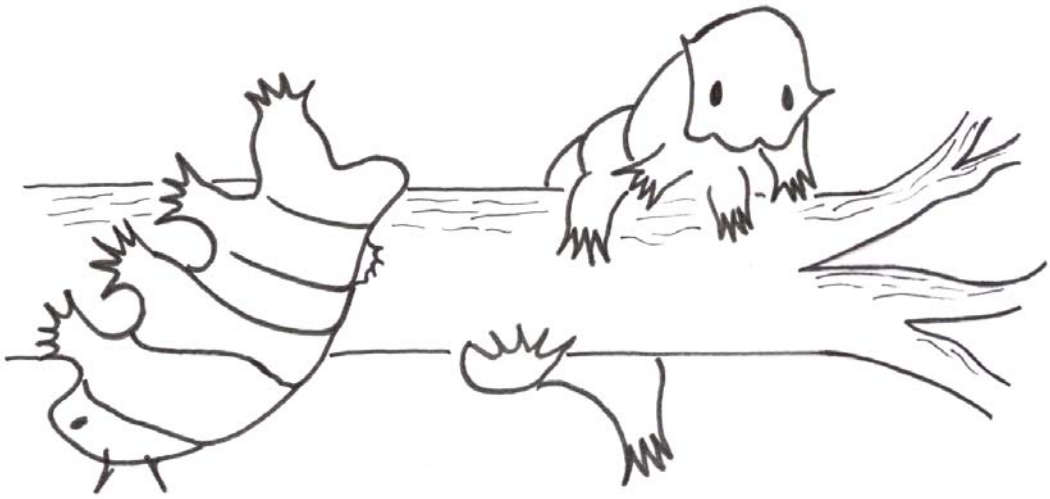
HÖGSKOLAN
KRISTIANSTAD



The effect of dehydration rates on anhydrobiotic survival and trehalose levels in tardigrades

Johanna Lundström
Linda Svensson

Examensarbete i biologi, 20 poäng
Biologi- och Geovetenskapsprogrammet
Institutionen för Matematik och Naturvetenskap
Högskolan Kristianstad
Kristianstad 2006

Institutionen för matematik och naturvetenskap
Högskolan Kristianstad
291 88 Kristianstad

Dept. of Mathematics and Science
Kristianstad University
SE-291 88 Kristianstad
Sweden

The paradox of life without water, summarized by Crowe 1975:

"If metabolism actually comes to a halt in anhydrobiotic tardigrades, we are forced into a seemingly insoluble dilemma about the nature of life; if life is defined in terms of metabolism, anhydrobiotic tardigrades must be 'dead', returning to 'life' when appropriate conditions are restored. But we know that some of the organisms die while in anhydrobiotic state, in the sense that they do not revive when conditions appropriate for life are restored. Does this mean that they 'died' while they were 'dead'?"

Handledare/supervisor:
Ola Persson - Universitetslektor i kemi
K. Ingemar Jönsson - Docent i teoretisk ekologi

Examinator/examiner:
Johan Elmberg - Professor i zooekologi

Författare/authors:
Johanna Lundström
Linda Svensson

Svensk titel:
Uttorkningshastighetens påverkan på anhydrobiotisk överlevnad och trehaloshalt hos tardigrader.

English title:
The effect of dehydration rates on anhydrobiotic survival and trehalose levels in tardigrades.

Abstract
The disaccharide trehalose is an important factor in many anhydrobiotic organisms, as it has a strong stabilizing effect on proteins and enzymes under desiccation. Trehalose has the ability to maintain biological structures at low water levels that would otherwise lead to irreversible denaturation. The rate at which anhydrobiotic tardigrades desiccate influence the probability to survive during a dry period, and this has earlier been documented in studies on nematodes. In this study we experimentally evaluated the effect of desiccation rate on trehalose production and anhydrobiotic survival in two tardigrade species, by using different relative humidities in desiccators. In *R. coronifer* the analysed trehalose level increased significantly with higher relative humidity during desiccation. In *M. cf. hufelandi* no such increase was observed. The survival increased at higher humidities and the revival rate showed the same pattern. We suggest that total desiccation is necessary before revival at humidities lower than about 70%. The desiccation rate in nature is not well known. Our results show high survival in lower humidities which suggest that the animals are able to cope with a wide range of desiccation rates.

Abstrakt
Disackariden trehalos är en viktig faktor hos många anhydrobiotiska organismer, eftersom det har en starkt stabiliserande effekt på proteiner och enzymer under uttorkning. Trehalos har förmågan att bibehålla biologiska strukturer vid låga vattenhalter vilka annars skulle leda till irreversibla denatureringar. Hastigheten då anhydrobiotiska tardigrader torkar ut påverkar trehalosackumuleringen och sannolikheten för överlevnad under en torrperiod, detta har tidigare dokumenterats hos nematoder. I den här studien har vi experimentellt analyserat effekten av uttorkningshastighet på trehalosproduktion och anhydrobiotisk överlevnad hos två tardigradarter genom att använda olika relativa fuktigheter i desiccatorer. Hos *R. coronifer* ökade den analyserade trehaloshalten signifikant vid högre relativa fuktigheter under uttorkningen. Hos *M. cf. hufelandi* observerades ingen sådan ökning. Överlevnaden ökade vid högre fuktigheter och återupplivningshastigheten visade samma mönster. Våra resultat antyder att total uttorkning är nödvändig innan återupplivning kan ske vid fuktigheter lägre än runt 70%. Uttorkningshastigheten i naturen är inte väl känd. Våra resultat visar en hög överlevnad vid låga fuktigheter vilket tyder på att djuren har möjlighet att klara en stor spännvidd av uttorkningshastigheter.

CONTENTS

1. Introduction

1.1 Cryptobiosis

Cryptobiosis (Keilin 1959) represents a state of latent life induced by environmental extremes. It has been described as "the state in an organism, in which it does not show any visible signs of life and when its metabolic activity becomes very difficult to detect, or comes to cease all together" (Ramazzotti & Maucci 1983, Kinchin 1994). The problem whether an organism can revive after total cessation of life is of great scientific interest. It is also one of the oldest problems that caught the thinking man considering life, death and immortality. It is reflected in almost all religions, in some legends and also in fairy tales (Keilin 1959).

