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Survival and DNA degradation in anhydrobiotic tardigrades

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SUMMARY

Anhydrobiosis is a highly stable state of suspended animation in an organism due to its desiccation, which is followed by recovery after rehydration. Changes occurring during drying could damage molecules, including DNA. Using the anhydrobiotic tardigrade *Paramacrobiotus richtersi* as a model organism, we have evaluated the effects of environmental factors, such as temperature and air humidity level (RH), on the survival of desiccated animals and on the degradation of their DNA. Tardigrades naturally desiccated in leaf litter and tardigrades experimentally desiccated on blotting paper were considered. Replicates were kept at 37°C and at different levels of RH for 21 days. RH values and temperature, as well as time of exposure to these environmental factors, have a negative effect on tardigrade survival and on the time required by animals to recover active life after desiccation. DNA damages (revealed as single strand breaks) occurred only in desiccated tardigrades kept for a long time at high RH values. These results indicate that during the anhydrobiotic state, damages take place and accumulate with time. Two hypotheses can be formulated to explain the results: (i) oxidative damages occur in desiccated specimens of *P. richtersi*, and (ii) high temperatures and high RH values change the state of the disaccharide trehalose, reducing its protective role.

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Key words: Tardigrada, *Paramacrobiotus richtersi*, desiccation, humidity, temperature, survival, DNA damage, oxidation.

INTRODUCTION

Anhydrobiosis is a highly stable state of suspended animation in an organism due to its desiccation, followed by recovery by rehydration (Keilin, 1959). It represents a fundamental concept about the nature of living systems because, due to the absence of ongoing metabolism, an anhydrobiotic organism lacks all of the dynamic features of living organisms and a kind of resurrection routinely occurs (Clegg, 2001; Tunnacliffe and Lapinski, 2003). Anhydrobiosis is widespread in nature; it is present in a wide taxonomic variety of bacteria, yeasts, plants, protists and also metazoans (Alpert, 2005; Watanabe, 2006; Rebecchi et al., 2007).

From anhydrobiosis' discovery in the 18th century (Van Leeuwenhoek, 1702), a huge amount of data covering a wide range of topics has been published with the aim of discovering the secret of life without water (for reviews, see Clegg, 2001; Alpert, 2006). Among them, considerable emphasis has been devoted to elucidating the biochemical and molecular basis of desiccation tolerance, due to the fact that the removal of intracellular water induces several kinds of changes in the cellular environment (Senaratna and McKersie, 1986). The changes due to the loss of water could generate (i) alterations of the molecular organization, including nucleic acids; (ii) transient dysfunctions in enzymes and/or electron transport chains, which may lead to the production of free radicals or promote chemical reactions that normally do not occur in the hydrated state; (iii) oxidation damage to cellular components, with the rise of toxic elements responsible for the immediate death of the animals during rehydration (Womersley, 1987; França et al., 2007). All or many of these changes might lead to massive damages to the cellular components, which eventually result in death of the cell and, consequently, in the organism's death. The ability to survive desiccation involves a complex array of factors at every level of cell structure and function. In desiccation-tolerant uni- and multicellular organisms, the ability to tolerate the absence of water is related to the synthesis of bioprotectants such as trehalose, stress proteins and LEA proteins (Goyal et al., 2005; Tunnacliffe et al., 2005; Kikawada et al., 2006), in addition to the regulation of antioxidant defence (Jamieson, 1998; França et al., 2007).

Even though in the last decades several efforts have been devoted to analyzing damages caused by desiccation, and the ability of anhydrobiotes to repair these damages, further studies are necessary. Several studies have analyzed DNA damage in plants and prokaryotes able to desiccate, emphasizing the role of DNA repair in these organisms (Mattimore and Battista, 1996; Oliver, 1997) whereas DNA damage and DNA repair mechanisms in anhydrobiotic metazoans have received little attention (Jönsson, 2007; Gladyshev and Meselson, 2008; Neumann et al., 2009; Rebecchi et al., 2009). The study of anhydrobiotic processes could also provide important information about the mechanisms involved in aging, considering that efficiency of DNA repair, presence of antioxidant enzymes and rates of free-radical production are also involved in senescence processes (Holliday, 2004). Last but not least, our increased understanding of anhydrobiosis holds promise because it may lead to innovative technological applications (Crowe et al., 2005).

