Comparative studies on storage cells in tardigrades during starvation and anhydrobiosis

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Abstract The impact of starvation and anhydrobiosis on the number and size of the storage cells in the tardigrade species Milnesium tardigradum, Paramacrobiotus tonollii and Macrobiotus sapiens was investigated to gain more insight on the energetic side of anhydrobiosis. Storage cells are free floating cells within the body cavity of tardigrades and are presumed to store and release energy in form of glycogen, protein and fat to maintain a constant nutrient regime for the other tissues. The body size of the animals was not correlated with the size of the storage cells, however, Milnesium tardigradum the largest species analysed also had the largest storage cells. A reduction in the size of the storage cells is apparent in all three species after seven days of starvation. A seven-day period of anhydrobiosis leads to a decrease in cell size in Milnesium tardigradum but not in Paramacrobiotus tonollii and Macrobiotus sapiens. Although Macrobiotus sapiens was raised on green algae, and Milnesium tardigradum and Paramacrobiotus tonollii were fed with rotifers and nematodes this difference in nourishment was not reflected in the response of the storage cells to anhydrobiosis [Current Zoology 56 (2): 259–263, 2010].

Key words Tardigrada, cryptobiosis, Milnesium tardigradum, Paramacrobiotus tonollii, Macrobiotus sapiens

Tardigrades can be found across all continents and oceans, inhabiting terrestrial, freshwater and marine habitats, from the summit of mountains to lowlands, polar to tropic regions and from coastal to the deep sea (Nelson, 2002). Terrestrial tardigrades are well adapted to extreme environmental changes in their microhabitats and well known for their capabilities to survive for several years in an anhydrobiotic or cryobiotic state. In the anhydrobiotic state they can endure experimental conditions of low or high temperatures as well as immersion in organic solvents, or exposure to high doses of radiation and hydrostatic pressure (Westh et al., 1991; Ramløv and Westh, 1992; Westh and Kristensen, 1992; Seki and Toyoshima, 1998; Ramløv and Westh, 2001; Horikawa et al., 2006; Jönsson and Schill, 2007; Jönsson et al., 2008; Ono et al., 2008; Hengherr et al., 2009b). A longevity of 20 years has been reported for the species Echiniscus testudo stored under laboratory conditions (Jørgensen et al., 2007). Under more natural conditions they have the capacity to survive unfavourable periods by complete desiccation. In this anhydrobiotic state the tardigrades do not show measurable metabolism and form the so-called tun state (Baumann, 1922).

The body cavity of tardigrades is filled with storage cells, also called storage bodies. They are seen as the major repository of energy stored in form of lipid, peptides and glycogen (May, 1946-1947; Węglarska, 1957; Rosati, 1968; Węglarska, 1975; Szymanska, 1994; Jönsson and Rebecchi, 2002). Electron microscopic work on Macrobiotus richtersi Murray 1911 by Szymanska (1994) demonstrated diminishing reserve materials in storage cells during oogenesis which had an impact on the size of the cells, i.e. they became smaller as fat and protein were used for the completion of the egg development. Węglarska (1957) working with Dactylobiotus dispar Murray, 1907 (formerly: Macrobiotus dispar) had already noted that cells in starved animals diminish and observed that starvation over a long period lead to the re-absorption of these cells. For Richtersius coronifer Richters 1903 Jönsson and Rebecchi (2002) showed that cell size is a factor affecting survival during anhydrobiosis. The cells furthermore decreased in size implying enhanced energy consumption during entry into and emergence from anhydrobiosis.

In this study we explored the storage cell number and size of carnivorous Milnesium tardigradum Doyère 1849 and Paramacrobiotus tonollii Ramazzotti 1958 and the herbivorous Macrobiotus sapiens Binda and Pilato 1984 and compared data on animals fed ad libitum with those that had been starved or induced into anhydrobiosis to explore the energetic aspects of anhy-
drobiosis in the three species.

