrows, asterisks and circles indicate time points for measurement of survival, water content and osmolytes, respectively. Lower panels: experimental protocol investigating the effects of gradual (C) and acute desiccation (D) on survival and water content after harsh desiccation treatments. Arrows indicate the time for measurement of survival and water content. The key indicates the relative humidity (RH, %).

days and also on day 24. Samples for osmolyte measurements were taken after 0, 2, 4, 8, 14 and 24 days (Fig. 1A).

Acutely exposed cocoons were exposed directly to a RH of 91% for either 4 or 14 days, after which survival was determined. Water content was determined after 2, 4, 8, 16 and 24 h during the first day of exposure, and subsequently after 2, 4 and 14 days of exposure (Fig. 1B). Samples for measurements of osmolytes were only taken after 4 days of exposure.

To further explore the relationship between pre-treatment, water content and survival, a second experiment was conducted in which gradually and acutely desiccated cocoons were exposed to a series of harsh desiccation treatments after their initial exposure (Fig. 1C,D). The gradually desiccated cocoons were pre-acclimated for 14 days using the same desiccation protocol as in the first experiment after which they were exposed to a final 4 day exposure to desiccation at RHs from 91% to 78% (Fig. 1C). For comparison, the acutely desiccated cocoons were exposed directly to their final desiccation treatment between 91% and 78% RH (Fig. 1D). To obtain the nine levels of dehydration, NaCl solutions were used to create RHs down to 83% as described, whereas saturated solutions of $(NH_4)_2SO_4$ (ammonium sulphate) and $Na_2S_2O_3{\cdot}5H_2O$ (sodium thiosulphate) were used to create 81 and 78% RH, respectively (Weast, 1989). For each desiccation treatment, six replicates of one cocoon were used to establish the water content, and survival was scored from ten replicates of five cocoons.

Survival and water content

Cocoons used for determination of survival were placed in Petri dishes with tap water at 20°C and checked every week for 6 weeks. Survival was scored from the number of juveniles that emerged successfully during this period (one juvenile emerges from each cocoon). Water content of single cocoons was determined gravimetrically by weighing the cocoon immediately after sampling and after drying at 60°C for 24 h. Weight measurements were performed with a Cahn 4700 automatic eletrobalance, precise to 0.01 mg. Each sample for osmolyte measurements consisted of five cocoons. Six samples were obtained for each round of sampling. These samples were then stored at –80°C until extraction.

Identification of osmolytes

Samples for osmolyte identification were dried at 60°C for 24 h after which the dry mass was determined. The dried cocoons were then crushed and extracted in 0.25 ml ethanol using a rotating glass rod in a 0.5 ml Eppendorf tube. The sample was centrifuged at 20 000 ***g*** for 5 min at 4°C and the supernatant saved. This procedure was repeated three times, after which the pooled supernatants were dried at 60°C for 24 h. The dry sample was then stored at –80°C until further quantitative analysis.

Nuclear magnetic resonance spectroscopy (NMR) was used to screen for increases in osmolytes as a result of desiccation. Thus, NMR was performed on a sample from untreated control cocoons, cocoons after 4 days of acute desiccation and cocoons after 14 days of gradual desiccation; each sample consisted of 15 cocoons. The samples were resuspended in 650 μl of 50 mmol l^{-1} phosphate buffer made up in distilled H_2O (pH 7.4). The samples were vortexed and 600 μl were transferred to a 5 mm NMR tube. The buffer contained 50 mg l^{-1} of the chemical shift reference 3-(trimethylsilyl)-propionic acid-D4, sodium salt (TSP). NMR measurements were performed at 25°C on a Bruker Advance 400 spectrometer, operating at a ^1H frequency of 400.13 MHz, and equipped with a HX inverse probe. ^1H NMR spectra were acquired using a single-90°-pulse experiment with a Carr–Purcell–Meiboom–Gill (CPMG) delay added in order to attenuate broad signals from high molecular mass components. The total CPMG delay was 40 ms and the spin-echo delay was 200 μs. Water was suppressed by presaturation during the relaxation delay of 1.5 s. A total of 256 transients of 16 K data points spanning a spectral width of 24 p.p.m. were collected, corresponding to a total experiment time of 10 min. For assignment purposes a two-dimensional ^1H–^1H TOCSY spectrum with 80 ms mixing was acquired. Signals were assigned using the TOCSY spectrum and by comparison with known metabolite chemical shifts (Fan, 1996; Lindon et al., 1999; Malmendal et al., 2006).

