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Freeze tolerance, supercooling points and ice formation: comparative studies on the subzero temperature survival of limno-terrestrial tardigrades

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SUMMARY

Many limno-terrestrial tardigrades live in unstable habitats where they experience extreme environmental conditions such as drought, heat and subzero temperatures. Although their stress tolerance is often related only to the anhydrobiotic state, tardigrades can also be exposed to great daily temperature fluctuations without dehydration. Survival of subzero temperatures in an active state requires either the ability to tolerate the freezing of body water or mechanisms to decrease the freezing point. Considering freeze tolerance in tardigrades as a general feature, we studied the survival rate of nine tardigrade species originating from polar, temperate and tropical regions by cooling them at rates of 9, 7, 5, 3 and 1°C h^{-1} down to –30°C then returning them to room temperature at 10°C h^{-1}. The resulting moderate survival after fast and slow cooling rates and low survival after intermediate cooling rates may indicate the influence of a physical effect during fast cooling and the possibility that they are able to synthesize cryoprotectants during slow cooling. Differential scanning calorimetry of starved, fed and cold acclimatized individuals showed no intraspecific significant differences in supercooling points and ice formation. Although this might suggest that metabolic and biochemical preparation are non-essential prior to subzero temperature exposure, the increased survival rate with slower cooling rates gives evidence that tardigrades still use some kind of mechanism to protect their cellular structure from freezing injury without influencing the freezing temperature. These results expand our current understanding of freeze tolerance in tardigrades and will lead to a better understanding of their ability to survive subzero temperature conditions.

Key words: tardigrada, differential scanning calorimetry, DSC, supercooling point, SCP, cooling rate, cold tolerance

INTRODUCTION

Tardigrades can be found in a diversity of habitats including marine, freshwater and terrestrial ecosystems ranging from the deep sea to the highest mountains (Nelson, 2002). Many limno-terrestrial tardigrade species are able to tolerate harsh environmental conditions in any developmental stage (Schill and Fritz, 2008) by entering a cryptobiotic state which is induced by desiccation (anhydrobiosis), lack of oxygen (anoxybiosis), osmotic pressure (osmobiosis) or low temperatures (cryobiosis) (Keilin, 1959). In this state metabolism is undetectable and life comes to a reversible standstill until activity is resumed under favourable conditions (Keilin, 1959). In the anhydrobiotic state, tardigrades and other cryptobiotes such as nematodes and rotifers show extraordinary tolerance of physical extremes including high and subzero temperatures, ionizing radiation, alcohols and high pressure (Horikawa et al., 2006; Jönsson et al., 2008; Jönsson and Schill, 2007; Ramløv and Westh, 2001; Rebecchi et al., 2007; Seki and Toyoshima, 1998).

To survive exposure to temperatures below the freezing point of their body fluids, cold tolerant organisms mainly use either the freeze avoidance or freeze tolerance strategy (Storey and Storey, 1996). While freeze avoiding organisms depress the temperature of spontaneous freezing (supercooling point, SCP) by using antifreeze proteins and other cryoprotectants (e.g. sugars and polyols) (Danks et al., 1994; Duman, 2001), ice formation of the extracellular body water is tolerated by freeze tolerant organisms and is often triggered at high temperatures by ice nucleating proteins (Lee and Costanzo, 1998; Ramløv, 2000). In many freeze tolerant organisms, polyols and sugars are accumulated to protect membranes and proteins against phase transition and to control the ice fraction size and minimum cell volume resulting from freeze concentration and osmotic dehydration (Ramløv, 2000; Sinclair et al., 2003; Zachariassen, 1985). Ice active proteins are also sometimes present and may act as recrystallization inhibitors, which prevent the growth and redistribution of ice crystals once these have formed (Duman, 2001; Wharton, 2003).

A third survival strategy termed cryoprotective dehydration has been observed in recent studies on the arctic springtail *Onychiurus arcticus* (Worland and Block, 2003; Worland et al., 1998) and the Antarctic midge *Belgica antarctica* (Elnitsky et al., 2008). In this case, desiccation occurs due to the difference in water vapour pressure between the animal's supercooled body fluids and ice in its surroundings (Elnitsky et al., 2008; Holmstrup et al., 2002; Worland and Block, 2003; Worland et al., 1998).