Cryptobiosis is used by different organisms to survive during times of unfavourable environmental conditions (Crowe & Crowe 1992, Jönsson 2003). Often it occurs only at certain stages in the organism's life-cycle, but in tardigrades, rotifers and nematodes latent states can occur at any stage of the life-cycle. Cryptobiosis has been divided into different forms depending on the inducing environmental factor (Kinchin 1994). Anhydrobiosis (cryptobiosis induced by dehydration) is the most studied form of cryptobiosis and it has been of interest to scientists ever since Antony van Leeuwenhoek first observed it about 300 years ago (Leeuwenhoek 1702, Bradbury 2001). Other forms of cryptobiosis are cryobiosis (low temperatures), anoxybiosis (lack of oxygen) and osmobiosis (concentration changes in the solution the organism lives in) (Kinchin 1994, Bertolani et al. 2004, Wikipedia 2006). Cryptobiosis can prolong the life-span from a few months up to many years, but the active life-span remains unaffected (Kinchin 1994). Several groups of organisms are able to enter cryptobiosis, for example tardigrades, rotifers, nematodes, bacteria, fungi and some higher plants (Keilin 1959).

1.2 Anhydrobiosis

Anhydrobiosis is initiated by desiccation (Kinchin 1994) and the most important effects are a reduction of metabolism to the extent that it is no longer detectable, no mobility, no reproduction and no development (Crowe & Crowe 1992, Jönsson 2003). Organisms that are able to go into anhydrobiosis throughout their whole life cycle are called holo-anhydrobiotic, a group to which tardigrades belong. Anhydrobiosis is an escape in time from adverse conditions, instead of an escape in space made by animals that are able to move away from hostile environments (Womersley 1981, Jönsson 2005).

To survive desiccation there are two defence mechanisms; protection mechanisms and repair mechanisms. Protective mechanisms can take place both at the beginning of dehydration, when protective compounds are synthesized, and during rehydration when these protectants undergo catabolism. Studies on anhydrobiotes have shown a correlation between their ability to survive desiccation and the production of specific sugars (Crowe & Crowe 1992). The most studied sugar is trehalose, a protective substance, which accumulates for hours after dehydration has started. This anabolic activity probably depends on the animals' remaining metabolism during this time (Wright 2001). The relation between results of experimental desiccation and natural ones is unclear, since the rate in nature is poorly known (Jönsson 2001).

When once again exposed to water the animals rehydrate and resume an active life (Crowe et al. 1992, Goldstein & Blaxter 2002). However, the number of revivals for a single animal is limited and so is the time the animal is able so stay dehydrated. The time it takes for different species to show the first signs of movement when rehydrated varies, but it is generally

proportional to the duration of anhydrobiosis. The longer the animal has been dry, the longer it takes for it to come back to life. Animals that have spent a few days in the dry state may need only a few minutes, while the ones having been dehydrated for years could take a few hours (Ramazzotti & Maucci 1983). Previous data propose the limit for staying dehydrated may be within a decade. To survive over this long period the animal requires mechanisms to protect the cells from devastation in forms of oxidation and radiation (Jönsson & Bertolani 2000). A radiation experiment made by Jönsson et al. (2005) suggested that repair-mechanisms might be of importance. The animals showed the same radiation tolerance both in a hydrated and dehydrated state. The actual damage to DNA by desiccation and radiation has so far not been quantified.

1.3 Trehalose

During anhydrobiosis tardigrades may lose not only their "free water", meaning water solutions of the cytoplasm, but also their "bound water". This bound water is needed for the animals to be able to maintain the structure of important hydrated macromolecules such as proteins, membrane phospholipids and nucleic acids. The lost water has to be replaced to prevent any damage. The replacement is preferably a compound that will maintain structures during dehydration and that is easily removed during rehydration (Womersley 1981, Kinchin 1994, Wright 2001). In the early 70's John Crowe discovered that almost all animals that survive desiccation synthesize large amounts of the disaccharide trehalose (Fig. 1) during drying (Crowe 2002, Bradbury 2001). Presumably trehalose plays an important role as a membrane protectant during desiccation (Kinchin 1994, Luzardo et al. 2000, Bradbury 2001, Wright 2001). Trehalose is a disaccharide of glucose. It is chemically stable and non-reducing (Behm 1997, Wright 2001) and therefore less damaging to cells and tissues than the monomer glucose (Watanabe et al. 2004). The practical and commercial use of trehalose is important both as a protectant of food and to preserve viability of frozen cells, tissues and embryos (Behm 1997, Wright 2001, Ma et al. 2005).

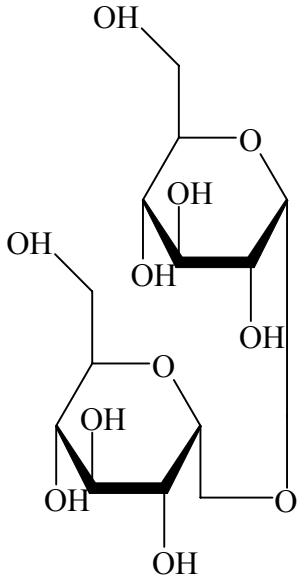


Figure 1. The chemical structure of α-α-trehalose (Behm 1997) (α-D-Glucopyranosyl-α-D-glucopyranoside, ($C_{12}H_{22}O_{11}$) after The Merck Index (2001).

The "water replacement hypothesis" suggests that disaccharides protect the anhydrobiotic organism by stabilizing the dry membrane and inhibit protein denaturation and aggregation (Ma et al. 2005). In cells without protection against dehydration, the head groups of the phospholipids are not kept separated as water is removed. The resulting closer approach destroys the structure and function of the membrane. As water is added there will be leakage of liquid cell contents as consequence (Potts 2001, Oliver et al. 2002). Cells that are able to survive dehydration contain sugars that interact with the polar head groups of the phospholipids through hydrogen bonding. In that way they retain their distances and there will be no leakage as water is added (Hoekstra et al. 1997, Crowe 2002, Oliver et al. 2002). The "vitrification hypothesis" proposes the membrane to be stabilized by vitrified sugar. As sugar creates this form of glass the movements of macromolecules are reduced. By doing so, both molecular and cellular structures are protected while their activity is restrained (Hoekstra et al. 1997, Sun & Leopold 1997, Bradbury 2001, Crowe 2002). To survive a total desiccation both water replacement and vitrification mechanisms might be involved (Ohtake et al. 2004).