Among anhydrobiotic metazoans, tardigrades, or water bears, are particularly attractive animal models to study anhydrobiosis. They are able to perform anhydrobiosis at any stage of their life cycle, from egg to adult (Bertolani et al., 2004; Schill and Fritz, 2008). Tardigrades are little known and neglected but they are invertebrates present in many environments (sea, freshwater, soil), frequently inhabiting very unstable habitats (e.g. the surface of lichens and mosses and ephemeral aquatic habitats) or extreme habitats (e.g.

glaciers and deserts) (Bertolani et al., 2004). Anhydrobiotic tardigrades are able to withstand several physical and chemical extremes [very low and high temperatures, high pressure, organic solvents and cosmic radiation (Jönsson et al., 2005; Horikawa et al., 2006; Rebecchi et al., 2007; Horikawa et al., 2008; Jönsson et al., 2008; Rebecchi et al., 2009)]. The survival of desiccated tardigrades under atmospheric oxygen conditions can be long (nine years) but seems not to exceed 20 years (Baumann, 1927; Guidetti and Jönsson, 2002; Rebecchi et al., 2006; Jørgensen et al., 2007). This means that during anhydrobiosis some damages occur leading to the death of tardigrades (Rebecchi et al., 2006), even though desiccation does not seem to have an effect on their aging and longevity because under alternating periods of desiccation (seven days) and of active life (seven days) the time spent in anhydrobiosis is ignored (Hengherr et al., 2008a).

In this paper, using the anhydrobiotic eutardigrade *Paramacrobiotus richtersi* (Murray 1911) (Parachela, Macrobiotidae) as a model organism, we have evaluated the effects of environmental factors, such as temperature and air humidity level, on the survival of desiccated animals and the degradation of their DNA.

MATERIALS AND METHODS

The terrestrial eutardigrade *Paramacrobiotus richtersi* (Murray 1911) was utilized. The population of *P. richtersi* considered here is bisexual and amphimictic and comes from hazel leaf litter collected dry at Formigine [Modena, Northern Italy; N 44°34.253′, E 10°50.892′, 80 m above sea level (a.s.l.)].

Heat stress at 37°C

For this experiment hazel leaf litter containing naturally desiccated tardigrades was used. Thirty replicates of 0.5 g of dry leaf litter each were run. Each replicate was placed in an open glass Petri dish (3 cm in diameter). Fifteen replicates were put in an oven at 37°C and 30–40% relative humidity (RH) (stressed animals), while 15 replicates were kept under lab temperature and air humidity (control animals). After 7, 14 and 21 days from the beginning of the experiment, five replicates of leaf litter of both sets (treatment and control) were hydrated and all tardigrades were collected (see below). Finally, tardigrade viability was evaluated. In addition, to evaluate the viability of tardigrades within leaf litter immediately before the beginning of the experiment, five replicates of 0.5 g of leaf litter each were evaluated.

To collect tardigrades from their substrate, leaf litter was slowly hydrated at room temperature. Leaf litter of each replicate was sprinkled with tap water for 15 min and then submerged in water for 30 min. Later, tardigrades were extracted from each replicate by sieves (250 μm and 37 μm mesh size) under running water and all animals were picked up with a glass pipette. Finally, they were observed under a stereomicroscope (Leica, Metzlar, Germany) to check their viability.

Heat stress at 37°C combined with different values of air RH

For this experiment hazel leaf litter containing naturally desiccated tardigrades (experiment A) and tardigrades experimentally desiccated on blotting paper (experiment B) were considered. The heat stress temperature was 37°C. The values of air RH utilized were: 80%, 50%, 20% and 0–3%. The last RH value was obtained using silica gel placed within a desiccator.

For experiment A, three RH values (80%, 20% and 0–3%) were considered. For each RH value, five replicates of 0.5 g of dry leaf litter each were used. Each replicate was placed in an open glass Petri dish (3 cm in diameter) and then put in a climate chamber (CHL 700, Angelantoni Industrie, Massa Martana, Perugia, Italy) at 37°C and at one of the three RH values. The experiment ran for 21 days. After this period the leaf litter in each replicate was hydrated and all tardigrades were collected. Finally, tardigrade viability was evaluated.

For experiment B, four RH values (80%, 50%, 20% and 0–3%) were tested. For each RH value, five replicates, each with 10 tardigrades experimentally desiccated on blotting paper, were used. Replicates were placed in a climate chamber at 37°C and at one of the four RH values. The experiment ran for 21 days. At the end of the experiment, tardigrades were rehydrated to check their viability. The following protocol was used to experimentally desiccate tardigrades. Firstly, tardigrades were extracted from leaf litter (see above method) and kept in water for 24 h at 16°C without any food sources. Then, they were forced into anhydrobiosis by placing each group of animals on blotting paper (5×5 mm in size) with 9 μl of water and exposing them initially to 80% RH and 20°C for 24 h and then to 50% RH at 20°C for 24 h in a climate chamber. To rehydrate desiccated tardigrades, water droplets were added onto each paper square every 10 min for a total of 50 min. After rehydration, tardigrades were maintained in water at 16°C and examined under a stereomicroscope to verify their viability.

