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ORIGINAL RESEARCH

Reduced ageing in the frozen state in the tardigrade *Milnesium inceptum* (Eutardigrada: Apochela)

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Abstract

Tardigrades can survive harsh environmental conditions, such as drought and low temperature. To withstand freezing, they enter cryobiosis, a state of biological organization in which metabolic activity slows down or comes reversibly to a standstill. Thus, cryobiosis resembles anhydrobiosis, where tardigrades (and a few other invertebrate groups) undergo extreme desiccation and appear not to age in the dry state. The lack of ageing in the anhydrobiotic state, the so-called 'Sleeping Beauty' hypothesis, is assumed also to pertain to cryobiosis, but this has not been investigated. To test this, a group of tardigrades was subjected to sub-zero temperature treatment by alternating weekly periods of freezing at −30°C and feeding at 20°C. The temporarily frozen tardigrades lived twice as long as the control group, but both control and temporarily frozen groups had similar lifespans if the time spent frozen was excluded. This represents the first demonstration that the 'Sleeping Beauty' hypothesis applies to cryobiosis, meaning that tardigrades do not age while frozen.

Introduction

The severity, seasonality, unpredictability and variability of environmental condition determine the life cycle patterns of invertebrates, especially not only in polar and alpine regions but also in temperate regions. At high altitudes and latitudes, ice formation is an important variable determining the survival of freshwater fauna and terrestrial fauna that depend on the humidity of substrates such as lichens, mosses and leaf litter. There are various documented strategies that explain how tardigrades and other arthropods survive low temperatures. Besides freeze avoidance, where the animal depresses the temperature of spontaneous freezing by using antifreeze proteins or other cryoprotectants, it is also possible to tolerate ice formation of the extracellular body water (Duman, 2001; Storey & Storey, 1996; Zachariassen et al., 2002; Zachariassen et al., 2004). Furthermore, some organisms are able to survive by cryoprotective dehydration, where desiccation occurs due to the difference in water vapour pressure between the supercooled body and the ice in the surroundings (Elnitsky et al., 2008; Hayashi & Wharton, 2010; Lopez-Martinez et al., 2009; Smith et al., 2008; Wharton, 2003; Wharton et al., 2005) or even by tolerating intracellular freezing (Sinclair & Renault, 2010; Wharton et al., 2005; Wharton & Ferns, 1995).

Some Tardigrades can survive freezing while either hydrated or dehydrated (Guidetti, Altiero, Bertolani, et al., 2011; Halberg et al., 2009; Hengherr et al., 2009; Møbjerg & Neves, 2021; Wright, 2001; Wright et al., 1992) and can tolerate ice formation within their body; such tardigrades should therefore be regarded as freeze-tolerant organisms. The survivorship and the amount of molecular damage accumulated after a cryptobiotic event are directly related to (i) the time spent in the cryptobiotic state, (ii) the environmental abiotic parameters at which the animals are maintained during the cryptobiotic state, (iii) and the abiotic and biotic conditions present during the initial or final phases of the cryptobiotic process (Guidetti, Altiero, & Rebecchi, 2011). The knowledge of freeze-tolerance abilities is based on studies performed on a few tardigrade species. Guidetti, Altiero, Bertolani, et al. (2011) showed a species-specific cryobiotic performance which is related to the microhabitat in which each tardigrade species dwells and consequently to its ecological traits. Tardigrade species living in xeric habitats (e.g. *Ramazzottius oberhaeuseri* and *Paramacrobiotus richtersi*) better withstand freezing than those living in hygrophilous habitats (e.g. *Hypsibius dujardini*), while true limnic species (e.g. *Dactylobiotus parthenogeneticus*) do not show any cryobiotic ability (Guidetti, Altiero, Bertolani, et al., 2011).

In general, cell ageing is closely related to metabolism and cell damage. But what happens when metabolic activities in particular change in cryptobiotic organisms? In cryptobiotic organisms, three possible scenarios have been proposed for the influence of cryptobiosis on the internal clock, which counts the time of ageing and controls the associated metabolic processes. The first model, called 'Sleeping Beauty', states that there is no ageing during the inactive period, while the other two models suggest that ageing does occur in this state, either at a slower rate than normal or at the same rate as in the active state (Ricci & Caprioli, 2005; Ricci & Pagani, 1997). The 'Sleeping Beauty' model has been shown to apply to tardigrades in the anhydrobiotic state (Hengherr et al., 2008). Despite the fact that freezing and drying are not identical stress vectors (Crowe et al., 1990), there is also the possibility of three scenarios during cryobiosis in tardigrades: ageing and metabolism of the frozen cells and thus of the tardigrades either progresses, slows down or stops.