Other studies have since Crowe's discovery suggested that while trehalose probably is the main substance for surviving desiccation, there may also be other factors involved in cell protection and recovery (Bradbury 2001, Oliver et al. 2001, Wright 2001, Oliver et al. 2002, Ma et al. 2005). Some of these are different heat-shock proteins (Ma et al. 2005) that are produced as a response to environmental stress such as dehydration. These proteins may also be synthesized in unstressed cells, and their role is for example to regulate other proteins (Ramlöv & Westh 2001). Although trehalose serves as a protective molecule in many desiccation-tolerant Metazoans, and levels up to 20% dry weight (dw) have been documented, tardigrades seem to need only a moderate level for a good protection (Westh & Ramlöv 1991). There are also anhydrobiotic animals that lack trehalose completely, e.g., rotifers (Lapinski & Tunnacliffe 2003).

1.4 Tardigrades and anhydrobiosis

In 1773 German Goeze made one of the first recorded observations of "little water bears", later on named tardigrades. The Italian professor Spallanzani gave them their current name tardigrades because of their way to walk, *tardi* being Italian for *slow* and *grado* meaning *walker* (Ramazzotti & Maucci 1983). In 1790 Swedish Carl von Linnaeus included tardigrades in Systema naturae (Kinchin 1994). Even though the phylum Tardigrada was discovered about 230 years ago, these invertebrates have until this day been little studied (Goldstein & Blaxter 2002). All tardigrades are small animals, almost always invisible to the naked eye and usually their body length does not exceed 1 mm (Schmidt-Rhaesa 2001). Many tardigrade species are more or less transparent (Goldstein & Blaxter 2002) and often colourless or grey, but some species may be brown, yellow, orange, pink or red (Ramazzotti & Maucci 1983). The tardigrade phylum is usually divided into two classes, forming the Heterotardigrada and Eutardigrada. Heterotardigrada, in which genus Echiniscoides is found, are sometimes described as "armoured" tardigrades who possess a carapace. The Eutardigrada on the other hand have no dorsal plates and are therefore called "naked" tardigrades. Both genera Richtersius and Macrobiotus, investigated in this study, belong to the latter group (Ramazzotti & Maucci 1983, Kinchin 1994). For further details regarding the systematics of tardigrades and their relationship to other phyla, see Appendix 1.

An easy way of grouping tardigrades ecologically is to call them either aquatic or terrestrial. Aquatic tardigrades live in marine, fresh and brackish waters and beach sediments. The largest number of known species is however found among so called semi-terrestrial tardigrades that live in mosses, lichens, liverworts and some other plants, and they also inhabit leaf litter in forests. All tardigrades must nevertheless be considered aquatic animals even though they live in terrestrial environments. To be able to live an active life they need to be surrounded by water, which gives them a moist environment (Ramazzotti & Maucci 1983). About 935 tardigrade species have been described but new species are continuously added (Guidetti et al. 2005). The occurrence of tardigrades in Sweden is uncertain, but so far the list includes 95 species (The Swedish Museum of Natural History 2006). Tardigrades are especially abundant in temperate and polar zones and some tardigrade species are even said to be cosmopolitans. They are found everywhere, from the Arctic to the Antarctic, while others are only found in a few areas. The most interesting character of tardigrades is their ability to enter cryptobiosis, which happens when the surrounding environment becomes too harsh (Ramazzotti & Maucci 1983).

Anhydrobiosis as a phenomenon was discovered by the first observers of tardigrades when they realised that seemingly dead animals in dehydrated sediments came alive again when exposed to water. Spallanzani stated in the 18th century that tardigrades are a group of animals

that can enjoy the advantages of "resurrection after death" (Kinchin 1994). Anhydrobiotic tardigrade species living in terrestrial habitats are only active when surrounded by a film of water (Bertolani et al. 2004) since they require moisture for gas exchange (Suzuki 2003). Because of that, terrestrial tardigrades are only active during limited periods, for example during rainfall, heavy dew, melting ice or snow, or a high degree of humidity (Ramazzotti & Maucci 1983). Entering the anhydrobiotic state, the tardigrades start to produce cell protecting substances and reduce their metabolism while they loose most of their free and bound water (Sun & Leopold 1997, Bertolani 2004). During the dry out the movements are reduced, the legs are drawn back and more or less disappear into the body (Crowe 1971). The formation of this so called tun (Fig. 2), reduces the surface area to about half that of the hydrated animal (Jönsson 2001), and this seems to be characteristic for animals capable of going into anhydrobiosis. Nematodes for example, coil their bodies while rotifers contract their bodies when entering the dormant state (Kinchin 1994).

(a) (b)

Figure 2. *R. coronifer* in the hydrated state (a) and after tun formation (b). Photo: K. Ingemar Jönsson.

The tun can survive for several years without water (Jönsson & Järemo 2003) as well as being resistant to for example high pressure and both high and low temperatures (Jönsson 2003). Tun formation is thought to protect the internal organs from physical damage during the time following both dehydration and rehydration (Crowe 1971). A slow, uniform desiccation rate similar to the one in nature allows them to gradually dry up into a tun while a too quick desiccation may cause a contraction into an irregular shape and the animal dies (Ramazzotti & Maucci 1983). Dehydration slowly continues until the tardigrade reaches equilibrium for a given relative humidity (RH). At this moment the amount of water retained is very low (Wright 2001). Humidity-related parameters regarding tardigrades have since long been discussed. It has for many years been known that tardigrades will enter anhydrobiosis successfully only if they are dehydrated in high relative humidities (Wright 1988). Crowe (1972) did show some of the effects of relative humidity on survival and desiccation rate. He found the highest number of survivals when the animals were dried at relative humidities over 70%. Studies on revival times have shown great variation and become longer and more variable after rapid desiccation. Also important is that different species of tardigrades have different tolerance to dehydration (Wright 1988).