Animal viability analysis

For all tests and controls, coordinated and active movements of the body (locomotion performance) constituted the criterion to evaluate tardigrade viability after hydration. Locomotion performance was evaluated after 1 h (t_1) and 24 h (t_{24}) from the beginning of hydration. At t_1, mobile (live) animals were isolated, enumerated and fixed in Carnoy's fluid (methanol:acetic acid, 3:1), while the immobile tardigrades were kept in water at 16°C for 24 h. After that period these animals were re-examined and mobile tardigrades were separated from the immobile ones, which were considered dead. Finally, all dead and live animals were enumerated and separately fixed in Carnoy's fluid.

Final survival was calculated by adding the number of animals found active 1 h after rehydration with the number of animals found active 24 h after rehydration. The difference between final survival and survival recorded 1 h after rehydration represents the 'Δ recovery'. The higher the value of Δ recovery, the greater is the number of animals that need a longer time to recover active life. Statistical analyses on recoveries after 1 h and 24 h, final survivals and Δ recoveries were carried out with Mann–Whitney *U*-test and Pearson correlation tests. SPSS 9.0 software (SPSS Inc., Chicago, IL, USA) was used.

Genomic DNA assay

Genomic DNA was extracted from desiccated tardigrades on blotting paper (see above) and kept for 21 days at 37°C and three different RH conditions: 20% RH, 50% RH, 80% RH. As a control, replicates of dry tardigrades at the end of the desiccation process as well as replicates of active hydrated tardigrades were used. For double strand damages assay, three replicates of 30 tardigrades each were used, while for single strand damages three replicates of 90 tardigrades each were used

Genomic DNA of both desiccated and active tardigrades was extracted by grinding the specimens in the presence of an extraction buffer solution (Tris HCl 50 mmol l^{-1}, pH 7.8, NaCl 400 mmol l^{-1}, EDTA 20 mmol l^{-1}, SDS 0.5%; TNES) (Sunnucks and Hales, 1996). After the homogenization, proteinase K (final concentration 0.01 μg μl^{-1}) and RNAase (final concentration 0.005 μg μl^{-1}) were

added, and the mixture was incubated at 55°C overnight. Cell debris was then precipitated with NaCl (final concentration 1 mol l^{-1}), and DNA was recovered using a standard sodium acetate/absolute ethanol precipitation. DNA pellets were then washed with 70% ethanol and re-suspended in 30 µl of a buffer solution (Tris HCl 10 mmol l^{-1}, pH 8, EDTA 1 mmol l^{-1}; TE). Controls with no DNA were also processed with the samples to check for contamination. The amount of DNA extracted from 30 tardigrades ranged from 300 to 540 ng, because at least 50 ng are needed to visualize double strand breaks (Sutherland et al., 2003).

For the double strand breaks study, DNA samples were resolved in 1.2% agarose gels in a buffer solution (Tris HCl 40 mmol l^{-1} pH 7.8, EDTA 1 mmol l^{-1}, acetic acid 20 mmol l^{-1}; TAE) over 90 min at 40 V. The evaluation of single strand damages was carried out with a DNA-denaturing gel electrophoresis method, adapted from Sambrook and Russell (Sambrook and Russell, 2001). DNA pellets were re-suspended as above and resolved in 1.2% agarose gels in alkaline buffer (NaOH 50 mmol l^{-1}, EDTA 1 mmol l^{-1}). Electrophoresis was run at 3 V cm^{-1} overnight in the same alkaline buffer. The gels were then neutralized in TrisHCl 1 mol l^{-1} and NaCl 1.5 mol l^{-1} for 45 min, stained in a 0.5 µg ml^{-1} ethidium bromide solution and photographed under UV lights. EZ Load 1 kb Molecular Ruler (Bio-Rad, Hercules, CA, USA) DNA marker was used as a molecular standard. For both types of electrophoresis, lack of DNA integrity was assessed by the presence/absence of DNA smears. A *P. richtersi* genomic DNA digested with *EcoRI* was used as positive control (see Fig. S1 in supplementary material).

RESULTS

Response of desiccated tardigrades after heat stress at 37°C

A mean of 25.8 (s.d.=6.6) *P. richtersi* specimens have been extracted in control leaf litter replicates, and a mean of 24.5 (s.d.=7.2) animals have been extracted in stressed replicates.

Final survival rates of both stressed and control animals was always high (more than 80%), independent of the duration of the experiment (7, 14 and 21 days; Figs 1 and 2). Final survival rates of tardigrades extracted from leaf litter immediately before the beginning of the experiment was 83.4% (s.d.=9.9).

