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Ice crystallization and freeze tolerance in embryonic stages of the tardigrade *Milnesium tardigradum*

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ABSTRACT

In tardigrades, tolerance to low temperature is well known and allows them to cope with subzero temperatures in their environment. Although the ability to tolerate freezing body water has been demonstrated in some tardigrades, freeze tolerance of embryonic stages has been little studied, although this has ecological significance. In this study, we evaluated the subzero temperature survival of five different developmental stages of the eutardigrade species *Milnesium tardigradum* after freezing to −30 °C. Embryos were exposed to five different cooling rates between room temperature and −30 °C at 1 °C/h, 3 °C/h, 5 °C/h, 7 °C/h, and 9 °C/h followed by a warming period at 10 °C/h. The results showed that the developmental stage and the cooling rate have a significant effect on the hatching rate. Less developed embryonic stages were more sensitive to freezing at higher freezing rates than more developed stages. Differential Scanning Calorimetry (DSC) was used to determine the temperature of crystallization (*Tc*) in single embryos of the different developmental stages and revealed no differences between the stages. Based on the calorimetric data, we also conclude that the ice nucleation is homogeneous in embryonic stages in tardigrades, as also recently shown for fully developed tardigrades, and not triggered by nucleating agents.

1. Introduction

One of the most important abiotic factors in determining the state of activity and geographic distribution of organisms is temperature (Doucet et al., 2009). Tardigrades, a group of hydrophilous micrometazoans, inhabit diverse habitats in terrestrial, freshwater and marine environments at all latitudes and altitudes (Nelson, 2002). Some tardigrade species that occupy very unstable habitats like mosses, lichens and intertidal zones exhibit a remarkable tolerance to physical extremes including almost complete desiccation, ionizing radiation, vacuum, high pressure and low and high temperatures (Hengherr et al., 2009a; Hengherr et al., 2009b; Horikawa et al., 2006; Jönsson et al., 2008; Jönsson and Schill, 2007; Ramløv and Westh, 1992; Ramløv and Westh, 2001; Westh and Kristensen, 1992).

Colonization of habitats where low temperature periods occur frequently requires certain adaptations that reduce the impact of cold and freezing stress. Physiological based work on the mechanisms, predominantly in insects, has highlighted several strategies including freeze tolerance, freeze avoidance, protective dehydration and rapid cold hardening (Clark and Worland, 2008). Freeze avoidance is associated especially with the production of antifreeze proteins (AFP) and small organic molecules such as polyhydroxy alcohols and sugars that are involved in depressing the temperature in spontaneous freezing (supercooling) which would be lethal for freeze susceptible organisms (Danks et al., 1994; Doucet et al., 2009; Duman, 2001). In contrast to the freeze-avoiding organisms, controlled ice formation of the body water is tolerated by freeze-tolerant organisms (Lee and Costanzo, 1998). As has been shown for the gall fly *Eurosta solidaginis* (Fitch, 1855), many freeze-tolerant organisms also accumulate cryoprotectants such as sugars, polyhydroxy alcohols and amino acids to maintain the cellular contents in an unfrozen state (Danks et al., 1994; Storey and Storey, 1996). Even AFPs can be found in freeze-tolerant species where they inhibit ice recrystallization and thus prevent the formation of large ice crystals and reduce tissue damage (Doucet et al., 2009). However, some organisms like the Antarctic nematode *Panagrolaimus davidi* Timm, 1971 even survive freezing of the intracellular compounds (Smith et al., 2008; Wharton et al., 2005; Wharton and Ferns, 1995; Wharton et al., 2003).

The tolerance of tardigrades to low temperatures is well known (Sømme, 1996; Wright, 2001) and the ability to survive freezing body water has been demonstrated in some tardigrade species characterising them as freeze-tolerant (Halberg et al., 2009; Hengherr et al., 2009a; Westh and Hvidt, 1990; Westh et al., 1991).

Ice formation in freeze-tolerant organisms usually occurs at relatively high subzero temperatures (−5 to −10 °C) due to the presence of ice nucleating agents (INA) in the extracellular fluid (Block, 1991;

Abbreviations: DSC, Differential scanning calorimetry; INA, Ice nucleating agent; AFP, Antifreeze protein; RH, Relative humidity; RT, Room temperature; SCP, Supercooling point; *Tc*, Temperature of crystallization.

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Duman, 2001). This leads to a slow process of ice formation where the growth of ice crystals can be controlled, resulting in smaller, and potentially less damaging initial ice crystals upon freezing. However, one of the most detailed studies on freeze tolerance of tardigrades (Hengherr et al., 2009a) revealed that tardigrades show much lower freezing temperatures (around −20 °C) and provided evidence that the ice nucleation is likely to be homogenous and not triggered by INAs.

The previous studies on freeze tolerance in tardigrades have contributed important information on our current understanding of their ability to cope with low temperatures in their environment. However, the freeze tolerance of embryonic stages has been little studied, although, in terms of their widespread distribution, this is an important ecological aspect.

In the first part of our study, we evaluated the subzero temperature survival of five different developmental stages of the eutardigrade species *Milnesium tardigradum*, subjected to different cooling rates. Differential Scanning Calorimetry (DSC) was used in the second part to determine the nucleation temperature and type in the embryonic stages to determine whether the crystallization is triggered by nucleating agents.