Quantification of osmolytes

The concentration of sorbitol, glucose, alanine, betaine, mannitol and trehalose of cocoons was determined before and during dehydration. Sorbitol, glucose, alanine, betaine and possibly mannitol were identified as candidate osmolytes from the NMR spectra whereas trehalose was included because of its well known role as a major inducible osmolyte in several other dehydration-tolerant organisms. Sorbitol, glucose, trehalose and mannitol were quantified spectrophotometrically using commercial enzymatic kits from Megazyme International (Bray, Co. Wicklow, Ireland). Similarly, alanine was measured spectrophotometrically as described previously (Lowry and Passonneau, 1972). For these measurements the extracts were rehydrated with 200 μl distilled water and subsamples were used for measurements of the different osmolytes. All measurements were performed at 25°C and concentrations were calculated relative to known standards.

Betaine could not readily be measured with a similar method, and instead betaine concentration was quantified in the individual samples by use of NMR spectroscopy. The amount of betaine was assessed from the ratio of the TSP signal to the betaine signal, and the concentration was calculated relative to standards of known concentration. To perform these measurements using NMR, a sub-sample of the rehydrated sample was lyophilized and treated as described above in the NMR section.

Statistics

Student's *t*-tests were used to test for differences in survival and water content between gradually and acutely exposed cocoons after both short and long exposures to 91% RH. Mann–Whitney rank sum tests were used in cases lacking normality. Differences in osmolyte content were analysed using a one-way ANOVA for each osmolyte individually. Here a *post hoc* Bonferroni test was used to separate groups that differed significantly from the hydrated control. Similar tests were used to assess the estimated embryo osmolyte concentrations. Again a *post hoc* Bonferroni test was used to separate groups that differed significantly. A one-way ANOVA was also used to test for differences in survival after the 'harsh' dehydration treatments. Differences in water content following the 'harsh' dehydration treatments were tested at each level of dehydration using a *t*-test, since unequal variance did not allow for a two-way analysis of variance. Survival data were arcsin transformed and all statistics were calculated using SigmaStat 2.03. An effect was considered significant at the $P<0.05$ level and all data are presented as means ± s.e.m.

RESULTS

Effect of acute and gradual desiccation on water content and survival

Water content of *D. octaedra* cocoons declined rapidly over the first 4 h of acute exposure to 91% RH and had reached equilibrium by 8 h at ~0.35 mg water mg^{-1} dry mass (Fig. 2A). In the gradual