No significant differences in final survival rates were recorded between stressed and control tardigrades at any survival check. No significant differences were recorded among final survival rates both in stressed and control animals independently of the duration of the experiment (7, 14 and 21 days). In addition, no significant differences were recorded among final survival rates of both stressed and control tardigrades with respect to the final survival rates recorded before the beginning of the experiment.

The Δ recovery of animals stressed at 37°C was a function of the days spent in the oven (Pearson's correlation test: P=0.004; Fig. 3). The Δ recovery of animals stressed for 21 days was significantly higher than that of the animals stressed for seven days (Mann–Whitney U-test: P=0.034) and that of tardigrades collected in leaf litter before the beginning of the experiment (Mann–Whitney U-test: P=0.034). The higher the Δ recovery, the greater is the number of animals that need a longer time to recover active life. No statistical differences were recorded in the Δ recoveries among controls.

Response of desiccated tardigrades after the combined effects of heat stress at 37°C and different RHs

In experiment A (naturally desiccated tardigrades within leaf litter), a mean of 23.4 (s.d.=9.2) *P. richtersi* specimens was extracted from leaf litter replicates. The highest final survival rate (76.4%, s.d.=6.6) was recorded for animals kept at 0–3% RH, while the lowest (61.1%, s.d.=6.6) was recorded for animals maintained at 80% RH (Fig. 4). A significant inverse relationship was found between final survival rates and the RH values (Pearson's correlation test: P=0.003). The final survival rate of animals kept at 0–3% RH was significantly higher than those kept at 80% RH (Mann–Whitney U-test: P=0.009).

The Δ recovery was directly related to the RH values (Pearson's correlation test: P<0.001). The recovery recorded at 80% RH was significantly higher than that recorded at 0–3% RH (Mann–Whitney U-test: P=0.009) and at 20% RH (Mann–Whitney U-test: P=0.009). No significant difference was recorded in Δ recovery between 0–3% and 20% RH.

In tardigrades experimentally desiccated on blotting paper (experiment B), the highest final survival (82.0%, s.d.=17.9) was recorded for specimens maintained at 0–3% RH (Fig. 5). The final survival of animals maintained at 20% RH and 50% RH was 58.0% (s.d.=11.0) and 56.4% (s.d.=10.0), respectively. All animals kept at 80% RH died (Fig. 5).

An inverse relationship was found between final survival rates and RH levels (Pearson's correlation test: P<0.001). The final

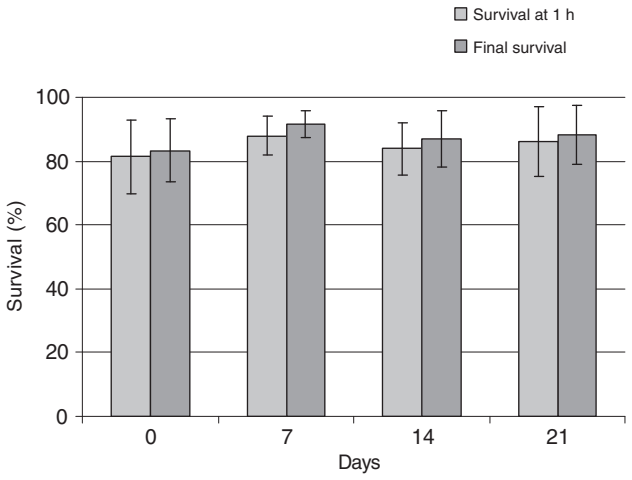

Fig. 1. Survival of desiccated specimens of *Paramacrobiotus richtersi* in control replicates. Zero days correspond to the beginning of the experiment. Each column represents mean value; bar on each column represents standard deviation.

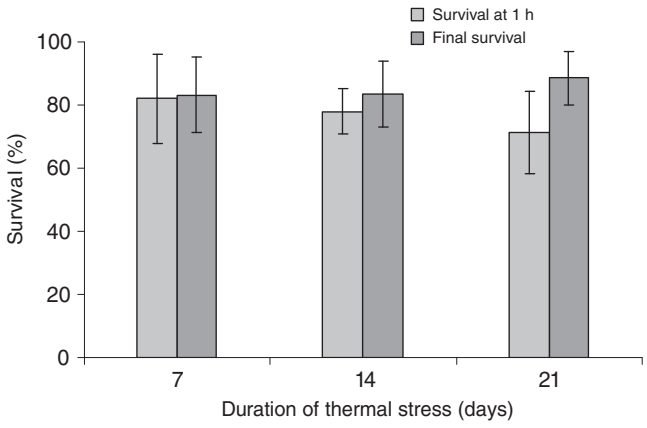

Fig. 2. Survival of desiccated specimens of *Paramacrobiotus richtersi* after heat stress at 37°C. Each column represents mean value; bar on each column represents standard deviation.