1.5 Objectives

The purpose of this study is to investigate the patterns of survival and trehalose production in connection with dehydration at different relative humidities. Hopefully this will gather some new information and enhance future research. According to previous studies trehalose may play a role for survival in tardigrades, but it is not known how or if the production of this sugar will change at different desiccation rates. However, if trehalose is an important factor for anhydrobiotic survival one would expect that trehalose synthesis covaries with anhydrobiotic survival. A too rapid dehydration may shorten the time the animals have to prepare for drying and so death may become unavoidable for many of the tardigrades. Since all species or tissues have evolved different adaptations to survive desiccation, further investigations showing interplays between different mechanisms might be of interest (Oliver et al. 2001). As far as we know no studies have been made on trehalose levels and survival response to different desiccation rates in tardigrades. Nor are there any previous studies on *E. testudo* on the aspect of trehalose content, but Wright (1988) used the species in his desiccation study and showed that it has a high ability to survive desiccation. Wright (1988) and Horikawa & Higashi (2004) studied survival after desiccation at different humidities in tardigrades and West & Ramlöv (1991) made analyses of trehalose content in one tardigrade species. Higa & Womersley (1993) have performed a study on survival and analysed trehalose content in nematodes after different desiccation rates. In our study the aspect of trehalose production is added to find a possible connection between synthesis of trehalose and the importance of this substance for the survival of tardigrades. Our study has focused on the following questions:

- Will the desiccation rate affect the revival time?
- Does anhydrobiotic survival vary with dehydration at different humidities, i.e. desiccation rates?
- Does anhydrobiotic survival and trehalose production covary?
- Does *E. testudo* contain trehalose in the dehydrated state?

We predict that lower humidities will increase the revival time, because low humidities may induce more cellular damage, requiring more time for repair. At higher humidities the damage to the cells will be minor which facilitates the revival. Due to the cell damage in lower humidities there will probably be a decrease in number of survivals. The reason is that the dehydration rate will be too fast in low humidities, this may give a breakpoint in survival. If the desiccation rate has minor impact on survival no distinct breakpoint will be shown, and there will be an overall increase in survival. If trehalose plays a major role in anhydrobiotic survival the level will covary with the survival results. Less influence by trehalose will not give an obvious relation.

We have worked together on all parts of this project and have both been responsible for, and performed, all parts of it.

2. Material and methods

2.1 Species

The species used in this project are described in detail in Kinchin (1994) and Ramazzotti & Maucci (1983).

Macrobiotus cf. hufelandi (Schultze, 1833)
Macrobiotus cf. hufelandi (Fig. 3), belonging to the Eutardigrada, was the first tardigrade species to be described and is actually a complex consisting of many similar species. Adult animals are between 300µm - 450µm long, nontransparent and may look white, grey or brown, while juveniles are colourless. Eyes are almost always present. This is the most common species and is called a cosmopolitan because it can be found everywhere in many different types of habitats, both terrestrial and sometimes freshwater habitats. *M. cf. hufelandi* exists everywhere tardigrades are found except on New Zealand (Kinchin 1994, Ramazzotti & Maucci 1983).

Figure 3. A hydrated tardigrade of species *M. cf. hufelandi*. Photo: K. Ingemar Jönsson.

Richtersius coronifer (Richters, 1903)
Richtersius coronifer, formerly known as *Adorybiotus c.* or *Macrobiotus c.*, is a large eutardigrade species that may be up to 1000µm long. They are seldom colourless, but instead often yellow or orange. Large eyespots are visible. This species is fairly common and has been found both in Europe, South America and in the Arctic (Kinchin 1994, Ramazzotti & Maucci 1983).

Echiniscus testudo (Doyére, 1840)
Echiniscus testudo (Fig. 4) belongs to the class Heterotardigrada and is part of the genus *Echiniscus* which is known to be very insensitive to dry habitats. The animals are typically brown or red and up to 360µm long. Red eyespots are present. *E. testudo* seems to prefer very dry environments with long periods of desiccation. They are found in many places all throughout the world, including Europe, South America, Spitsbergen Islands and Asia (Kinchin 1994, Ramazzotti & Maucci 1983).

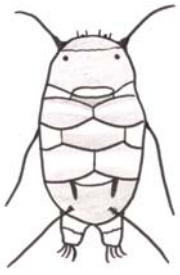


Figure 4. A hydrated *E. testudo* showing the carapace, after Kinchin (1994).

2.2 Moss samples

Moss samples (*Orthotrichum cupulatum*) (Fig. 5b) were collected from two different places in the Alvar habitat (Fig. 5a) at the island of Öland, Sweden, in January and April 2006. Alvaret is an extreme environment with sunny and dry summers where only the hardiest organisms will survive (Andersson 2005). The samples were stored at room temperature until dry and then kept in a plastic or paper bag in a refrigerator for up to two months, until use.

(a) (b)

Figure 5. The extreme and harsh environment in Alvaret at Öland, Sweden (a), where for example the moss *Orthotricum cupulatum* (b) is found. *O. cupulatum* has proved to be a suitable environment for tardigrades. Photo: K. Ingemar Jönsson.

2.3 Extraction of tardigrades

Tardigrades were extracted from moss material by using plastic funnels filled with distilled water. Tap water was not used since the water in Kristianstad is relatively hard which could possibly have a negative effect on the survival of tardigrades (O. Persson, personal communication). A short piece of transparent hose with a small tube in one end was put at the narrow end of the funnel. In order to obtain as pure samples as possible, the moss was put upside down on two layers of mesh; a textile material with a metal net (mesh size 1 mm) underneath it. The funnel was filled with plenty of distilled water and the soaked moss was left for 20-24 hours. During these hours of soaking the tardigrades fell off the moss material down through the textile and metal net, by their own movements, and ended up in the small tube. The next day this tube was emptied into a small glass bowl, fresh distilled water was added and the animals were observed in a stereo microscope. Living tardigrades of each species described above were collected one by one using an Irvin loop or a Pasteur pipette to eliminate contamination of the samples. The animals were placed in groups of 25 on 2x2 cm microscope slide squares, after Horikawa & Higashi (2004). All superfluous water was removed with a Pasteur pipette and blotting-paper.

2.4 Dehydration

The slides with aggregated tardigrades were placed in salt solution desiccators with fixed relative humidities (% RH). The desiccators were kept at room temperature (approx. 20^{o}C). A hygrometer was used to confirm that the correct relative humidity had been established. The relative humidities used in this experiment were 0%, 33%, 45%, 58%, 65%, 69%, 75%, 79% and 85%. Phosphorus pentoxide (P_2O_5) was used for 0% RH, magnesium chloride ($MgCl_2$) for 33% RH, potassium carbonate (K_2CO_3) for 45% RH, magnesium nitrate ($Mg(NO_3)_2$) for 58% RH, ammonium nitrate (NH_4NO_3) for 65% and 69% RH (different water concentrations), ammonium chloride (NH_4Cl) for 79% RH, and sodium chloride (NaCl) for 75% and 85% RH (different water concentrations) (Greenspan 1977). Samples were left in the desiccator for 48 hours to make sure the animals were completely dehydrated (K.I. Jönsson, personal communication).