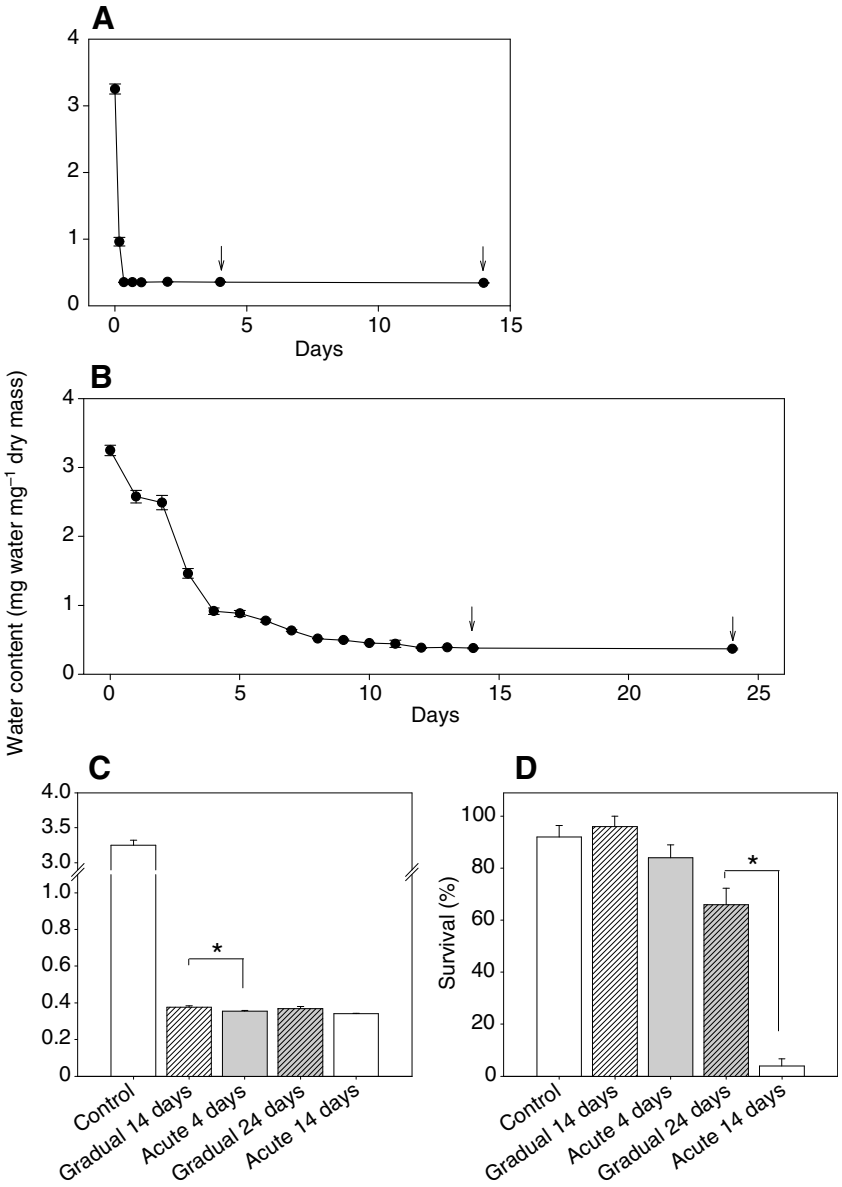

Fig. 2. Water content of *D. octaedra* cocoons during acute (A) and gradual (B) desiccation. The final desiccation strength was 91% RH for both groups. Water content (C) and survival (D) are shown after 4 and 14 days exposure at the final desiccation strength. Student *t*-tests were employed to test for differences in water content and survival between gradually and acutely exposed cocoons after both short- and long-term exposure. Values are means ± s.e.m. and asterisks indicate significant differences.

desiccation treatment, cocoon water content fell gradually for the first 10 days, by which time they had reached a stable level at ~0.38 mg water mg^{-1} dry mass (Fig. 2B). The water content of the gradually desiccated cocoons was significantly higher (P<0.05) than the acutely exposed cocoons when both groups were sampled after 4 days at 91% RH (Fig. 2C). A similar tendency existed after 24 days of gradual desiccation and 14 days acute desiccation, although not statistically significant (P=0.064). There was only minor mortality associated with 4 days of acute desiccation at 91% RH or 10 days of gradual desiccation followed by 4 days at 91% RH (Fig. 2D). Here, the slightly better survival of the gradually desiccated treatment group was not significant after the short exposure (P=0.08; Mann-Whitney test). However, there was a marked benefit of the gradual desiccation treatment when the exposure time was extended to 14 and 24 days, for the acutely and gradually exposed cocoons, respectively (P<0.001).

A second set of experiments were conducted to further explore the relationship between acute and gradual dehydration with regard to survival and water content. Here the cocoons were exposed to a series of harsh exposures from 91 to 78% RH (Fig. 3A). The acutely exposed cocoons showed significantly decreased survival at RHs below 89% (one way ANOVA; P<0.001) and below 85% RH acute desiccation caused 100% mortality. In the gradually exposed cocoons the survival was significantly affected at RHs below 83% (one way ANOVA; P<0.001), but even at these harsh RHs mortality did not rise above 40% (Fig. 3A). Water content of the cocoons decreased with lowered RH for both the acute and gradual exposures, but the gradually exposed cocoons had a significantly higher water content at practically all desiccation levels (Fig. 3B). To investigate the importance of water content for survival, we plotted these two parameters against each other for the two treatment groups (Fig. 3C). It is evident that gradual desiccation increases survival more than can be explained by water content alone.

Osmolyte accumulation

NMR analysis of extracts showed that sorbitol and to a lesser extent glucose, alanine and betaine, all increased in response to desiccation (Fig. 4). Interestingly, sorbitol concentrations remained low until