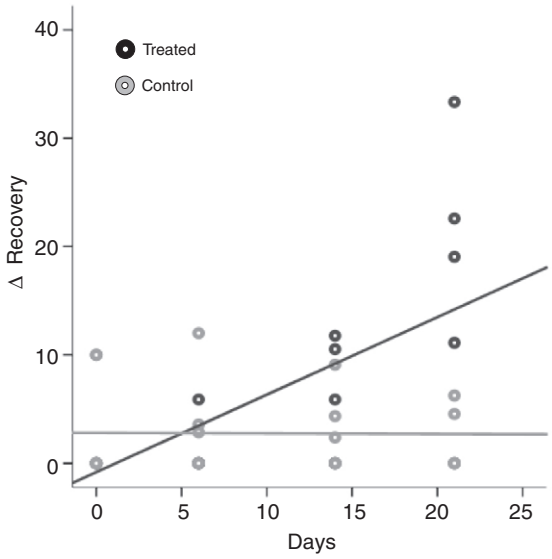

Fig. 3. *Paramacrobiotus richtersi*. Δ Recoveries recorded in control and treated replicates. Lines represent linear curves of adaptation to total data.

survival at 0–3% was significantly higher than that recorded at 20% RH (Mann–Whitney *U*-test: P=0.039) and at 50% RH (Mann–Whitney *U*-test: P=0.026).

The Δ recovery of desiccated animals kept at 50% RH was significantly higher (Mann–Whitney *U*-test: P=0.007) than that of tardigrades maintained at 20% RH. No significant differences were recorded in Δ recovery between 50%–(0–3)% RH and between (0–3)–20% RH. Finally, no relationship was found between Δ recovery and RH levels.

DNA degradation

No visible damages were observed in the double strand DNA of desiccated specimens of *P. richtersi* kept for 21 days at 37°C and 20%, 50% or 80% RH and in controls (active hydrated or desiccated tardigrades; Fig. 6). All lanes showed no smearing, which is typically associated with DNA degradation. No visible damages were observed in the single strand DNA of controls (active hydrated tardigrades or desiccated ones; Fig. 7) and in tardigrades kept for 21 days at 20% RH. However, smearing was observed in desiccated

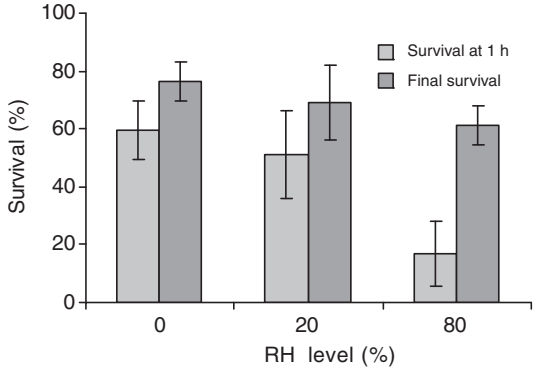

Fig. 4. Survival of *Paramacrobiotus richtersi* specimens naturally desiccated within leaf litter and exposed to different values of air relative humidity (RH) and to heat stress at 37°C for 21 days (experiment A). Each column represents mean value; bar on each column represents standard deviation.

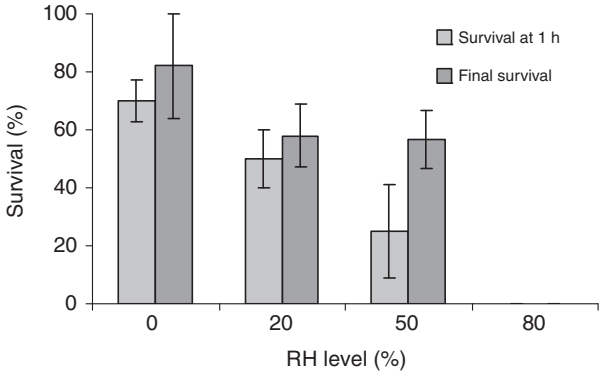

Fig. 5. Survival of *Paramacrobiotus richtersi* specimens experimentally desiccated on blotting paper and exposed to different values of air relative humidity (RH) and to heat stress at 37°C for 21 days (experiment B). Each column represents mean value; bar on each column represents standard deviation.

tardigrades kept for 21 days at 37°C and 50% or 80% RH, indicating the presence of single strand breaks.