2.5 Survival count

The survival study of *R. coronifer* and *M. cf. hufelandi* was made in six to nine replicates at each RH. After 48 hours the microscope slide was removed from the desiccator, and distilled water was added to the aggregated group of animals in three droplets (approx. 300µl) with a Pasteur pipette following Horikawa & Higashi (2004). Several previous experiments with two moss living tardigrade species, one being *M. cf. hufelandi*, showed that an active tardigrade uses approximately 10^{-3}µl oxygen per hour (Ramazzotti & Maucci 1983). To avoid anoxybiosis it is important to use fresh oxygen-rich water and to study the animal long enough to confirm its revitalization (Nelson 2002, Jönsson et al. 2004). The animals were studied under a stereo microscope every 15 minutes for one hour and the number of surviving tardigrades was noted. The criterion of survival that we used was any small movement of the legs. The animals were left in water for another two hours and after a total revival time of three hours the animals were studied again for a final survival count. A three hour revival time is according to Horikawa & Higashi (2004) long enough to receive a reliable result.

2.6 Trehalose analyses

Sample preparation

After 48 hours in the desiccator, four microscope slides with approximately 25 animals on each slide were removed. The slides were placed under a stereo microscope and the animals were transferred into an Eppendorf tube using a scalpel and a needle. Consequently each Eppendorf tube contained about 100 animals. The tubes were placed in a Memmert Model 400 oven at 80°C and after 24 hours the temperature was raised to 100°C for another 24 hours to remove all the excess water from the samples before preparation for GC analyses. All tubes were then transferred to a desiccator containing the drying agent phosphorus pentoxide (P_2O_5), which has a strong attraction for water (Jones & Atkins 2002), to maintain a completely dry environment until the next step.

The extraction began by adding 200µl 40% ethanol to each Eppendorf tube, which contained approximately 100 animals. The tube was put in 100°C water for one minute. The animals and the ethanol, together with another 2 x 100µl 40% ethanol used to rinse the tube, were transferred to a homogenizer and ground manually for 1 minute. The homogenate plus two 100µl 40% ethanol washings were combined and transferred back to the Eppendorf tube. The tube was centrifugated for three minutes at 14.5 x 1000 rpm in an Eppendorf Minispin Plus. The supernatant was then transferred to a new Eppendorf tube and to the remaining pellet 200µl 95% ethanol was added. The tube was placed in an ultra sonic bath Branson 2210 for one minute to dissolve the pellet and then in 100°C water for one minute. The contents of the tube were once again homogenized and the tube was washed with 100µl 95% ethanol. After the grinding, the mixture and two washes of 100µl 95% ethanol were transferred back to the Eppendorf tube which was then centrifugated. The supernatant was combined with the supernatant from the first homogenisation and the pellet was once again resuspended, this time with 20% ethanol. The same procedure as described above was repeated with 20% ethanol. The Eppendorf tube with the three supernatants containing the extracts was placed in an oven at 80°C for 4 hours until half the volume had evaporated. The temperature was then raised to 110°C for approximately 15 hours to evaporate all the solvent. When all the solvent had evaporated the tube was washed with 2 x 50µl 20% ethanol and 1 x 50µl 95% ethanol. The final extracts were transferred to a 0.3ml glass vial (60.03-FIV, Scantec Lab, Partille, Sweden), together with the internal standard (see below).

Weight measurements
To allow quantification of trehalose (% trehalose per tardigrade dry weight), a few samples of each species were weighed. When completely dry the animals were easily moved from the Eppendorf tube to a pre-weighed foil pan and weighed on a Mettler M3 microbalance (Mettler Instrumente AG). The mean weight for each species was then calculated to make a quantification of the level of trehalose possible.

Standard solutions
A sorbitol solution was added as an internal standard used for quantitative measurements. First a 0.4% stock solution, containing commercial α-Sorbitol (Sigma-Aldrich, St. Louis, USA) and 40% ethanol, was prepared and then this stock solution was diluted to a 0.008% standard solution. 50µl of the standard was added to each vial containing the final extracts, corresponding to approximately 4% of the dry weight of 100 tardigrades. The vial was placed in an oven at 80^{o}C for 24 hours in order to evaporate all the solvent. For the external standards commercial α-Sorbitol and trehalose (0.04%) (Sigma-Aldrich, St. Louis, USA) was used.

GC analyses of extracted compounds
A seal (50 X 11-AC7, Scantec Lab, Partille, Sweden) was put on the vial and 100µl Sigma-Sil-A (Sigma-Aldrich, St. Louis, USA) was added with a gas-tight Hamilton syringe in a fume cupboard. The Sigma-Sil-A is a premixed reagent, containing chlorotrimethylsilane, pyridine and hexamethylsilazane, able to form volatile trimethylsilylcarbohydrate derivates suitable for gaschromatography (Westh & Ramlöv 1991). Analyses were made with a Finnigan Trace GC-MS (ThermoQuest, Milan, Italy) and the computer software used was Xcalibur^{TM} (Home Page version 1.2). The samples were injected on a BPX-5 column (30 m x 0.25 mm, SGE, Ringwood, Australia) where helium was used as a carrier gas. The GC-MS was run in SCAN mode. The temperature program for the GC oven was: 100^{o}C for 2 minutes, 10 ^{o}C/min to 250 ^{o}C, and a final hold at 250 ^{o}C for 5 minutes. After half of the analyses where finished the program was changed to 100^{o}C for 2 minutes, 10 ^{o}C/min to 250 ^{o}C, and a final hold at 250 ^{o}C for 11 minutes since important peaks were lost at the end of the short program. Quantifications were based on peak areas (Hallgren et al., in press) by using the internal standard solution containing 0.008% sorbitol. See Appendix 3 for an example of a chromatogram. The response factor for trehalose was calculated by using the external standard containing sorbitol and trehalose in the same concentrations (0.04%). Response factor = Area $_{Trehalose}$ / Area $_{Sorbitol}$. The level of trehalose in the dehydrated tardigrades could then be calculated.