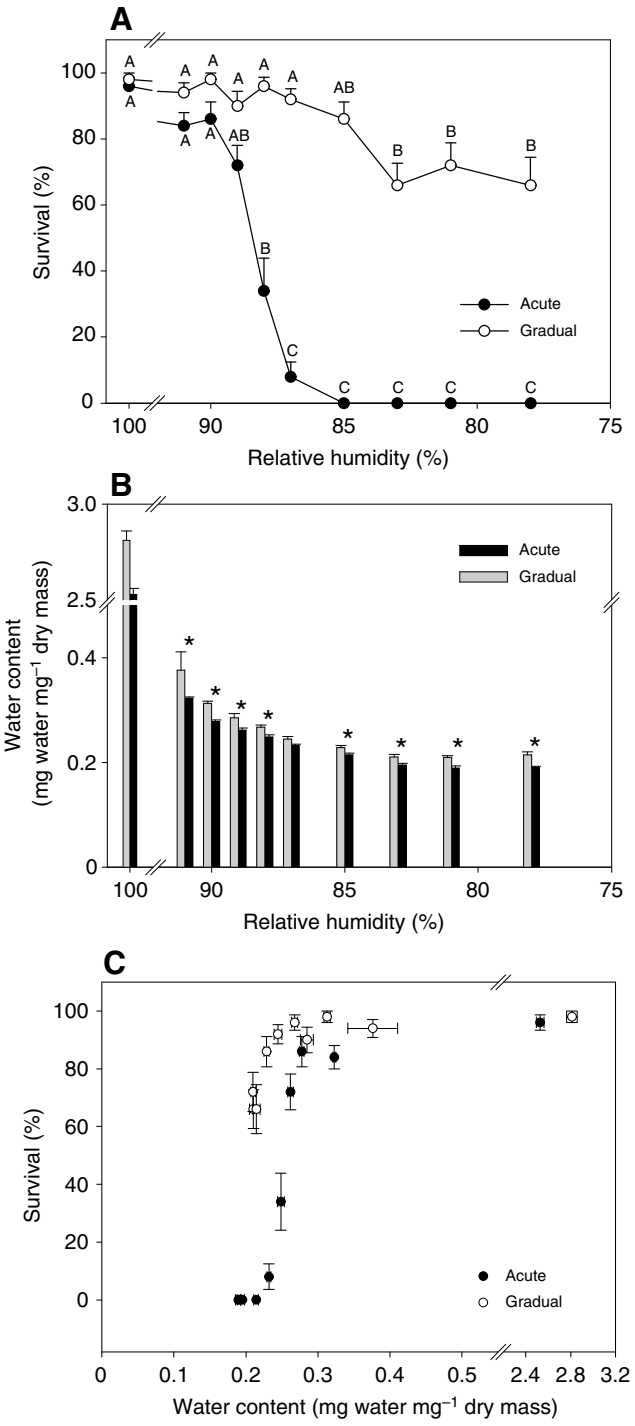

Fig. 3. (A) Survival following severe desiccation in *D. octaedra* cocoons. Cocoons were either exposed acutely (filled circles) or given a 14 day gradual pre-acclimation to desiccation before the final exposure to severe desiccation stress (open circles). Different letters indicate significant differences in survival within each treatment (one-way ANOVA). (B) Water content of acutely and gradually desiccated cocoons following severe desiccation stress. Asterisks indicate a significant difference in water content between pre-acclimated and acutely exposed cocoons (separate *t*-tests for each desiccation level). (C) Survival *versus* water content of cocoons exposed acutely (filled circles) and cocoons that were pre-acclimated by gradual desiccation (open circles). All values are means ± s.e.m.

day 8 in the gradual desiccation treatment where it rose to 2.2 μg mg^{-1} dry mass and maximized at day 14 (3.4 μg mg^{-1} dry mass) before falling back to 1.8 μg mg^{-1} dry mass at day 24. There was no significant increase of sorbitol in the acutely desiccated cocoons (Fig. 5A). Glucose concentration seemed to increase before sorbitol. Thus, glucose remained low during the gradual desiccation until day 4 when there was a small, non-significant rise. Glucose was significantly higher than control at day 8 but had fallen back to control levels at day 14 and 24. Glucose was also significantly higher than control levels in the acutely desiccated cocoons where it reached a concentration of 1.5 μg mg^{-1} dry mass after 4 days. Alanine concentration rose to the maximum concentration of 0.24 μg mg^{-1} dry mass at day 8 and 14 in the gradually exposed cocoons (Fig. 5B) but there was no significant increase in the acutely desiccated cocoons. There were no significant changes in trehalose, mannitol or betaine concentrations in either of the desiccation treatments (Fig. 5B).

Calculation of osmotic contributions of osmolytes in embryos

Previous studies have shown that osmolytes only accumulate in the embryo of the cocoon (Holmstrup, 1995). To determine the concentration of osmolytes in the embryos it was necessary to establish a general relationship between cocoon mass and embryo mass. This was done by measuring the length and width of 10 cocoons and their embryos under a stereomicroscope. The cocoons are ellipsoid in shape and the embryos are cylindrical. The volume, V, was therefore calculated with the formulas, $V=4/3\pi abc$ (abc=length, height and width) and $V=\pi r^2 h$ (r=radius; h=height) for cocoon and embryo, respectively. The average volume of a cocoon was 3.97 mm^3 and the average volume of an embryo was 0.17 mm^3, thus the average embryo mass was estimated to be 4.3% of cocoon mass.

The osmotic activity of osmolytes depends of course as much on the water content in dehydrated embryos as it does on their concentration on a dry mass basis. The water content of the cocoons can be divided into two fractions; osmotically inactive water (OIW) and osmotically active water (OAW). OAW is readily removed during desiccation whereas OIW is bound by structures such as membranes and proteins, and even during severe dehydration it is not readily removed from the tissues (Holmstrup and Westh, 1994).