2. Material and methods

2.1. Organisms

To study the low temperature tolerance of embryonic stages of tardigrades the cosmopolitan eutardigrade *M. tardigradum* Doyère, 1840 (Apochela, Milnesiidae) was used. Specimens were originally collected from dry moss in Tübingen, Germany, where low temperatures frequently occur during wintertime (Sträßer, 1998; Wallén, 1977). A laboratory culture of *M. tardigradum* was scaled up for growth in plastic culture dishes with 4 mm layers of 3% agar (peqGOLD Universal agarose, peqLAB Erlangen, Germany) covered with a thin layer of Volvic™ water (Danone Waters Deutschland, Wiesbaden, Germany) at 20 °C and a 12 h light 12 h dark cycle, according to Schill et al. (2004) and Hengherr et al. (2008b). The bdelloid rotifer *Philodina citrina* Ehrenberg, 1832, cultured separately in Volvic™ water and raised on the green algae *Chlorogonium elongatum* Dang. 1897 was provided as food. Egg-containing exuvia were collected every 24 h and placed in 24 well-plates with Volvic™ water. After 5 days, five different developmental stages were available in the well-plates. The stage classification according to Suzuki, (2003) was used. Stage 1 was the blastula stage, 24 h after egg deposition. Stage 2 was 24 h later and showed morphogenetic movement and produced a ventro-lateral cleft. Stage 3, after 72 h, was characterized by an increasing transparency. Occasional rotatory movements represent stage 4 after 96 h. After 120 h (stage 5) the mouthparts became visible and the development was almost completed. Therefore stage 5 was followed by hatching within a few hours. For the cooling experiments exuviae of each developmental stage were split into 25 cohorts containing 40 embryos each.

2.2. Subzero temperature treatment

Each cohort was transferred in a water droplet into microliter tubes (Sarstedt, Nümbrecht, Germany). Afterwards a micropipette was used to reduce the remaining water to a residual volume of approximately 1 to 2 µL. To provide constant cooling and thawing rates a Reichert AFS automatic freeze substitution system (Leica, Munich, Germany) was used. Starting at room temperature the embryonic stages were cooled rapidly (15 °C h^{-1}) from room temperature (RT) to 4 °C. Subsequently they were exposed to cooling rates of 9, 7, 5, 3 and 1 °C h^{-1} down to −30 °C, held there for 60 min, followed by a thawing rate of 10 °C h^{-1} up to room temperature according to Hengherr et al. (2009a). After the subzero temperature treatment the embryonic stages were placed into 24 well-plates with Volvic™ water at 20 °C. The embryos were checked each day under a stereomicroscope and hatched tardigrades were recorded. To provide a measure of embryo freeze tolerance the subsequent hatching success of the different developmental stages was compared. Additional five cohorts (40 embryos each) of each developmental stage were not subjected to low temperature and therefore used as controls.

2.3. Differential scanning calorimetry

The kinetics of ice formation associated with subzero temperature exposure of embryonic stages of *M. tardigradum* were studied with Differential Scanning Calorimetry. Individual embryo-containing eggs ($N=15$) of the 5 different developmental stages were transferred in separate single droplets of water into 40 µL aluminium pans. Residual water around each egg was removed with a filter paper directly before the pans were hermetically sealed and placed into the calorimeter (DSC1 equipped with an Intercooler II, Mettler-Toledo, Gießen, Germany). The samples were weighed before and after the DSC run to ensure that no water loss has occurred. The calorimeter was calibrated using indium as an upper temperature enthalpy standard (melting point 156.6 °C, enthalpy 28.71 Jg^{-1}) and the melting point of ice as lower temperature check. The standard protocol starting at 25 °C and cooling rapidly (10 °C min^{-1}) to 5 °C and at 1 °C min^{-1} from 5 °C to −30 °C returning to 5 °C at the same rate was used for all measurements with the calorimeter head under a stream of dry nitrogen as purge gas.

The total water content of the eggs was determined by deducting the dry mass (after drying for 24 h at 60 °C and 33% relative humidity (RH)) from the fresh mass using a fine scale Supermicro S4 2405 (Sartorius, Göttingen, Germany). The measurements were made for every single developmental stage on groups ($N=5$) with 20 eggs.

To determine the type of nucleation we plotted the temperature of crystallization (Tc) as a function of water volume together with available data from earlier studies on nucleation temperatures of pure water samples, freeze-avoiding insects and freeze-tolerant eutardigrade species (Hengherr et al., 2009a; Mackenzie et al., 1977; Wilson et al., 2003; Zachariassen et al., 2004).

2.4. Statistics

Differences in the hatching rates of the developmental stages and the cooling rates were tested for significance using a one way repeated measures ANOVA followed by a Bonferroni *t*-test as an all pairwise multiple comparison procedure. All statistics were carried out with the software SigmaStat 3.5 (Systat Software GmbH, Erkrath, Germany) and the significance levels were set as $P>0.05$ (not significant) and $P\leq 0.05$ (significant).

3. Results

3.1. Survival after subzero temperature treatment

The post freeze survival and hatching after the exposure to low temperatures at different cooling rates and developmental stages is given in Table 1 and Fig. 1. Both factors, stage and cooling rate, have a significant effect on the hatch rate (Tables 2 and 3). Stage 1, the blastula stage, showed in general a poor survivorship and hatching rate, irrespective to the cooling rate. Their best hatch rate (12%, s.d. ± 4.5) was found after cooling them with 1 °C h^{-1}, their lowest (3.8%, s.d. ± 4.3) at 5 °C h^{-1}. All cooling conditions resulted in a delayed development and a low hatch rate related to the control (Fig. 1A). Stage 2 also showed a low survivorship after all cooling treatments. The only significant increase was observed after the 9 °C h^{-1} treatment. Compared to the delayed development in stage 1, the development of the stage 2 embryos appears a bit quicker, except at the cooling rates 1 °C h^{-1} and 5 °C h^{-1} which show a similar delayed development as embryos of stage 1 (Fig. 1B). A significant increase in hatch rate of 87% (s.d. ± 2.7) with cooling rates of 1 °C h^{-1} was observed in stage 3 embryos, in