DISCUSSION

Our results indicate that temperature and RH values, as well as the time of exposure to these two environmental factors, have a strong impact on tardigrade survival and/or on the time required to recover active life after desiccation. In fact, even though the final survival of desiccated specimens of *P. richtersi* was not affected by a thermal stress of 37°C, either for short (seven days) or for longer periods (up to 21 days), the temperature associated with the time of exposure had an evident effect on the Δ recovery of animals. Δ Recovery increased

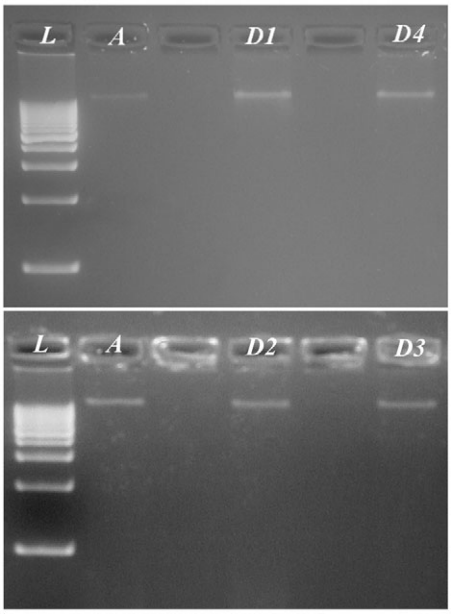

Fig. 6. Genomic DNA electrophoresis gel in standard conditions showing the absence of double strand breaks in *Paramacrobiotus richtersi*. *L*=ladder size standard (EZ Load 1 kb, Bio-Rad); *A*=active animals; *D1*=dry animals immediately after the end of experimental desiccation process; *D2*=experimentally desiccated animals kept for 21 days at 37°C and 20% RH (relative humidity); *D3*=experimentally desiccated animals kept for 21 days at 37°C and 50% RH; *D4*=experimentally desiccated animals kept for 21 days at 37°C and 80% RH.

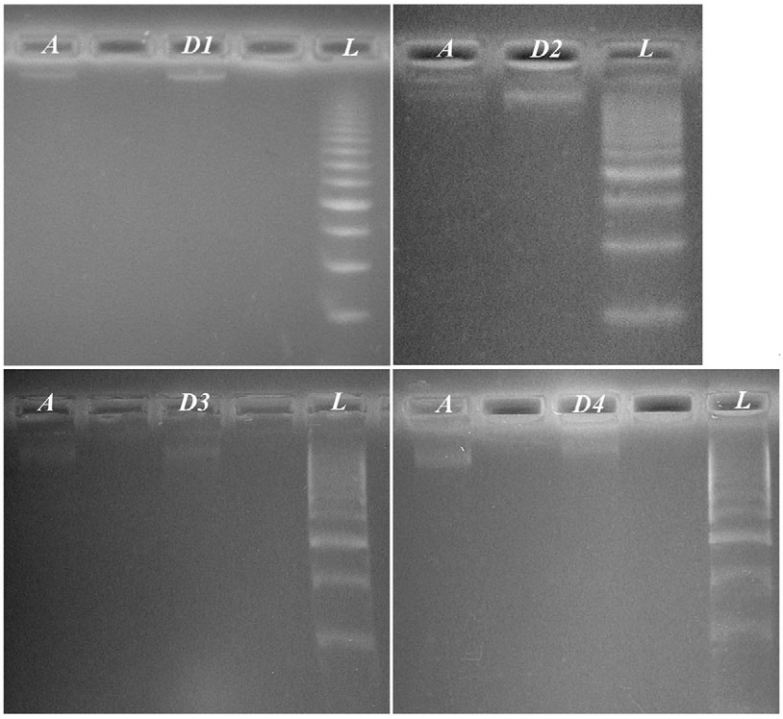

Fig. 7. Genomic DNA electrophoresis gel in alkaline conditions in *Paramacrobiotus richtersi*. The presence of single strand breaks (smearing) is detected in *D3* and *D4* lanes. *L*=Ladder size standard (EZ Load 1 kb, Bio-Rad); *A*=active animals; *D1*=dry animals immediately after the end of experimental desiccation process; *D2*=experimentally desiccated animals kept for 21 days at 37°C and 20% RH (relative humidity); *D3*=experimentally desiccated animals kept for 21 days at 37°C and 50% RH; *D4*=experimentally desiccated animals kept for 21 days at 37°C and 80% RH.

with the amount of time spent in the dry state. A positive correlation between the time spent in the anhydrobiotic state and the time required for recovering active life after rehydration was reported in another tardigrade species (Crowe and Higgins, 1967), in nematodes (Bhatt and Rhode, 1970; Crowe and Madin, 1975; Wharton, 2002) and in rotifers (Jacobs, 1909). In addition, mortality and Δ recovery increased proportionally with the increase of RH values at which the desiccated specimens of *P. richtersi* were kept. Humidity level and temperature, as well as the oxygen concentration, play an important role in the prospect for long-term anhydrobiotic survival also in rotifers (Örstan, 1998; Caprioli and Ricci, 2001). In accordance with our data, exposure to 75% RH of the desiccated rotifer *Macrotrachela quadricornifera* for a long time (20–40 days) caused a high mortality rate (about 100%) (Caprioli and Ricci, 2001). Nevertheless, these authors obtained contrasting results because the exposure to very low RH value (about 0%) for seven days led to a high decrease of rotifer survival (Caprioli and Ricci, 2001).