1 Material and Methods

1.1 Tardigrade culture

Cultures were grown from animals derived from moss samples from Tübingen, Germany (M. tardigradum), Eugene, Oregon, USA (P. tonollii) and Rovinj, Croatia (M. sapiens). The tardigrades were kept in a controlled environment with a constant temperature of 20 ± 1 °C and a light/dark cycle of 12 h. The animals were reared on Petri dishes filled with a small layer of agarose (3%) and covered with spring water (Volvic™ water, Danone Waters Deutschland GmbH, Frankfurt, Germany). Milnesium tardigradum and P. tonollii, carnivorous species, were fed the bdelloid rotifer Philodina citrina and small nematodes (Panagrellus sp.), which had been raised on the alga Chlorogonium elongatum in our own laboratory cultures. Macrobiotus sapiens, an herbivore, was fed C. elongatum. For all experiments animals of large size and in good physical condition were taken directly from the culture. Females with developing eggs were excluded. To determine the average size of the animals, ten of each species were photographed when they were at maximum stretch with a Nikon DS-Fil camera (Nikon GmbH, Düsseldorf, Germany) attached to an Olympus SZH10 stereomicroscope (Olympus Deutschland GmbH, Hamburg). The body length was determined with the ‘measurement’ option integrated in the Nikon Software NIS-Elements D 2.30, SP4 (Nikon GmbH). To get maximally stretched animals the tardigrades were treated with a solution that leads to a maximal stretching (100 mL 96% EtOH, 30 mL 40% formaldehyde, 5 mL acetic acid, 200 mL aqua dest) according to Greven (1980). Survival rates during starvation were determined by keeping fifteen animals the tardigrades were treated with a solution that proved to be a good stain for the storage cells. During fixation and staining the cover slips were placed in wet chambers to prevent desiccation.

One-Way ANOVA was used for analysis of cell number. Statistics were performed with the software Sigma-Stat 3.5 (Systat Software GmbH, Erkrath, Germany). To compare the different treatments within species One-Way ANOVA was used for analysis of cell number. Because data of cell size showed no normal distribution a Kruskal-Wallis One Way Analysis of Variance on Ranks was applied. A multiple comparison procedure (Student-Newman Keuls method) was added to isolate the groups that differ from each other. All data for one species and one treatment were used as controls for all the experiments. For the comparative analysis animals were starved for seven days by preventing access to food. The second group of animals were induced into anhydrobiosis by the drying regime as previously described elsewhere (Hengherr et al., 2008a). Briefly, the animals were transferred to open microliter tubes which were placed into small plastic chambers with 85% relative humidity for two days. After this period the tubes were transferred into chambers with 33% relative humidity to completely dry the animals for seven days. The described humidities were achieved by a constant saturation vapour pressure over a saturated salt solution. These anhydrobiotic animals were re-hydrated in Volvic™ water (usually 15 to 30 minutes) until they resumed activity and analysed immediately.

To count the storage cells, the tardigrades were transferred to a cover slip with 425–465 mOsm/kg cell culture medium (Ex-cell™ 405, Sigma-Aldrich, Munich, Germany). Using the stereomicroscope the storage cells were released by cutting the tardigrades into two pieces with a scalpel. This procedure allowed the cells to float freely out of the body cavity into the surrounding cell culture medium. The carcasses were moved slightly with the scalpel until all storage bodies were removed. The cells were fixed for 20 min. by adding a small amount of 4% paraformaldehyde (PFA) to cell culture medium on the cover slip to give a final concentration of approximately 1% PFA. The cells were then stained with 4 mM SYBR14 dye (Invitrogen, Karlsruhe, Germany) for 10 mins, in previous experiments SYBR14 has proved to be a good stain for the storage cells. During fixation and staining the cover slips were placed in wet chambers to prevent desiccation.