Tsujimoto and colleagues (Tsujimoto et al., 2016) managed to recover viable, fertile tardigrades that had been frozen for over 30 years. Therefore, either the 'Sleeping Beauty' or the slow ageing model is likely to describe the effects of cryobiosis on tardigrade longevity, because the normal, hydrated lifespan of tardigrades is no more than 2 years (Altiero et al., 2006; Altiero et al., 2018; Hengherr et al., 2008; Lemloh et al., 2010; Rebecchi et al., 2006; Suzuki, 2003). The purpose of the current experimental study was to analyse the effect of cryobiosis on the internal clock of *M. inceptum* (Morek et al., 2019) by comparing whether periodically frozen animals have a longer lifespan than active animals in cultures. This should lead to a better understanding of the mechanisms of cryobiosis and its effects on tardigrades.

Materials and methods

Tardigrade culture

Laboratory-reared tardigrades of the species *Milnesium inceptum* Morek, Suzuki, Schill, Yankova, Georgiev, Marley, Michalczyk, 2019 (Eutardigrada: Apochela) were used in this study. They were cultured and scaled up for growth on agar plates (3%; Lonza, Rockland, ME, USA) covered by a thin layer of Volvic® water (Danone Waters, Wiesbaden, Germany). The bdelloid rotifer *Philodina citrina* Ehrenberg, 1832, raised on the green algae *Chlorogonium elongatum* (P.A. Dangeard) Francé 1897, was provided as food. Freshly hatched tardigrades were also fed with *C. elongatum*. To obtain a group of individuals of the same age, all previously laid eggs were removed under the binocular microscope (SZH10 Research Stereo, Olympus, Hamburg, Germany), and, subsequently, newly laid eggs were collected from the culture and hatched on separate agar plates. This procedure was repeated several times to obtain 716 individuals in total for the study. Twenty-eight days after hatching, the tardigrades were separated into smaller cohorts, each on a small petri dish (Ø 3.5 cm) with a layer of agarose (3%) and covered with Volvic® water.

Sub-zero temperature treatment

The 28-day-old tardigrades were subjected to sub-zero temperature treatment by cooling on agar plates from room temperature (RT, 20°C) down to −30°C within 15 h in a freezer. The temperature end point of −30°C was chosen to ensure that they were completely frozen, since the supercooling point, which is the temperature at which spontaneous crystallization cannot be further suppressed, of this tardigrade species is −22°C (Hengherr et al., 2009). The freezing process was monitored with a temperature data logger (LOG 32T, TFA Dostmann GmbH & Co. KG, Wertheim, Germany). The specimens were kept for 7 days at −30°C without temperature fluctuations to avoid recrystallisation of the existing ice crystals in the animals, after which the animals were allowed to slowly warm within an insulated box to room temperature overnight. Live and dead animals were recorded after thawing. Tardigrades were assumed to be dead if there was no visible movement within 24 h. The survival and mortality rates on each individual plate in the insulated box during an experiment were recorded. We ran three independent freezing experiments and started in experiment E1 with six plates (E1-1 to E1-6) and 143 specimens in total, in experiment E2 with nine plates (E2-1 to E2-9) and 226 specimens in total and in experiment E3 with seven plates (E3-1 to E3-7) and 172 specimens. Following the thawing period, we started the feeding period, in which the active animals were fed for 7 days. At the end of the feeding period, the numbers of live and dead animals were documented, and the same sub-zero temperature treatment imposed again. The weekly change from freezing to feeding period was repeated until all animals were dead. The control group, which was never frozen, included six plates (C1–C6), each with 25 specimens.

Statistics

The statistical significance of differences in the lifespan between the sub-zero temperature treatment cohorts and the control cohorts was tested using Kaplan–Meier Fisher's Exact test, which performed using OASIS2 (Online Application for Survival Analysis 2) (Han et al., 2016). $P < 0.05$ was considered as a statistically significant level.