2.7 Statistics
All statistical analyses were performed with the computer software SYSTAT (Version 11.0, Systat Software Inc.). The non-parametric Kruskal-Wallis (K-W) one-way analysis of variance and sub type Mann-Whitney U-test (U) was used to evaluate variation in survival and trehalose between dehydration groups and between species. In correlation analyses we used Spearman rank correlation. Non-parametric analyses were used because the data were not normally distributed. All statistical tests were two-tailed.

3. Results

3.1 Samples

The number of different relative humidities, number of replicates made in each humidity (mean value in brackets) and the number of animals in each replicate of the two species used in the survival study is shown in Table 1. Also shown is the number of humidities, number of replicates made in each humidity and the mean number of animals in each replicate of the three species used in the trehalose study. The number of replicates made on hydrated tardigrades and the mean number of animals in each hydrated replicate is also displayed. Some of the dry animals were weighed on a microbalance and the table shows the number of samples weighed of each species, the mean number of animals in each sample and the mean weight with standard deviations in µg for the three species.

Table 1. Number of animals and replicates between dehydration groups and species in the survival study. The mean number of replicates/humidity is in brackets. Also shown is the number of animals and samples between dehydration groups and species in the trehalose study. Only eight humidities were used for *M. cf. hufelandi* and *R. coronifer* in the trehalose study due to a lack of animals at the end of the study. *E. testudo* was not found in sufficient numbers and only two humidities were tested regarding their trehalose content. Hydrated samples and the weight samples for quantification of trehalose are also illustrated (standard deviation in brackets). Approximately 8000 tardigrades were collected during this study.

	M. cf. hufelandi	*R. coronifer*	*E. testudo*
No of humidities (survival)	9	9	-
No of replicates/ humidity	6-9 (7)	6-9 (7)	-
No animals/ replicate	25	24	-
No of humidities (trehalose)	8	8	2
No of samples/ humidity	3	2	1
No animals/ sample	101	100	300
No of hydrated samples	3	2	-
No of animals/ sample	101	99	-
No of weight samples	3	5	3
No of animals/ sample	101	102	100
Mean weight (µg)	0.56 (±0.07)	1.46 (±0.26)	0.30 (±0.06)

3.2 Revival rate

At all humidities the proportion of active animals increased with time after rehydration began. *R. coronifer* needed longer time to revive than *M. cf. hufelandi* after rehydration. *M. cf. hufelandi* dehydrated at RH above 75% reached their maximum survival number after 60 minutes, and then the curve is flattened out. At lower humidities the proportion of active animals steadily increased to the last count at 180 minutes. This pattern was not observed with *R. coronifer* where the proportion increased steadily with time at all humidities. Figures 6 and 7 show the revival rate for *M. cf. hufelandi* and *R. coronifer*, respectively. Note that the scale on the x-axis, representing time, is discontinuous. See Appendix 2 for the proportions and standard deviations in numbers.

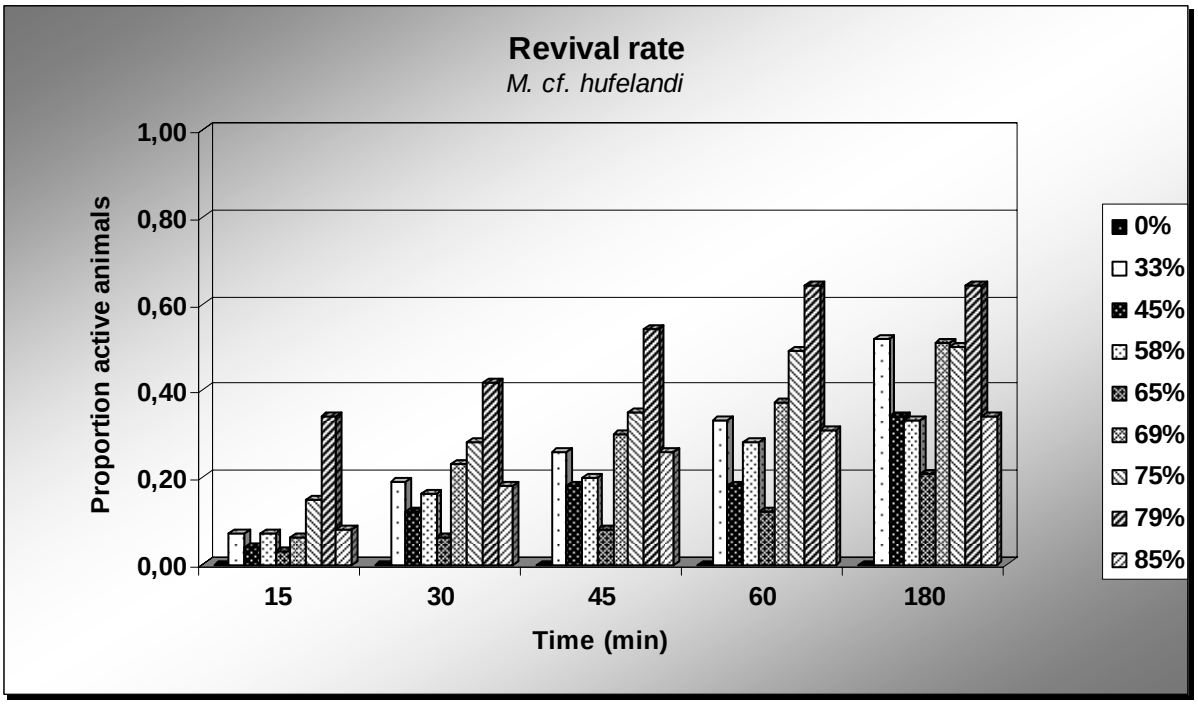


Figure 6. The proportion of active animals of *M. cf. hufelandi* at different times after rehydration. Each bar represents the mean value of six to nine replicates at each count event. Note that the x-axis (time) is discontinuous.