To count the storage cells, the cover slips were photographed with fluorescence on the inverted microscope using the filter-set: BP450-490 / BS510 / BP515-565. The freely available software “daime” (Daims et al., 2006) was used to count the cells. Ten animals per species and condition were used.

1.3 Statistics

Statistics were performed with the software Sigma-Stat 3.5 (Systat Software GmbH, Erkrath, Germany). To compare the different treatments within species One-Way ANOVA was used for analysis of cell number. Because data of cell size showed no normal distribution a Kruskal-Wallis One Way Analysis of Variance on Ranks was applied. A multiple comparison procedure (Student-Newman Keuls method) was added to isolate the groups that differ from each other. All data for one species and one treatment were defined as one group.
and compared with each other with the described tests. For all tests significance was accepted for $P \leq 0.05$.

2 Results

The storage cells of the three species proved to be quite disparate with respect to size and number. As a consequence, the standard deviations (s.d.) of the mean values were high. This indicated there were slightly different states of ingestion, digestion and defecation at the point of sampling. The storage cells within individual animals also showed a heterogeneous size distribution.

*Milnesium tardigradum*, the largest ($801.74 \pm 37.63 \mu m$, mean with *SD*) species of tardigrades used in this study, also had the largest storage cells ($214.14 \pm 70.39 \mu m^2$, Fig. 1, $P<0.001$). The smaller species, *M. sapiens* (body length: $476.36 \pm 30.23 \mu m$) and *P. tonollii* (body length: $628.54 \pm 78.40 \mu m$) had smaller storage cells ($50.06 \pm 19.07 \mu m^2$ and $79.79 \pm 30.88 \mu m^2$, respectively). Although one may hypothesise that the number of storage cells is a positive correlation to the overall size of a tardigrade species, in fact, *P. tonollii* contained more cells ($1068.80 \pm 323.90$) than *M. tardigradum* ($394.90 \pm 136.46$), which clearly demonstrates that such a correlation does not exist in the three analysed species.

Seven days of food deprivation led to significantly smaller cells in all three species (Fig. 1). The most considerable decrease in cell size occurred in the smallest species, *M. sapiens* (-69.8%), whilst the cell size of *P. tonollii* was reduced by 46.41% and by 14.52% in *M. tardigradum*. Starvation over this period did not lead to a significant decline in the number of the storage cells (Fig. 2).

In the experiment with animals that had been induced into anhydrobiosis *M. tardigradum* clearly differed from *P. tonollii* and *M. sapiens* with respect to the change of the storage cell size. The difference between the storage cell size of the control group ($214.14 \pm 70.39 \mu m^2$) and the dehydrated group ($151.51 \pm 43.03 \mu m^2$) in *M. tardigradum* was statistically significant, whereas *P. tonollii* and *M. sapiens* showed no statistical difference (Fig. 1).

The investigation of the survival rate of the three species analysed showed the expected decrease during prolonged starvation. *Milnesium tardigradum* reached 50% mortality after twenty days, whilst *M. sapiens* and *P. tonollii* reached 50% mortality after 30 days (Fig. 3).
3 Discussion

In earlier publications values for the size of the storage cells for other eutardigrade species were derived from images taken on electron microscopes or light microscopes (Table 1). *M. tardigradum* currently appears to have the largest storage cells, and *M. sapiens* the smallest after a period of starvation. The high variability in number and size of the storage cells reflects the versatile role these ‘organs’ play in the life of the tardigrade.

Eutardigrades show excellent tolerance to starvation and it has been reported that they survive several weeks of starvation (Ramazzotti and Maucci, 1983). The three species in this investigation also show this remarkable ability, which was not affected by their preferred food source (i.e. herbivore or carnivore). This is especially demonstrated when comparing the 50% mortality values to the normal longevity of the three species (*M. tardigradum* 82.7 ± 2.7 days, Hengherr et al. 2008a; *M. sapiens* 83.0 ± 3.5 days and *P. tonollii* 69.0 ± 45.1 days, Lemloh, 2008).