Some organisms including the Antarctic nematode *Panagrolaimus davidi* even survive intracellular freezing (Smith et al., 2008; Wharton and Ferns, 1995; Wharton et al., 2003). Freeze tolerance in the tardigrade *Richtersius* (*Adorybiotus*) *coronifer*, originating from polar regions, has been reported previously by Westh and colleagues (Westh and Kristensen, 1992), with survival of freezing of more than 80% of the body water during exposure to subzero temperatures. However, whether tardigrades also tolerate intracellular freezing or keep the cytoplasm in a liquid state either by synthesizing cryoprotectants or by cryoprotective dehydration is unknown.

Tardigrades are well known to survive freezing in the dehydrated state (Wright, 2001) but are also reported to survive

exposure to –196°C fully hydrated (Ramløv and Westh, 1992; Sømme and Meier, 1995). However, there have been very few experimental studies concerning the ability of tardigrades to survive natural freezing conditions in a hydrated state (Ramløv and Westh, 1992; Sømme and Meier, 1995; Westh and Hvidt, 1990; Westh et al., 1991). In fact, few detailed studies have been undertaken on cold tolerance as a general feature in any limno-terrestrial organism.

This study presents new data on the effect of various cooling rates on the survival of nine different tardigrade species originating from polar, temperate and tropical areas. SCPs of individual tardigrades were obtained for the first time using differential scanning calorimetry (DSC) and the effects of gut content and acclimation to low temperatures on the SCP were investigated. While SCP measurements in previous studies were obtained using groups of tardigrades, which might be influenced by the nucleation activity of the surrounding water resulting in one single freezing event for the whole group, this study provides the first data on SCPs of individual tardigrades.

MATERIALS AND METHODS

Tardigrades

Laboratory reared and field collected tardigrades were used in this study. Animals of the Eutardigrada species *Macrobiotus sapiens* Binda and Pilato 1984 were collected in Rovinj, Croatia. Specimens of *Milnesium tardigradum* Doyère 1840 and *Paramacrobiotus richtersi* Murray 1911 were collected in Tübingen, Germany. The species *Macrobiotus tonollii* Ramazzotti 1956 was from Eugene, OR, USA (all temperate habitats). *Paramacrobiotus richtersi* 'group 1' was from Nakuru and *Paramacrobiotus richtersi* 'group 2' was from Naivasha, Kenya (tropical habitats). *Paramacrobiotus richtersi* 'group 3' was from Fairbanks, AK, USA (polar habitats). The species used have been characterized by Guidetti and colleagues (Guidetti et al., 2009). Laboratory cultures of the eutardigrade species *M. tardigradum*, *M. tonollii*, *M. sapiens*, *P. richtersi*, *P. richtersi* 'group 1', *P. richtersi* 'group 2' and *P. richtersi* 'group 3' were scaled up for growth on agar plates (3%; peqGOLD Universal Agarose, peqLAB, Erlangen, Germany) covered by a thin layer of Volvic® water (Danone Waters, Wiesbaden, Germany) according to Schill et al. (Schill et al., 2004) and Hengherr et al. (Hengherr et al., 2008). The bdelloid rotifer *Philodina citrina*, raised on the green algae *Chlorogonium elongatum*, was provided as food. Freshly hatched tardigrades were also fed with *C. elongatum*. In addition to rotifers and green algae, adult tardigrades were also fed with the nematode *Panagrellus* sp.

As we have yet to establish a laboratory culture of the herbivorous heterotardigrade species *Echiniscus granulatus* Doyère 1840 and *Echiniscus testudo* Doyère 1840 (both from temperate habitats), we separated individuals directly from moss samples originating from Tübingen (*E. granulatus*) and Munich (*E. testudo*), Germany, using a pipette and a dissecting microsope. Both localities were considered to be temperate.