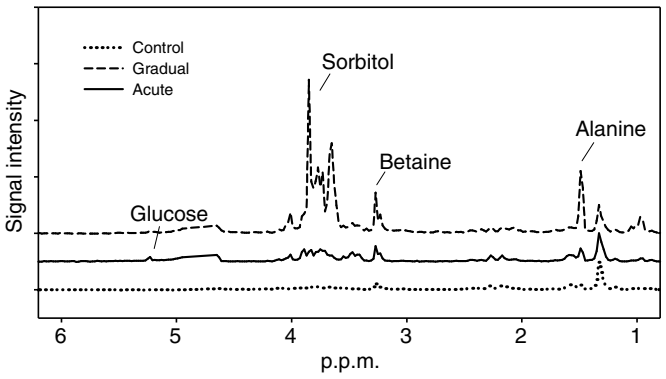

Fig. 4. Representative ^{1}H NMR spectra (displaced along the vertical axis to improve clarity) showing the major metabolites in *D. octaedra* cocoons. The three traces show untreated and fully hydrated cocoons (dotted line), cocoons gradually desiccated over 10 days with a final 4 day exposure to 91% RH (dashed line) and cocoons exposed acutely to 91% RH for 4 days. Well-resolved signals from the metabolites discussed in this study are assigned.

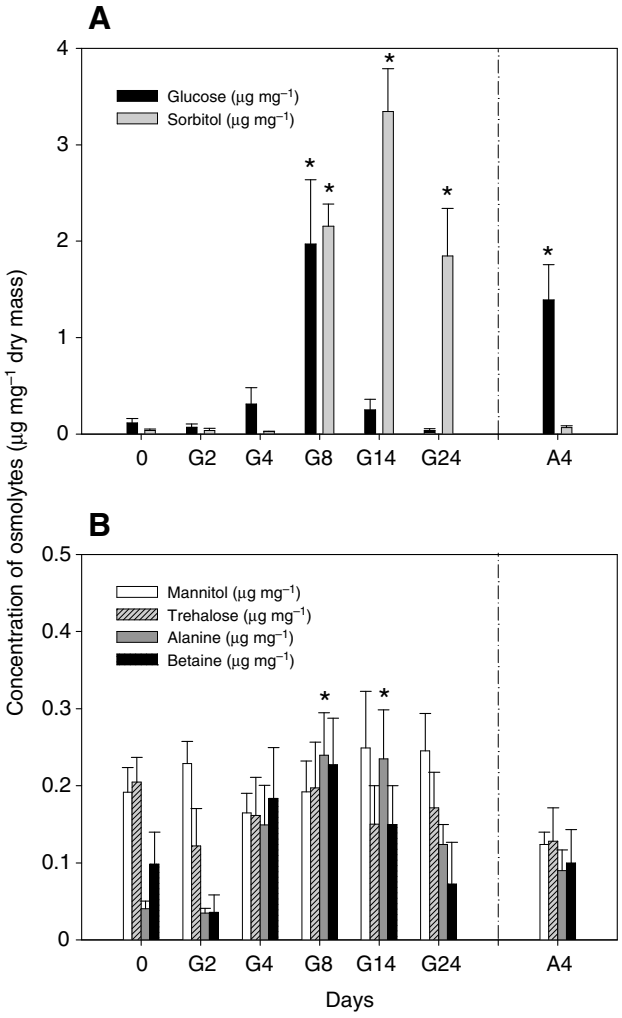

Fig. 5. Concentrations of (A) sorbitol and glucose, and (B) alanine, betaine, trehalose and mannitol in *D. octaedra* cocoons. Samples were taken from untreated controls (0), during a gradual pre-acclimation to desiccation stress at 91% RH (G2–G24) or after a 4 day acute exposure to 91% RH (A4). Samples during the gradual pre-acclimation were taken after 2, 4, 8, 14 and 24 days (see Fig. 1). Asterisks indicate significant difference from day 0 (P<0.05). All values are means ± s.e.m.

It is therefore necessary to calculate the OAW fraction for a given water content. In the present study, OIW was estimated to be 0.12 mg water mg^{-1} dry mass, which was the lowest observed water content of cocoons after an acute dehydration (data not shown). This results in a conservative estimate of the osmotic contributions of the osmolytes when contents (μg mg^{-1} dry mass cocoon) are converted to molal concentrations (mol kg^{-1} embryo OAW).