In a previous study on *D. dispar* re-absorption of the storage cells was detected (Weglarska, 1957). In our study, seven days of starvation was not sufficient to observe a decline in the number of storage cells, which would imply re-absorption, for *M. tardigradum, M. sapiens* or *P. tonollii*. This suggests the animals had not reached the point at which energy usage required the storage cells to be emptied and re-absorbed. This is supported by the additional data from the long-term starvation experiments which showed that seven days of starvation did not produce a significant decline in survival rates.

The analysis of size of the storage cells after anhydrobiosis did reveal a difference between the three species. Cell size decreased in *M. tardigradum* while the size of the storage cells remained unchanged in both the *Macrobiotus* species in comparison with the control of active animals fed *ad libitum*. We suppose that the nutrient intake was not the cause of the observed differences as both *M. tardigradum* and *P. tonollii* had a similar food source (i.e. *P. citrina, Panagrellus* sp.). For the species *Richtersius coronifer*, Jönsson and Rebecchi (2002) found a decrease in the size of the storage cells after anhydrobiosis (-14 % after a period of 12 days). They concluded that the energy requirements of entering and exiting the dehydrated state was utilising the stored material. The actual duration of the anhydrobiotic state should not influence the size of the storage cells as virtually no metabolism takes place during anhydrobiosis (Pigoń and Węglarska, 1955). *M. tardigradum* like *R. coronifer* displayed a reduction in cell size, and it is therefore probable that the stored energy of the cells was being utilised during entry and exit from anhydrobiosis. In contrast, neither of the analysed *Macrobiotus* species showed a change in storage cell size. This suggests different genera have different energy budgets by which the process of anhydrobiosis is managed. How these processes and energy budgets differ remains unclear as the process of anhydrobiosis is still not fully understood. Recent work has shown that *M. tardigradum* exhibits unique responses to dehydration without the use of trehalose (Hengherr et al., 2008b), has a very high tolerance to heat when in the anhydrobiotic state (Hengherr et al., 2009b), and shows a novel method of surviving low temperatures (Hengherr et al., 2009a). Starved animals of *Macrobiotus* species (Baumann, 1922) and *M. sapiens* (unpublished data) have been observed entering and exiting anhydrobiosis several times in succession without causing obvious changes or vitiations to the animals. This implies that in this genus the energy source for anhydrobiosis is independent of the storage cells.

<table>
<thead>
<tr>
<th>Species</th>
<th>Diameter (µm)</th>
<th>Area (µm²)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dactylobiotus dispar</td>
<td>6-13</td>
<td>28.27-132.73</td>
<td>Weglarska, 1957</td>
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<tr>
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<td>78.54-113.10</td>
<td>Rosati, 1968</td>
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<td>29.22-107.51</td>
<td>Szymskas, 1994</td>
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<td>15.12-53.95</td>
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<tr>
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<td>42.76-82.75</td>
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<td>151.51-343.93</td>
<td>present publication</td>
</tr>
</tbody>
</table>

Published minimum (min.) and maximum (max.) values are underlined, while additional numbers were calculated for better comparability. Measurements were performed on animals in different physiological states (*D. dispar*: encysted animals, *M. hufelandi*: well fed animals, *M. richtersi*: animals undergoing oogenesis, *R. coronifer*: anhydrobiotic animals).

Table 1 Comparison of storage cell size from different tardigrade species
This study compared the response of starvation and anhydrobiosis in three tardigrade species focusing on the storage cells as the main energy store in tardigrades. These cells are potentially important in mediating the protection of the other tissues by, for example, producing protective metabolites. In these experiments the response to starvation and anhydrobiosis was found to be independent of diet. Further studies are required to elucidate the different responses of storage cells in different species that are capable of anhydrobiosis to unravel the process of survival during dehydration.

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