The increase in mortality and/or in Δ recovery occurring in *P. richtersi* specimens during a period of anhydrobiosis indicates that in desiccated animals some kind of damage takes place and that these damages are accumulated with time. Because repair mechanisms are absent during anhydrobiosis, the amount of accumulated damage in a desiccated state is a function of the time spent in anhydrobiosis. This damage accumulation limits the longevity of anhydrobiotes and probably induces a precocious senescence that could explain the presence of an upper limit for long-term anhydrobiotic survival.

One of the survival strategies against desiccation damage is the accumulation of high concentrations of a small non-reducing disaccharide such as trehalose (e.g. in bacteria, fungi, lower plants and some invertebrates) (Clegg, 2001). The role of this sugar in desiccation-tolerant organisms is twofold. First, trehalose plays a role in the protection of cells and biomolecules by replacing water that is normally bonded to hydrogen. Second, it is involved in the formation of a glassy matrix in the cytoplasm (Wolkers et al., 2002). In the African chironomid *Polypedilum vanderplanki*, high temperatures and high moisture levels reduced the survival rate of desiccated larvae due to the change from the glassy to the rubbery state of trehalose (Sakurai et al., 2008). The presence of trehalose in desiccated tardigrades has been known for a long time (Westh and Ramløv, 1991), and recently this disaccharide has been detected in *P. richtersi* specimens (Hengherr et al., 2008b). Although the lower amount of trehalose detected in *P. richtersi* (0.1% of the dry body weight) (Hengherr et al., 2008b) with respect to the amount detected in *P. vanderplanki* larvae (20% of the dry body weight) (Watanabe et al., 2004), we can hypothesize that the increase in mortality and in Δ recovery of anhydrobiotic tardigrades could be given by the loss of the bioprotective trehalose function due to exposition to high temperature and humidity level.

Loss of water increases the ionic concentration [which can lead to the formation of reactive oxygen species (ROS)] and the susceptibility of biomolecules to the attack of oxygen (Oliver et al., 2001; França et al., 2007), especially at high temperature (Wright, 2001). Oxidation damage was involved in the reduced viability of dried invertebrates (Crowe, 1975; Örstan, 1998; Clegg et al., 2000), including the tardigrade *Paramacrobiotus areolatus* (Murray 1907) (Crowe and Madin, 1974), yeast (Pereira et al., 2003) and prokaryotes (Potts, 1994). In our experiments, tardigrades exposed to higher temperatures and higher humidity levels, in addition to oxygen, showed higher levels of damage in terms of increased mortality and/or Δ recovery. This suggests another possible cause of the occurrence of damages in *P. richtersi* specimens. Oxidative stresses can be responsible for the death of the animal and for the longer recovery time probably related to the time necessary to repair oxidation damages. As suggested by Wright (Wright, 2001), this implies that optimal conditions against aging in anhydrobiotic organisms are those in which oxidative reactions are minimized. These conditions are represented by low temperature, low P_{O_2}, and low water content (in our experiment low RH values). In agreement with this statement, several papers have reported that long-term viability of desiccated tardigrades increases considerably when animals are kept at –22°C or –80°C (Sømme and Meier, 1995; Newsham et al., 2006; Rebecchi et al., 2007). The long-

term viability of desiccated tardigrades could also be influenced by the kind of substrate on which the animals are kept desiccated (Guidetti and Jönsson, 2002). In our experiments, all animals desiccated on papers and kept at 80% RH died whereas those desiccated within leaf litter had a good final survival. These results could be due to a screening effect of leaf litter because leaf layers could prevent or reduce the action of environmental factors (e.g. oxygen and humidity) that lead to oxidation phenomena.

Oxidative stress may be highly destructive in anhydrobiotes in which the activity of antioxidant defences could be reduced (Wright, 2001) and the metabolic activity is absent. The increase in efficiency of antioxidant defences, including enzymes and antioxidants, seems to increase anhydrobiotes' survival by avoiding the production of ROS (Oliver et al., 2001; França et al., 2007). A very recent study indicates that in desiccated *P. richtersi* specimens an increase in antioxidant enzyme systems occurs (Rizzo et al., 2009).