Osmolyte accumulation in embryos

The calculated concentrations of osmolytes in embryos show that sorbitol, glucose, mannitol, alanine and betaine were elevated at specific times during the gradual and acute dehydration (Table 1). Glucose concentration was slightly elevated at day 4, but reached a maximum concentration of 0.635 mol kg^{-1} OAW at day 8 before falling to 0.114 mol kg^{-1} OAW at day 14. Glucose concentration of the acutely desiccated cocoons rose to 0.779 mol kg^{-1} OAW. The concentration of sorbitol reached a maximum of 1.539 mol kg^{-1} OAW at day 14 and had fallen to 0.950 mol kg^{-1} OAW at day 24. Mannitol concentration rose to approximately 0.12 mol kg^{-1} OAW at day 14 and remained at this level until day 24. The rise in mannitol concentration was not due to *de novo* synthesis but rather to a concentration effect from water loss. Trehalose concentration was also slightly elevated because of the reduced water content during both the gradual and acute exposure, but this was not statistically significant. Alanine rose to a maximum of 0.167 mol kg^{-1} OAW at day 14, whereas the concentration in embryos of the acutely exposed cocoons was only 0.078 mol kg^{-1} OAW. The highest concentration of betaine, 0.113 mol kg^{-1} OAW, was found at day 8 and embryos of the acutely exposed cocoons contained almost as much betaine, 0.086 mol kg^{-1} OAW as the gradually exposed embryos. This estimated total osmolality of these measured compatible osmolytes peaked at day 14 in the gradually exposed 91% RH group at a concentration of 2.073 mol kg^{-1} OAW. This was almost twice the maximum seen in the 91% RH acute group where the calculated osmolality reached 1.052 mol kg^{-1} OAW after 4 days.

DISCUSSION

Osmolyte accumulation in embryos

Little is known about desiccation-induced accumulation of protective osmolytes in earthworm cocoons apart from a single study where sorbitol was shown to accumulate in five earthworm species including *D. octaedra* (Holmstrup, 1995). The present study

Table 1. Estimated concentrations of osmolytes in embryos during the gradual desiccation to 91% RH (day 2, 4, 8, 14 and 24) and acute desiccation at 91% RH (day 4)

	Gradual desiccation						Acute desiccation
Osmolytes	Day 0	Day 2	Day 4	Day 8	Day 14	Day 24	Day 4
WC (mg mg^{-1} dry mass)	3.25	2.49	0.92	0.52	0.40	0.37	0.35
OIW (mg mg^{-1} dry mass)	0.12	0.12	0.12	0.12	0.12	0.12	0.12
OAW (mg mg^{-1} dry mass)	3.13	2.37	0.80	0.40	0.28	0.25	0.23
Glucose (mol kg^{-1} OAW^{-1})	0.005±0.002^{a}	0.004±0.002^{a}	0.050±0.027^{a}	0.635±0.214^{b,c}	0.114±0.051^{c}	0.019±0.009^{a}	0.779±0.205^{b}
Sorbitol (mol kg^{-1} OAW^{-1})	0.001±0.001^{a}	0.002±0.001^{a}	0.001±0.001^{a}	0.694±0.074^{b}	1.539±0.205^{c}	0.950±0.255^{b}	0.037±0.010^{a}
Mannitol (mol kg^{-1} OAW^{-1})	0.008±0.001^{a}	0.012±0.002^{a}	0.026±0.004^{a}	0.061±0.013^{a,b}	0.114±0.034^{c}	0.125±0.025^{bc}	0.069±0.009^{ab}
Trehalose (mol kg^{-1} OAW^{-1})	0.004±0.001^{a}	0.003±0.001^{a}	0.011±0.005^{a}	0.028±0.009^{a}	0.034±0.014^{a}	0.025±0.010^{a}	0.003±0.003^{a}
Alanine (mol kg^{-1} OAW^{-1})	0.003±0.001^{a}	0.003±0.000^{a}	0.037±0.013^{ab}	0.119±0.027^{b,c}	0.167±0.045^{c}	0.098±0.020^{a,c}	0.078±0.023^{a,c}
Betaine (mol kg^{-1} OAW^{-1})	0.006±0.002^{a,b}	0.003±0.001^{a}	0.046±0.016^{a,c}	0.113±0.027^{b,c}	0.106±0.027^{b,c}	0.058±0.034^{a,b,c}	0.086±0.024^{a,b,c}
Sum (mol kg^{-1} OAW^{-1})	0.027±0.003^{a}	0.027±0.003^{a}	0.171±0.037^{a}	1.650±0.319^{b}	2.073±0.305^{b}	1.276±0.311^{b}	1.052±0.288^{a,b}

Values are means ± s.e.m.; different letters indicate significant differences between concentrations.