Subzero temperature treatment

Groups of 40 individuals of each species were transferred in a water droplet into microtubes. Subsequently, the remaining water was reduced using a micropipette, leaving a residual water volume of 1–2 µl. Constant cooling and thawing rates were achieved using a Reichert AFS automatic freeze substitution system (Leica, Munich, Germany). Laboratory studies have used a variety of cooling rates, usually distributed around 1°C min^{-1} to investigate cold hardiness in insects, though these cooling rates have been criticized as too fast compared with natural cooling rates (Sinclair et al., 2003). Natural cooling rates around 3 and 6°C h^{-1} have been recorded in ecological studies on e.g. *Drosophila melanogaster* (Kelty and Lee, 1999). To study the cold tolerance of tardigrades under ecologically relevant cooling rates, we used cooling rates of 9, 7, 5, 3 and 1°C h^{-1}. The tardigrades were exposed to the cooling rates starting at room temperature (RT, 20°C) down to –30°C, followed by a warming period at 10°C h^{-1} up to RT. Alive and dead animals were recorded after thawing. Tardigrades were assumed to be dead if there was no movement visible within 2 h of rehydration. Four replicates per species were used for each cooling treatment (see Table 1).

Calorimetry

A Mettler-Toledo differential scanning calorimeter (DSC820, Mettler-Toledo, Leicester, UK) was used to measure the SCP (=temperature of crystallization, T_c) and quantity of water freezing in tardigrades as they were cooled at 10°C min^{-1} from 25°C to 5°C and at 1°C min^{-1} from 5°C to –30°C. To obtain crystallization points for each individual the animals were placed in separate single droplets of water in aluminium pans. Residual water around each individual was removed with filter paper directly before the pans were hermetically sealed and placed in the calorimeter. After the determinations, the animals were checked visually for any indication of dehydration resulting from a poorly sealed pan, which would have a large effect on the SCP; data for such samples were not included in this study. The calorimeter was calibrated using indium as an upper temperature and enthalpy standard (melting point 156.6°C, enthalpy 28.71 J g^{-1}) and the melting point of ice as a lower temperature check. A standard temperature program starting at 25°C and cooling rapidly (10°C min^{-1}) to 5°C then to –30°C at 1°C min^{-1} and returning to 5°C at the same rate was used for all measurements. The quantity of water freezing in the animals (osmotically active water) was calculated from the freeze exotherm using an enthalpy value of 334.5 J g^{-1}.

The total body water of the animals was determined by deducting the dry mass (after drying for 24 h at RT over silica gel) from the fresh mass. The measurements were made on groups (N=4) with 3–8 animals.

To obtain the SCP of tardigrades with empty guts, animals of all species were starved over 2 days before starting the calorimetric analysis, whereas individuals of *P. richtersi* 'group 3' (N=15) and *P. richtersi* (N=15) were additionally fed before measurement to study the effect of food content in the gut on the SCP. To test the influence of cold acclimatization on the SCP, individuals of all species were cold adapted at 15°C for 24 h, followed by 48 h at 8°C and a further 48 h at 4°C before starting the DSC measurements. The resulting SCPs were compared with the SCPs of starved animals. SCPs were obtained for 15–31 animals per species in each treatment. The exact number of animals used (N) for each SCP determination is indicated in Table 2.

Statistics

The statistical significance of differences in the SCPs and frozen body water was tested using a Kruskal–Wallace one way ANOVA followed by an all pairwise multiple comparison procedure (Dunn's method; SigmaStat 3.5, Systat Software GmbH, Erkrath, Germany). The survival rate after different cooling rates was tested on significant differences using a one way repeated measures ANOVA and a Tukey test as an all pairwise multiple comparison procedure. Significance levels were $P>0.05$ (not significant) and $P\leq0.05$ (significant).

Table 1. Percentage survival (means±s.d.) of the tested tardigrade species after cooling at different rates from room temperature (RT) down to –30°C with a constant thawing rate of 10°C h^{-1} up to RT again

Species	9°C h^{-1}	7°C h^{-1}	5°C h^{-1}	3°C h^{-1}	1°C h^{-1}
M. sapiens	47.5±17.1	43.8±14.9	0.0±0.0	0.0±0.0	27.5±10.4
P. richtersi 'group 1'	90.0±10.8	77.5±10.4	32.5±11.9[c]	16.3±14.9	27.5±5.0[a]
P. richtersi 'group 2'	23.8±8.5	7.5±5.0	5.0±4.8	15.0±10.8	38.8±11.9
P. richtersi 'group 3'	20.0±4.8	3.8±4.8	1.3±2.5	8.8±2.3	15.0±7.1
P. richtersi	92.5±9.6	66.3±8.5	55.0±14.7[c]	61.3±19.3	72.5±6.5[a,b]
M. tonollii	35.0±8.2	0.0±0.0	27.5±13.2	35.0±8.2	76.3±12.5[a]
M. tardigradum	95.0±4.1	86.3±8.5	71.3±8.5	71.3±20.2	86.3±7.5
E. granulatus	53.1±3.2	46.9±7.5	22.5±11.4	52.5±8.4	60.6±4.3
E. testudo	46.9±5.5	38.8±6.3	19.4±5.5	17.5±5.4	42.5±4.6