Changes occurring in the cellular environment due to loss of water could cause damage to biological molecules, including DNA. DNA damage has been found in desiccated bacteria and plants (Mattimore and Battista, 1996; Oliver, 1997; Battista et al., 1999; Billi, 2009) and only very recently in desiccated metazoans (Neumann et al., 2009). Our experiments on dry *P. richtersi* (Parachela, Macrobiotidae) specimens show that DNA damage due to the desiccation did not occur in recently desiccated animals (two days) but DNA lesions (revealed as single strand breaks) occurred in anhydrobiotic tardigrades kept for a long time at high oxidative conditions (e.g. 50% and 80% RH and high temperature). Instead, minor DNA damage has been recorded in hydrated specimens of *Milnesium tardigradum* Doyère 1840 (Apochela, Milnesiidae) right after two days of anhydrobiosis (Neumann et al., 2009). Therefore, the anhydrobiotic process *per se* does not seem to induce DNA damages or it induces only minor DNA damage. Differences in the presence of DNA damages between *M. tardigradum* and *P. richtersi* could be related, other then to their different phylogenetic histories, to the absence of trehalose in the former species (Hengherr et al., 2008b). In fact, trehalose plays a major role in protecting cells against oxidation damages during dehydration or during direct oxidative stress (Benarourdj et al., 2001; Alvarez-Peral et al., 2002; Pereira et al., 2003; Shirkey et al., 2003). In *M. tardigradum*, Neumann et al. (Neumann et al., 2009) recorded DNA damages only after a prolonged anhydrobiotic period, while our experiments on *P. richtersi* demonstrated that DNA damage can also occur in short periods, when desiccated animals are kept at high oxidative conditions, namely 50%–80% RH and high temperature. Therefore, the appearance of DNA damage and its accumulation in anhydrobiotic tardigrades is a function of the time spent in a desiccated state and the environment in which they are kept. Similarly, a time-dependent increase in DNA damage, revealed as an increase in DNA double strands breaks, has been identified in the bacterium *Deinococcus radiodurans*. Its prolonged desiccation (six weeks) causes extensive DNA damage, indicating that *D. radiodurans* requires extraordinary DNA repair ability to survive desiccation (Mattimore and Battista, 1996), as in the cyanobacterium *Chroococcidiopsis* sp. (Billi et al., 2000). Therefore, the ability to repair DNA damage accumulated during desiccation is critical for the viability of desiccation-tolerant organisms, including metazoans as suggested by Jönsson (Jönsson, 2007) and Neumann et al. (Neumann et al., 2009). Pending further investigations, we can assume that the long time needed to recover active life (Δ recovery) in *P. richtersi* specimens upon prolonged desiccation is strictly related to the time necessary to activate the mechanisms to repair DNA damage. DNA repairs in anhydrobiotic animals could also have important evolutionary implications not only related to adaptive phenomena but also to the genome structure of organisms (Gladyshev and Meselson, 2008). The co-occurrence of live and dead *P. richtersi* specimens in experimental conditions in which DNA damage (e.g. 37°C and 50% RH) was recorded, indicating that not all tardigrades reacted in the same way when exposed to the same environmental conditions. Therefore, desiccated tardigrades could be able to protect themselves from desiccation-induced damage and from the environmental factors acting during the anhydrobiotic state. This ability could be related to the physiological conditions or life-cycle stages of animals. In addition, we found that DNA damage in *P. richtersi* occurs only at single strand, consequently it could be not lethal for tardigrades, considering that single strand breaks are the commonest DNA lesions often arising spontaneously in living cells (El-Khamisy and Caldecott, 2006). Lastly, the presence of dead animals under experimental conditions in which DNA degradation was not recorded (e.g. 37°C, 20% RH) indicates that during desiccation other molecules are damaged and that tardigrades are not able to repair these molecules after rehydration. According to Du and Gebicki (Du and Gebicki, 2004), proteins represent the initial target of oxidative damage and their oxidation precedes any detectable DNA damage. Although the presence of oxidative damages was not investigated here, it is possible that they occur in desiccation survivors, at least after rehydration.

Our results suggest that future studies on desiccation tolerance should be addressed to investigate the mechanisms used by small metazoans to prevent oxidation damage and the formation of free radicals, as well as the mechanisms implied in the restoration of DNA integrity upon prolonged desiccation. Knowledge of these mechanisms should lead to improved technologies in seed storage, gene banks, tissue engineering, cell transplantation, long-term preservation and archiving of pharmaceutical products and the preservation of dry foods.

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