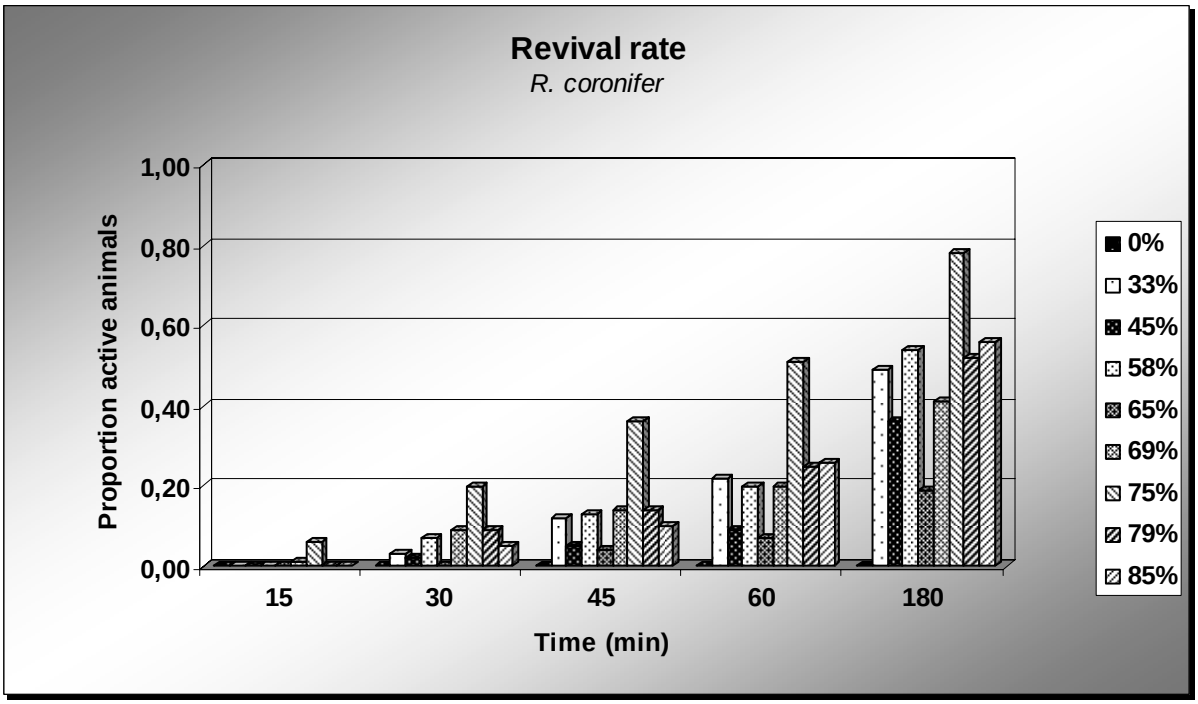


Figure 7. The proportion of active animals of *R. coronifer* at different times after rehydration. Each bar represents the mean value of six to nine replicates at each count event. Note that the x-axis (time) is discontinuous.

3.3 Survival and trehalose

The mean proportion (with standard deviations) of surviving animals for each humidity and the level of trehalose in dehydrated tardigrades, for species *M. cf. hufelandi* and *R. coronifer*, is shown in Figure 8 and 9 respectively. For *M. cf. hufelandi,* the animals dehydrated at 79% RH had the highest survival while the tardigrades dehydrated at 65% RH had the lowest survival. *R. coronifer* shows the highest survival at 75% RH, and they too show the lowest activity when dehydrated at 65% RH. KW is the Kruskal-Wallis statistica value, the higher value the bigger difference, df is the degree of freedom and n is the number of observations. Overall we found a significant difference in survival between dehydration groups (KW = 32.77, df = 8, $p<0.001$) in the statistical comparisons between the nine dehydration groups in *M. cf. hufelandi* (Table 2). We also found an overall significant difference between the nine dehydration groups within species *R. coronifer* (Table 3) (KW = 30.53, df = 8, $p<0.001$). Note that the trehalose level for *M. cf. hufelandi* (Fig. 8) dehydrated in 58% RH is missing, due to a malfunctioning GC-analysis. Trehalose level did not differ among dehydration groups in either species (*M. cf. hufelandi*: KW = 9.22, df = 7, $p>0.10$, *R. coronifer*: KW = 12.85, df = 8, $p>0.10$) and therefore no detailed analyses between different dehydration groups were performed. For a complete table of the mean numbers of survival and trehalose levels, see Appendix 2. We found no correlation between survival and dehydration groups in *R. coronifer* ($r_s=0.52$, $p>0.10$, n = 8), but between trehalose and dehydration rate (relative humidity) we found a strong positive correlation ($r_s=0.93$, $p<0.005$, n = 8). The same analyses in *M. cf. hufelandi* showed no significant association (survival contra dehydration rate: $r_s=-0.29$, $p>0.10$, n = 7, and trehalose contra dehydration rate: $r_s=0.05$, $p>0.10$, n = 7).