Four replicates (N=4), containing 40 animals each, were used per cooling rate and species. [a]Significant difference between 9 and 1°C h^{-1}, [b]significant difference between 3 and 1°C h^{-1}, and [c]significant difference between 9 and 5°C h^{-1}.

RESULTS

Low temperature survival

In our first experiment, the tardigrades were subjected to different cooling rates. Table 1 presents the survival rate of different species of tardigrades after cooling at different rates. Cooling at 9°C h^{-1} resulted in higher survival compared with slower cooling rates of 7, 5 and 3°C h^{-1}. No *M. tonollii* animals survived after cooling at 7°C h^{-1} but survival increased at a cooling rate of 1°C h^{-1}. All other species showed a decrease in survival with a reduction in cooling rate from 9 to 5°C h^{-1} and an increase again towards 1°C h^{-1}. Increased survival rates after cooling at 9°C h^{-1} compared with 1°C h^{-1} were seen for the temperate species *E. testudo*, *M. tardigradum*, *M. sapiens*, *P. richtersi*, the tropical species *P. richtersi* 'group 1' and the polar species *P. richtersi* 'group 3', being significantly different only in *P. richtersi* 'group 1' and *P. richtersi*. Increased survival after cooling at 1°C h^{-1} was shown by *P. richtersi* 'group 2' (tropical), *M. tonollii* and *E. granulatus* (both temperate), with *M. tonollii* showing the only significant difference.

Interspecific comparison of the survival rate after cooling at different rates revealed high variations. *M. tardigradum* showed the highest survival rate of 95.0±4.1% at 9°C h^{-1} (mean±s.d.) and the lowest survival rate of 71.3±20.2% at 3°C h^{-1}. However, no significant differences in survival between the different cooling rates were observed. Although *P. richtersi* and *P. richtersi* 'group 1' also showed high survival rates after cooling at 9°C h^{-1} (92.5±9.6% and 90.0±10.8%), their recovery ability dropped significantly to 55.0±14.7% and 32.5±11.9%, respectively, after cooling at 5°C h^{-1}. While the survival ability of *P. richtersi* 'group 1' decreased further to 16.3±14.9% (3°C h^{-1}) and only increased significantly to 27.5±5.0% after cooling at 1°C h^{-1}, the value of *P. richtersi* increased steadily with decreasing cooling rate to a survival of 72.5±6.5% after the 1°C h^{-1} treatment. Most of the other species showed a lower but generally similar pattern of survival. A total loss of the ability to survive after cooling rates of 5 and 3°C h^{-1} was observed in *M. sapiens* and after 7°C h^{-1} in *M. tonollii*.

Calorimetry

In the second experiment, SCPs (means±s.d.) of the individual specimens, measured by DSC, ranged between –23.7±3.9°C for *M. sapiens* and –11.5±2.2°C for *E. granulatus* in starved animals. Fig. 1 illustrates a representative thermogram with each peak indicating a freezing event of an individual specimen. Ice formation appears to be a rapid process with the freezing exotherm lasting less than 30 s in all species used in this study. Differences in the SCP of starved and fed animals were only observed between the two heterotardigrades and the eutardigrades tested in this study (Table 2). The SCP of *E. granulatus* was significantly different from the SCP of all other tested species except *E. testudo*. For the SCP of *E. testudo* significant differences from *M. sapiens*, *M. tonollii* and *M. tardigradum* were observed. Neither acclimation to low temperatures nor a well fed condition in *P. richtersi* (temperate) and *P. richtersi* 'group 3' (polar) affected the SCP significantly (Table 2).