WC, water content of cocoons. Osmotically inactive water (OIW) is set to the lowest observed water content of acutely desiccated cocoons. Osmolytes are assumed to be dissolved solely in osmotically active water (OAW), which is the difference between WC and OIW. RH, relative humidity.

confirmed the presence of sorbitol in *D. octaedra*, but also revealed that other osmolytes were accumulated in response to desiccation at 91% RH. Glucose was elevated in the gradually desiccated cocoons but apparently only transiently in the early phase of desiccation. When cocoons were dehydrated to ~0.5 mg water mg^{-1} dry mass, glucose disappeared and sorbitol increased significantly. A plausible explanation for this is that glucose was derived from glycogen *via* glucose 6-phosphate and further transformed to sorbitol by polyol dehydrogenase when the water content was so low that oxygen supply to the embryo became a limiting factor (Storey and Storey, 1991). When most OAW has been lost the embryo is encased in a sheath of water-reduced albuminous fluids which may greatly hamper the normal oxygen diffusion across the cocoon fluid–embryo surface interface. Sorbitol synthesis is not sensitive to oxygen limitation in the same way as glucose synthesis (Storey and Storey, 1991), and the observed increase of alanine, a well-known end-product of anaerobic metabolism (Hochachka and Somero, 1984), also suggests that desiccated embryos have experienced anaerobic or partly anaerobic conditions when water contents became low. However, there was no indication of increased lactate levels, although this is an anaerobic end-product in adult *D. octaedra* (S. Calderon, personal communication). The reduced rate of water loss in the gradually exposed cocoons resulted in a different pattern of osmolyte production than in cocoons exposed acutely to 91% RH. Apparently gradual desiccation gave the embryos sufficient time to accumulate sorbitol in high concentrations, which the acutely desiccated cocoons did not have the time to produce, or alternatively, the biochemical pathways were completely changed.

The accumulation of glucose in high concentrations is known in post-embryonic stages of *D. octaedra* subjected to freezing, desiccation or osmotic shock (Overgaard et al., 2007; Rasmussen and Holmstrup, 2002), and thus the occurrence of this carbohydrate was to be expected in dehydrated embryos. However, mannitol, trehalose, alanine and perhaps most interesting, betaine, were also found. No previous reports have shown the presence of these osmolytes in earthworms. Betaine is known from many organisms including marine animals and thought to have the potential to counteract the perturbing effects on protein structure of solutes such as Na^+ that may build up under osmotic stress (Yancey, 2005). Although betaine was not particularly elevated on a dry mass basis, it did occur in significant concentrations (>100 mOsm) in dehydrated embryos where also the original salts (Na^+, K^+ and Cl^-) must be highly concentrated and thus potentially damaging. It is therefore suggested that protection against protein destabilization may be offered by betaine and possibly other observed osmolytes under these circumstances.

Although other adaptations are also necessary, accumulation of trehalose in high concentrations has often been associated with tolerance of extreme desiccation in anhydrobiotic organisms such as tardigrades, nematodes and yeast (Crowe et al., 2002; Madin and Crowe, 1975; Westh and Ramløv, 1991). In *D. octaedra*, trehalose was only detected in negligible concentrations and did not respond to dehydration, whereas sorbitol was the primary osmolyte in the gradually dehydrated cocoons. The accumulation of sorbitol is restricted to the embryo and not found in the albuminous fluid of the cocoons, which should be taken into account when estimating the internal concentration (Holmstrup, 1995). Considering this, the total concentrations of osmolytes in the gradually desiccated embryos reached a level of 2 mol kg^{-1} OAW with sorbitol accounting for 75% of this estimate. The higher concentration of some of the osmolytes such as mannitol and trehalose was only caused by the lower water content and were not the result of a higher accumulation. However, the concentrations of osmolytes in the gradually exposed embryos were twice that in those acutely exposed (1 mol kg^{-1} OAW).

Effects of acute and gradual desiccation on survival

The ability to survive both short and prolonged periods of desiccation is an ecologically important trait for many soil invertebrates, especially those inhabiting the top layer of the soil surface. In the present study we clearly demonstrate that dehydration-tolerant cocoons of *D. octaedra* survive much better when exposed to a gradual dehydration compared to an acute dehydration. Thus, slow dehydration conferred a 70% survival in the gradually exposed cocoons at the lowest humidity used (78% RH), compared to no survival in the acutely exposed cocoons. Previous studies have also shown that slow dehydration may confer improved tolerance of a given desiccation level as compared to acute exposure to the same level of desiccation (Crowe and Madin, 1975; Hayward et al., 2007; Sjursen et al., 2001; Womersley and Ching, 1989). However, many studies of invertebrate desiccation tolerance use acute exposure regimes and only very few have used a gradual desiccation protocol similar to the present study. Given the marked difference in dehydration tolerance found between our two treatment groups we conclude that acute tolerance assays are likely to underestimate the actual dehydration tolerance of many species as the soil water is usually not readily removed over short timescales.