The control group and the experiment groups are compared with survival rate and recovery rate. With the records at end of each period, the survival rate of the group is calculated as follow:

$$\frac{(\#\text{of animals alive at the end of the period})}{(\#\text{of total animals})}$$

The recovery rate for every freezing period of each group is calculated as follows:

$$\frac{(\#\text{of animals alive after the freezing period})}{(\#\text{of total animals before the freezing period})}$$

Results

Longevity of frozen tardigrades

If the time spent frozen is included in the age of the tardigrades, the frozen groups survived longer than the control group (Fig. 1). The survival plots of each experimental group (E1, E2 and E3) showed significantly higher survival rates than all control group (Control). Every pairwise comparisons of control group and experimental groups (E) had a *P*-value of <0.05, but pairwise comparisons between experimental groups had a *P*-value of >0.05. Detailed statistical information on significance is included in the supplement (Table 1).

Considering only the activity period, in which the frozen period is excluded, longevity of the frozen groups was similar to that of the control group (Fig. 2). Specifically, the total activity periods of two of the three experimental groups (E2 and E3) were similar to those of the control group. In case of the experimental group E1, the mean longevity was observed longer than that of the control group but had similar maximum longevity. Pairwise comparisons of control group and experimental groups (E2 and E3) groups had a *P*-value of >0.05, and E1 experimental group had a *P*-value of <0.05 (Table 2). The recovery rates tend to decrease with the age of animals (Fig. 3).

The oldest animal in the frozen groups survived for 169 days, if time spent frozen was included, or 94 days if time in the frozen state was excluded, while the oldest animal in the control group lived for 93 days (Table 3). The mean longevity of the experimental group E1 was 80.41 days if time in the frozen state was excluded, whereas the control cohorts showed a mean longevity of 72.6 days. The experimental group E2 and E3 showed a mean longevity of 77.68 and 74.17 days, respectively.

Table 1 Statistics between survival plots of the frozen groups (E1, E2 and E3) and the control group

Condition	Chi-square	*P*-value	Bonferroni *P*-value
Control vs. E1	46.67	0	0
Control vs. E2	101.61	0	0
Control vs. E3	79.92	0	0
E1 vs. E2	0.95	0.33	0.99
E1 vs. E3	2.13	0.14	0.43
E2 vs. E3	0.07	0.79	1

Discussion

The survival of the tardigrade species *M. inceptum* after exposure to sub-zero temperatures underlines the remarkable ability of these invertebrates to cope with unfavourable environmental conditions. However, only a few studies of cryobiosis in tardigrades have been published (Guidetti, Altiero, Bertolani, et al., 2011; Westh & Hvidt, 1990; Westh & Kristensen, 1992; Westh & Ramlov, 1988). In the present study, the statistical analysis showed no difference between the periodically frozen groups. However, a significant difference was found between the control group and the periodically frozen groups when the frozen time is included. If the freezing period is excluded, the total lifespan of the experimental groups E2 and E3 is similar to that of the non-freezing control groups. This coincides with the earlier results of the anhydrobiosis experiments (Hengherr et al., 2008) where no ageing occurred in the cryptobiosis state.

The experimental group E1, in contrast to both groups E2 and E3 showed a longer total lifespan than the control groups when the time spent frozen is excluded (Fig. 2). However, the reasons underlying this change in mortality need to be examined in further studies.

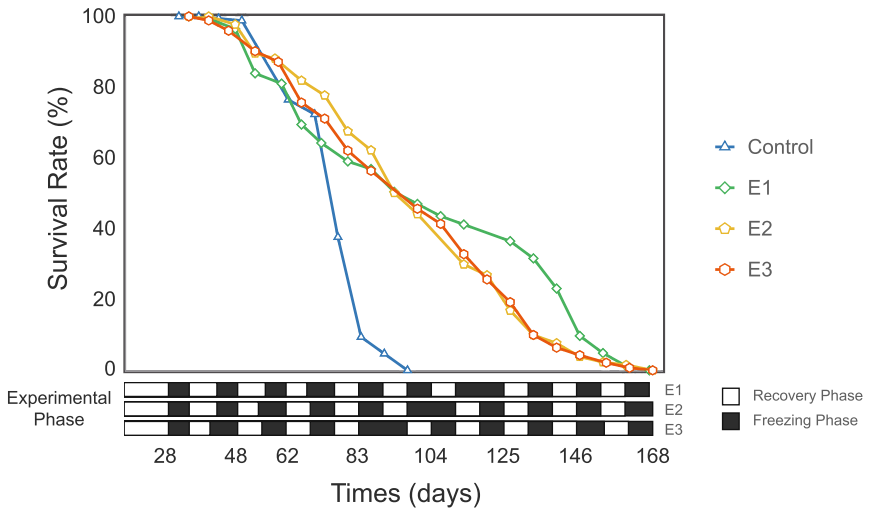

Figure 1 Age-specific survival rate of *Milnesium inceptum*. Age of the alternate frozen cohorts includes the time spent in cryobiotic periods. Each data point shows the survival rate of the periods. The alternate frozen cohorts showed longer longevity than the control cohort.