The total body mass (fresh mass) of the tested tardigrades ranged from 4.6±0.3 µg in *M. sapiens* to 7.9±0.7 µg in *M. tardigradum* with corresponding water masses from 3.7±0.3 µg in *M. sapiens* up to 6.5±0.1 µg as indicated in Table 3. A comparison of the amount of frozen body water between cold acclimatized tardigrades and animals kept at room temperature indicates a slight but not significant decrease in quantity in the cold acclimatized tardigrades (Table 2).

DISCUSSION

Despite the interspecific variations in the survival ability of tardigrades exposed to subzero temperatures, this study underlines the remarkable ability of all investigated species to cope with unfavourable environmental conditions at low temperatures regardless of their origin. Considering the survival rates and the calorimetry measurements, we may state that all tardigrades tested in this study can tolerate ice formation within their body and therefore belong to the group of freeze tolerant organisms with a high potential to supercool and the ability to survive temperatures below the SCP. Furthermore, our results support earlier investigations on the eutardigrade species *Richtersius coronifer* (Ramløv and Westh, 1992) that the cooling rate has a large influence on the survival of tardigrades. In contrast, however, most of our tested species not only showed an increased level of survival at the slowest cooling rate but also often showed an even better survival at the fastest cooling rate (9°C h^{-1}). Although we cannot completely explain the reasons for this effect, it might be due to the physical effects of rapid cooling and ice crystal formation.

Several studies have reported that the capacity to supercool decreases with increasing body mass (Johnston and Lee, 1990; Lee and Costanzo, 1998). Although our data partly support these results, with the smallest eutardigrade in our study (*M. sapiens*) having the lowest (though not significantly so) SCP of all tested eutardigrades, it may be challenged by the presence of the much higher SCP of the equally small heterotardigrades *E. granulatus* and *E. testudo*.

It has been documented that some arthropod species shed their mid-gut during moulting, involving the complete evacuation of the gut contents, which would otherwise initiate ice nucleation at a relatively high temperature. It is known that this process decreases the SCP in some arthropods (Lee and Costanzo, 1998; Worland and Convey, 2008; Worland et al., 2006). As it was not possible to culture