The higher survival was probably linked to the higher water content in the gradually exposed cocoons which in turn may be linked to the accumulation of osmolytes, which by their colligative properties would have reduced the water loss and increased the water content at equilibrium of embryos. However, survival in the gradually desiccated cocoons was also higher when compared at the same level of dehydration (i.e. WC being the same; Fig. 4C) suggesting that other non-colligative protective mechanisms also play an important role. An increased concentration of sugars and/or polyols is likely to enhance cellular and membrane integrity during both desiccation and freezing by replacing the primary water of hydration and through the formation of amorphous glasses (vitrification), thus stabilizing the structure of macromolecules and membranes (Crowe et al., 1992). Trehalose is proposed to function as such a ‘water replacement’ molecule and sorbitol and other osmolytes in earthworm embryos may have a similar function important to survival. However, research in anhydrobiosis has recently focused on mechanisms other than the production of trehalose and other compatible osmolytes; notably, desiccation-induced synthesis of chaperoning proteins such as LEA proteins, which could be a mechanism in earthworm embryos (Tunnacliffe and Lapinski, 2003).

The cocoons were transferred directly to water after the desiccation treatments. This rehydration regime exposes embryo cells to hypo-osmotic shock with the rapid influx of water. This recovery treatment was used because it is probably the best reflection of natural conditions where dehydrated cocoons will be abruptly surrounded by liquid water after rainfall. Owing to dehydration the intracellular osmotic pressure is extremely high (78% RH≈15 Osm) and the cells will quickly absorb water and swell. Indeed cellular leakage has been reported for other desiccation-tolerant organisms and model membrane vesicles during rapid rehydration (Cacela and Hincha, 2006), and problems caused by rehydration are arguably as important in the maintenance of cellular integrity and enzyme activity as those incurred from desiccation. However, both acutely

and gradually desiccated cocoons are assumed to be in osmotic equilibrium, and this hypo-osmotic shock is therefore similar for both experimental groups.

Water loss and survival

The highest loss of water in the present study was seen at 78% RH where the gradually and acutely exposed cocoons both lost more than 92% of their initial water content, but where the final water content in the gradually desiccated cocoons was 11% higher than that of the acutely exposed (0.215 and 0.190 mg water mg^{-1} dry mass, respectively). This RH was not tolerated by the acutely desiccated cocoons, but about 70% of the gradually desiccated cocoons survived. Holmstrup and Westh (Holmstrup and Westh, 1995) suggested that the lower critical water content for survival in *D. octaedra* cocoons was equal to the OIW content, thus coinciding with the loss of most, if not all OAW. Using differential scanning calorimetry, OIW of partly desiccated *D. octaedra* cocoons (with a total water content of 0.65 mg mg^{-1} dry mass) has been estimated as the 'unfreezable' fraction of water at –60°C to approximately 0.44 mg water mg^{-1} dry mass (Holmstrup and Westh, 1994) and much higher than the 0.215 mg mg^{-1} dry mass, at which gradually desiccated cocoons in the present study survived reasonably well. However, OIW is probably not a 'fixed' pool of water, and it has been suggested that a portion of the OIW of fully hydrated cocoons is 'released' and becomes OAW in response to the degree of dehydration (Holmstrup and Westh, 1994; Wharton and Worland, 2001). Inspection of data shown in Fig. 4B suggests that OIW of extremely dehydrated cocoons is somewhat lower than estimated by Holmstrup and Westh (Holmstrup and Westh, 1994) stabilising at about 0.2 mg mg^{-1} dry mass. Extrapolation of the data for gradually desiccated cocoons in Fig. 3C predicts that 100% mortality would occur at OIW levels just below 0.2 mg mg^{-1} dry mass suggesting that survival of *D. octaedra* cocoons is not possible if water loss exceeds loss of all OAW. Although the cocoons are able to survive loss of ~95% of their original water content they can probably not be categorised as a truly anhydrobiotic organism as this group of animals often tolerate the loss of 99% of their normal water content (Crowe et al., 1992). Nevertheless *D. octaedra* cocoons have an extreme desiccation tolerance and the present study shows that these cocoons have many physiological similarities to anhydrobiotic organisms, particularly when the desiccation occurs gradually as would be expected in a natural setting. We therefore propose that *D. octaedra* cocoons belong in a transition category between the desiccation sensitive and the truly anhydrobiotic organisms.

This research was supported by the Danish Research Agency (M.H. and M.B.), The Carlsberg Foundation (J.O.), The Danish National Research Foundation and The Danish Biotechnological Instrument Centre (A.M.). Hans Ramløv is thanked for helpful comments on analysis of osmolytes.

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