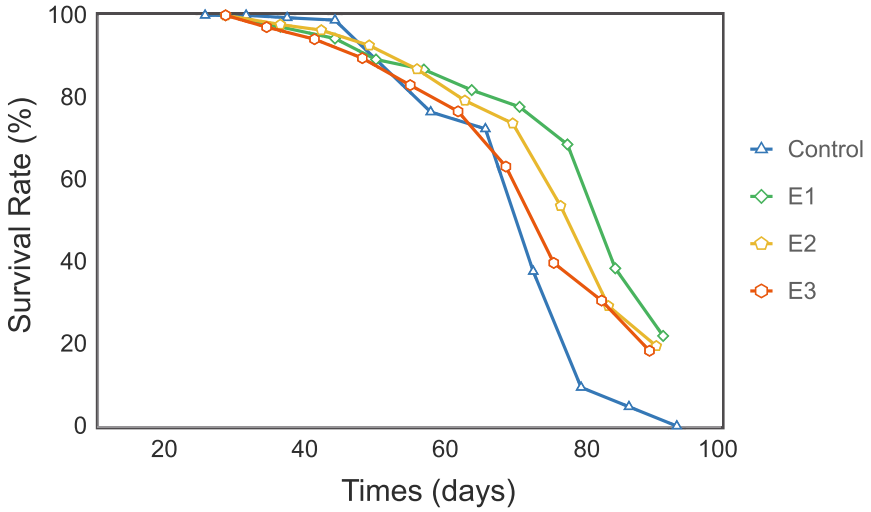

Figure 2 Age-specific survival rate of *Milnesium inceptum*. Time spent in cryobiotic periods is excluded from the age of alternate frozen periods. Each data point shows survival rate of the periods. The alternate frozen cohorts showed similar activity periods with the control cohort.

Table 2 Statistics between frozen period excluded survival plots of the frozen groups (E1, E2, and E3) and the control group

Condition	Chi-square	*P*-value	Bonferroni *P*-value
Control vs. E1	12.62	0	0
Control vs. E2	2.66	0.1	0.31
Control vs. E3	0.23	0.63	1
E1 vs. E2	3.93	0.05	0.14
E1 vs. E3	8.63	0	0.01
E2 vs. E3	6.9	0.01	0.03

Survival after a frozen period can be ascribed to factors acting during entering, staying in or leaving the frozen state. Until now, few experimental trials show that the freezing phase is probably the critical point for the transition into cryobiosis. Here, the death of the tardigrade can occur if freezing takes place too quickly and/or if certain biochemical and physiological processes cannot take place at all or at least not fast enough. Another important reason for an unsuccessful transition into cryobiosis may be an insufficient amount of energy

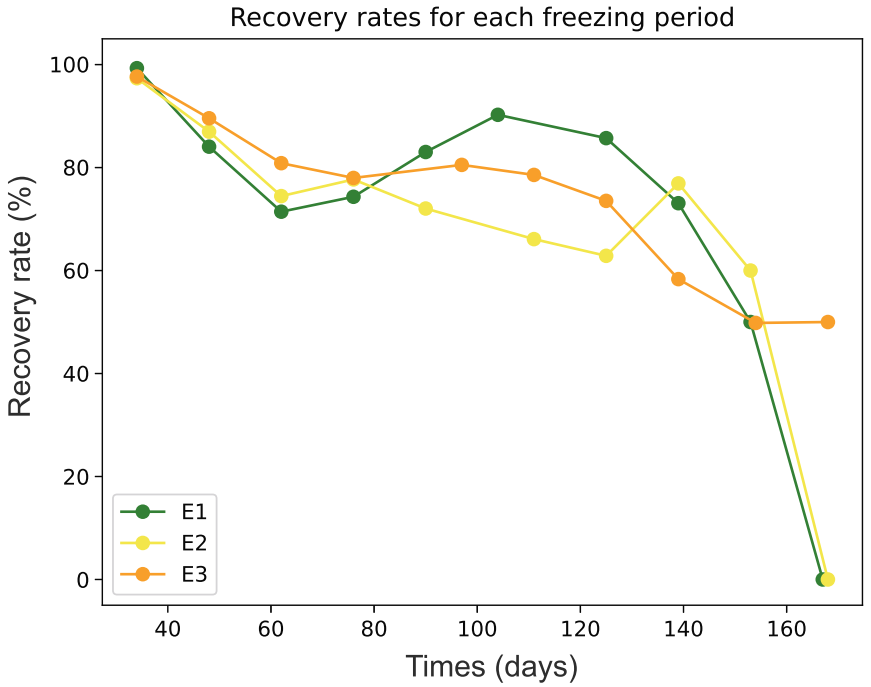

Figure 3 Graph showing the recovery rates of *Milnesium inceptum* tend to decrease with the age of animals.