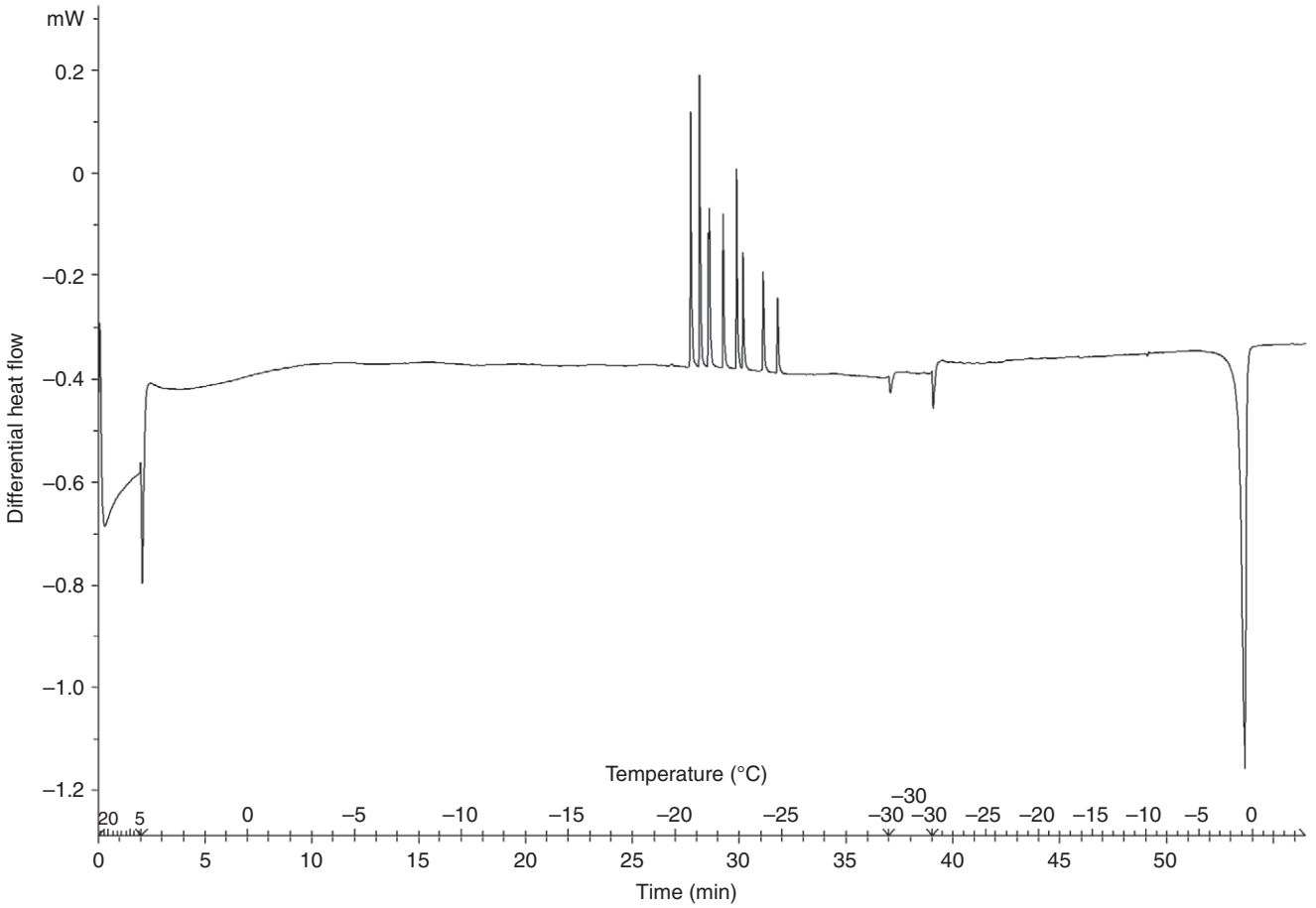

Fig. 1. Representative thermogram illustrating the freeze–thaw cycle of starved animals of *Paramacrobiotus richtersi* 'group 1' which were kept at room temperature and placed separately in the pan. Each peak in the cooling period indicates a freezing exotherm of an individual tardigrade. Mean values of the supercooling points are given in Table 2. The peak of the melting endotherm close to 0°C represents the melting of the frozen body water of all specimens together.

the two heterotardigrades *E. granulatus* and *E. testudo* and therefore starvation could not be performed, it is quite possible that some gut contents initiated ice nucleation, which may explain the higher SCP. Interestingly, our data for the eutardigrades *P. richtersi* and *P. richtersi* 'group 3', where no difference in SCP distribution of starved and well fed animals was observed, challenges an involvement of the gut contents as ice nucleators in tardigrades. This is in accordance with other studies made in insects where no effect of the gut content on the SCP distribution has been observed, thus making the role of feeding and nutritional condition as a SCP influencing factor contentious (Klok and Chown, 1998; Worland and Block, 1999). However, an effect due to moulting, as observed

Table 2. Calorimetry results of the tardigrades after a period of starvation, cold acclimatization and feeding

Species	SCP starved (°C)	*N*	SCP cold acclimatized (°C)	*N*	SCP fed (°C)	*N*	% of body water frozen during freezing of exotherm: Warm acclimatized	% of body water frozen during freezing of exotherm: Cold acclimatized
M. sapiens	–23.7±3.9[a,b]	28	–24.2±3.9[a]	16	n.t.		84.5±6.1	81.1±3.2
P. richtersi 'group 1'	–21.7±2.1[a]	20	–19.2±2.0[a]	18	n.t.		84.3±6.1	79.9±4.4
P. richtersi 'group 2'	–20.5±4.9[a]	31	–21.0±4.9[a]	18	n.t.		85.5±5.9	82.6±4.2
P. richtersi 'group 3'	–20.4±2.9[a]	15	–21.3±2.3[a]	15	–21.5±4.5	15	86.8±4.0	80.9±4.9
P. richtersi	–21.6±3.8[a]	27	–20.6±2.8[a]	19	–20.9±4.3	15	85.7±3.7	82.5±6.5
M. tonollii	–21.8±3.2[a,b]	28	–20.9±4.0[a]	18	n.t.		86.4±3.0	812.0±5.3
M. tardigradum	–22.0±3.5[a,b]	26	–22.6±2.8[a]	21	n.t.		85.1±7.8	80.5±4.5
E. granulatus	–11.5±3.0	15	–10.3±1.9	15	n.t.		83.8±8.1	78.0±5.4
E. testudo	–18.4±2.2	23	–16.7±3.9	17	n.t.		84.3±8.2	80.5±4.9

Values are presented as means ± s.d. (n.t., not tested). *N*=individual animals used for each supercooling point (SCP) determination. [a]Significant difference from SCPs of *E. granulatus*; [b]significant difference from SCPs of *E. testudo*.

Table 3. Total body mass and water mass (means±s.d.) of the tested tardigrade species

Species	Body mass, starved (μg)	Water mass, starved (μg)	Body mass, fed (μg)	Water mass, fed (μg)
M. sapiens	4.6±0.3	3.7±0.3	n.t.	n.t.
P. richtersi 'group 1'	6.2±0.8	4.9±0.7	n.t.	n.t.
P. richtersi 'group 2'	6.9±0.6	5.4±0.4	n.t.	n.t.
P. richtersi 'group 3'	5.7±0.4	4.5±0.3	5.6±0.5	4.6±0.4
P. richtersi	7.3±0.6	5.9±0.5	7.1±0.2	5.8±0.1
M. tonollii	5.0±0.3	3.9±0.2	n.t.	n.t.
M. tardigradum	7.9±0.7	6.5±0.1	n.t.	n.t.
E. granulatus	4.6±0.6	3.7±0.5	n.t.	n.t.
E. testudo	4.8±0.9	3.8±0.7	n.t.	n.t.

Results from groups (*N*=4) containing 3–8 animals (n.t., not tested).

in Collembola (Worland et al., 2006), cannot be excluded. Considering the fact that limno-terrestrial tardigrades live in habitats where frequent freeze and thaw cycles may occur, it might be energetically essential to retain gut contents during freezing periods and freeze tolerance may be crucial due to the possible hazards of inoculation from nucleators. However, it has been shown that tardigrades are capable of surviving fairly long periods without food (Ramazzotti and Maucci, 1983).

As no changes in either the melting point (data not shown) or the SCP distribution were observed in either the tardigrades kept at room temperature or those acclimatized to low temperatures, it is unlikely that low molecular weight cryoprotectants such as polyols are synthesized as observed in freeze tolerant insects (Worland, 2005; Zachariassen, 1985). The non-reducing disaccharide trehalose has been found in tardigrades of the genus *Richtersius*, *Macrobiotus*, *Paramacrobiotus* and *Echiniscus* (Hengherr et al., 2008; Westh and Ramløv, 1991) but, interestingly, carbohydrate analysis did not detect trehalose in active or anhydrobiotic *M. tardigradum* (Hengherr et al., 2008) which tend to show the highest survival. However, low concentrations of other small carbohydrates may still be involved in freeze tolerance.

Cold acclimation at 4°C resulted in a decreased amount of water in all tested tardigrade species of the present study as has been shown in winter acclimatized animals of *Richtersius coronifer* and *Amphibolus nebulosus* (Westh and Kristensen, 1992). This may reflect an increase in the amount of 'bound' water due to interactions between water and macromolecules, as investigated in insect larvae (Storey et al., 1981).

DSC studies on *R. coronifer* and *A. nebulosus* showed a SCP between –6.7 and –7.4°C during slow cooling (Westh and Kristensen, 1992; Westh et al., 1991). In subsequent experiments, Westh and colleagues (Westh et al., 1991) provided conclusive evidence for the existence of ice nucleating agents (INA) in *R. coronifer*. INAs consist of proteins or lipoproteins which initiate heterogeneous ice nucleation and freezing at temperatures typically between –5 and –10°C (Duman, 2001; Wright, 2001). A decrease of the SCP to –16°C after previous heating to 90°C and DSC analysis of gel filtered body fluids indicate that the ice nucleating activity is composed of proteins (Westh et al., 1991). In contrast, our DSC measurements show a much lower SCP in all species. Plotting the SCPs as a function of water volume together with available data on the nucleation temperature of pure water samples and freeze avoiding insects (Mackenzie et al., 1977; Wilson et al., 2003; Zachariassen et al., 2004) (Fig. 2) shows that the SCPs of *Macrobiotus*, *Paramacrobiotus* and *M. tardigradum* fit the regression lines of the earlier studies, indicating a homogeneous nucleation and that INAs are not present in these eutardigrades.