Table 3 Mean and maximum longevity (days) of *Milnesium inceptum* (E1, E2 and E3), including and excluding the time spent in cryobiosis and the control group

Experiment	Mean longevity (±SD)	Maximum longevity
Hydrated control	72.6	93
Alternate frozen (including cryobiotic periods)	99.60 (±1.70)	169
Alternate frozen (excluding cryobiotic periods)	77.42 (±2.55)	94

to enable the necessary metabolic processes. Analysis of storage cells after anhydrobiosis in *M. inceptum* showed a decreased cell size compared with the control (Reuner et al., 2010). Storage cells, also called storage bodies, are free-floating cells in the body cavity of tardigrades, and they are seen as the major repository of energy stored in form of lipid, peptides and glycogen. For the species *Richtersius coronifer*, a decrease in size of the storage cells was also found after anhydrobiosis (Jönsson & Rebecchi, 2002). These authors concluded that the energy required to enter and exit the dehydrated state depletes the stored material. A similar effect may pertain to tardigrades undergoing cryobiosis, but currently no data are available. It may also be possible that cellular damage and damage to biological molecules occurs during the transition to cryobiosis or during the state of cryobiosis (Guidetti, Altiero, Bertolani, et al., 2011). Cellular and molecular damage in frozen organisms caused by recrystallisation processes occurs at a constant or varying sub-zero temperature (Wharton, 2003). This involves a change in the size distribution of ice crystals as larger crystals grow at the expense of small crystals. This could be damaging to a frozen organism (Knight et al., 1988; Knight et al., 1995; Knight & Duman, 1986) and makes long-term survival very unlikely. In anhydrobiotic tardigrades, it was shown that the longer the anhydrobiotic phase lasted, the more damage was inflicted on the DNA that could not be repaired during anhydrobiosis (Neumann et al., 2009). We have a comparable situation with the frozen cells in which the repair enzymes cannot be active either.

There are various mechanisms in invertebrates that have evolved in the course of evolution to survive difficult environmental conditions. Especially in regions in which the temperature of some or all of the ground below the seasonally freezing and thawing layer remains continuously at or below 0 degrees Celsius for several consecutive years, some organisms may be preserved for a long time. Tsujimoto et al., 2016 reported a recovery of two individuals and development of an egg of the Antarctic tardigrade, *Acutuncus antarcticus*, from a frozen moss sample collected in Antarctica in 1983 and stored at −20°C for 30.5 years. This is the longest records of survival for tardigrades as animals or eggs (Tsujimoto et al., 2016). But long periods of survival in the frozen state are also found in other invertebrates. Rotifers are known to survive between 6 and 10 years in a frozen state, although we do not have any information about the supercooling point and whether the rotifers were actually completely frozen (Iakovenko et al., 2015; Shain et al., 2016). The longest time period of rotifer survival in a frozen state, and all animals in general, is published by Shmakova et al. (2021). They reported the survival of an obligate parthenogenetic bdelloid rotifer of the genus *Adineta*, recovered from northeastern Siberian permafrost radiocarbon-dated to ~24 000 years BP (Shmakova et al., 2021).

Freezing processes in general are a very complex physical process. Therefore, in freezing experiments with organisms that are frozen during their life in their environment, there are a number of unknown factors that make it difficult to draw a definite conclusion. The oldest animals in our experimental groups showed an almost identical longevity (time spent frozen is excluded), as the animals from the control group. This is a clear indication that the ageing process slows down or halts, as described by the 'Sleeping Beauty' hypothesis. Therefore the 'Sleeping Beauty' hypothesis is not only valid for anhydrobiosis (Hengherr et al., 2009) but also applies to cryobiosis, even though freezing and drying are not identical stress vectors (Crowe et al., 1990). Therefore, this study represents the first experimental evidence that tardigrades reduced ageing in the frozen state during cryobiosis. Although *M. inceptum* represents only one tardigrade species, these results are likely applicable to other species.

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