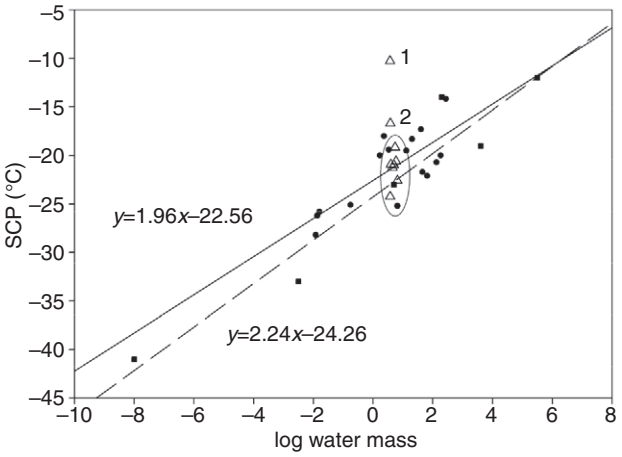

Fig. 2. Nucleating temperatures of the tested tardigrades plotted as a function of the logarithm of their water content. The data points are indicated as triangles. 1 indicates the data point of *E. granulatus* and 2 that of *E. testudo*. The ellipse indicates the cluster of all eutardigrades used in this study. The broken line is the linear regression line of the data points (squares) of pure water samples observed by MacKenzie and colleagues (MacKenzie et al., 1977) and Wilson and colleagues (Wilson et al., 2003). The solid line is the linear regression line of data points (circles) obtained from freeze avoiding insects [modified from Zachariassen et al. (Zachariassen et al., 2004)].

However, an involvement of INAs cannot be excluded in the case of *E. granulatus* and *E. testudo*. A further possible reason for higher SCPs might be the higher potential for inoculative freezing when using pooled animals with probably more surrounding water, as in the earlier studies (Westh and Hvidt, 1990; Westh and Kristensen, 1992; Westh et al., 1991), than when measuring individual specimens, resulting in distinct crystallization events for each individual with a low potential for inoculative freezing due to surrounding animals or water.

Although the invariant SCPs, the fast ice formation and the fast cooling rates tolerated by the tardigrades may lead to the assumption that they do not require metabolic and biochemical preparation prior to subzero temperature exposure, we must not exclude it. In fact, the increase in survival at slow cooling rates down to $1°C\,h^{-1}$ presented by all species may indicate that the animals synthesize e.g. ice active proteins or cryoprotective compounds to increase their survival ability. Considering the trend of all species to survive better after cooling at slow rates we may postulate higher survival rates below $1°C\,h^{-1}$. With slower rates, the tardigrades would have even

more time to synthesize or recruit protecting agents involved in regulating ice growth to conserve cell structure. Environmental cooling rates of around 0.6°C h^{-1} in temperate environments are not unusual (Sinclair, 2001). However, further detailed studies concerning cooling rates in typical field microhabitats such as moss cushions are required.

Extracellular ice formation will subject the cells and tissues to freeze dehydration. As a consequence of ice formation, intracellular solutes become more concentrated and the cells become osmotically dehydrated. Fast ice growth in tardigrades, which has also been reported by Westh and Kristensen (Westh and Kristensen, 1992), presents an enormous osmotic shock which, together with cell volume collapse, is probably the most likely cause of mortality in unprotected cells (Lee and Costanzo, 1998; Storey and Storey, 1996). Therefore tolerance to subzero temperatures in tardigrades may be related to tolerance to extreme dehydration, which also requires the ability to deal with wide variations in cell volume and osmolality of body fluids. Besides the osmotic shock, rapid ice formation increases the likelihood of intracellular freezing with its associated physical problems resulting in cellular damage due to rapid changes in cell volume and damage from growing ice crystals (Wright, 2001). Intracellular freeze tolerance, if it occurs, could provide a successful strategy for tardigrades to tolerate low temperatures and reduce transmembrane osmotic stress during freezing as has been demonstrated in the Antarctic nematode *P. davidi* (Smith et al., 2008; Wharton and Ferns, 1995; Wharton et al., 2003). However, studies concerning intracellular freezing in tardigrades have yet to be performed. Molecular and metabolic investigations at slow cooling rates as used in this experiment could provide an insight into the remarkable phenomenon of cold tolerance in tardigrades.

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