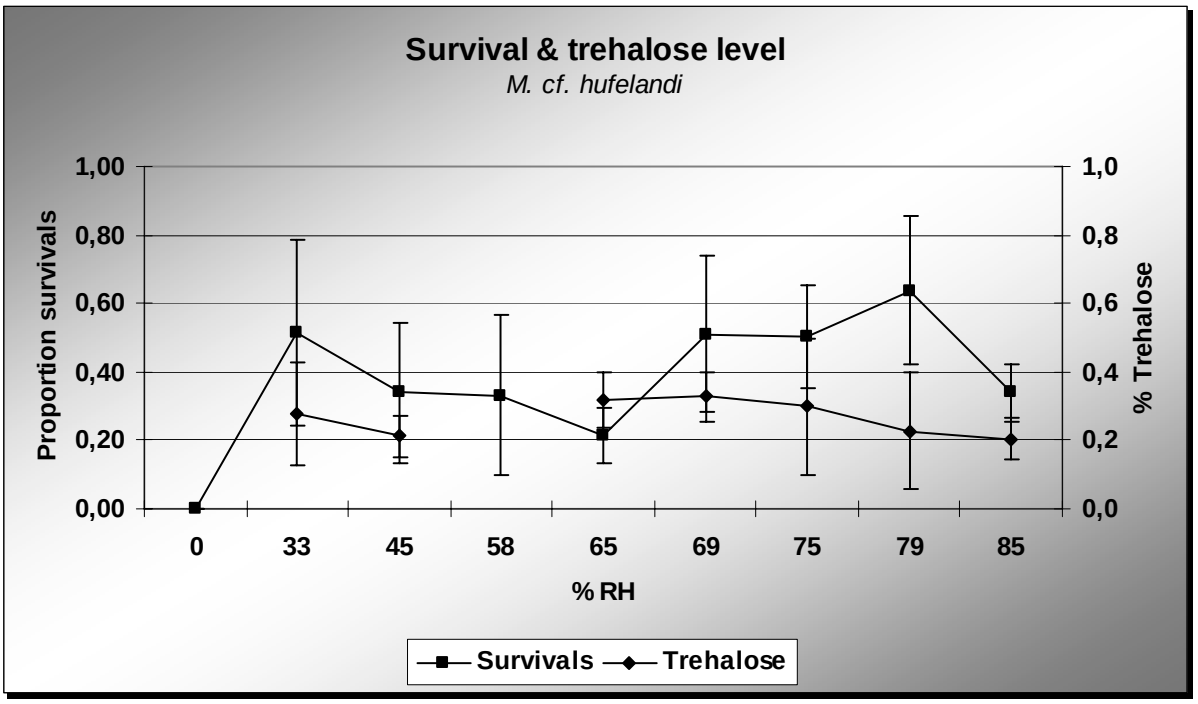


Figure 8. Recorded survival (180 minutes post-rehydration) and trehalose level (% dw) in *M. cf. hufelandi* at different humidities. The trehalose level at 58% RH is missing due to a malfunctioning GC-analysis. The line with squares represents the proportion of surviving tardigrades and the line with triangles represents the level of trehalose (% dw) in the tardigrades dehydrated at each humidity.

Table 2. Statistical analyses of survival between different dehydration groups in *M. cf. hufelandi*. The overall survival analysis gave KW = 32.77, df = 8, p<0.001, n = 66. The overall statistical analysis of trehalose in *M. cf. hufelandi* did not show any significance (KW = 9.22, df = 7, p>0.10, n = 23). (NS = Non Significant, * = p< 0.05, ** = p<0.01, *** = p<0.001).

% RH	0	33	45	58	65	69	75	79
0	-	-	-	-	-	-	-	-
33	***	-	-	-	-	-	-	-
45	***	NS	-	-	-	-	-	-
58	**	NS	NS	-	-	-	-	-
65	***	**	NS	NS	-	-	-	-
69	***	NS	NS	NS	**	-	-	-
75	**	NS	**	*	***	NS	-	-
79	**	NS	*	NS	***	NS	NS	-
85	**	NS	NS	NS	**	NS	NS	NS

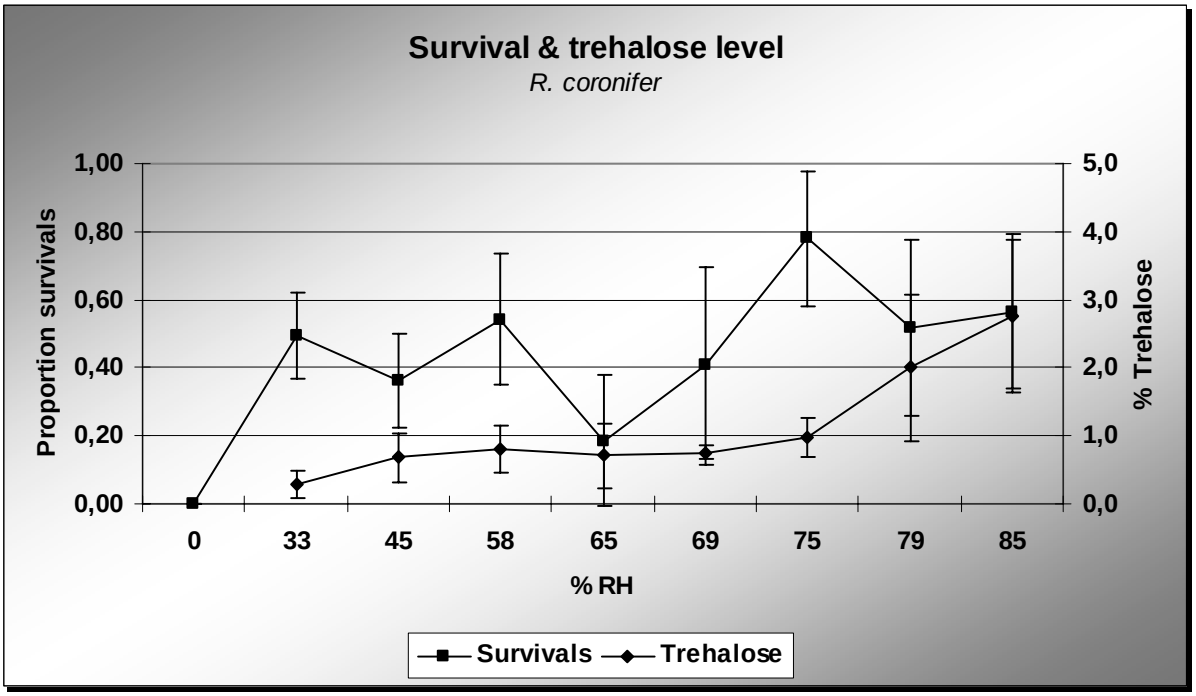


Figure 9. Recorded survival (180 minutes post-rehydration) and trehalose level (% dw) in *R. coronifer* at different humidities. The line with squares represents the proportion of surviving tardigrades and the line with triangles represents the level of trehalose (% dw) in the tardigrades dehydrated at each humidity.

Table 3. Statistical analyses of survival between different dehydration groups in *R. coronifer*. The overall survival analysis gave KW = 30.53, df = 8, p<0.001, n = 61. The overall statistical analysis of trehalose in *R. coronifer* did not show any significance (KW = 12.85, df = 8, p>0.10, n = 18). (NS = Non Significant, * = p< 0.05, ** = p<0.01, *** = p<0.001).

% RH	0	33	45	58	65	69	75	79
0	-	-	-	-	-	-	-	-
33	**	-	-	-	-	-	-	-
45	**	NS	-	-	-	-	-	-
58	**	NS	NS	-	-	-	-	-
65	*	***	**	***	-	-	-	-
69	*	NS	NS	NS	**	-	-	-
75	**	NS	*	NS	***	NS	-	-
79	**	NS	NS	NS	***	NS	NS	-
85	**	NS	NS	NS	**	NS	**	NS

We did not find any differences among species in survival or trehalose for specific dehydration groups, apart from group 75% RH, where *R. coronifer* had a significantly higher survival (Table 4).

Table 4: Statistical analyses of survival and trehalose between *R. coronifer* and *M. cf. hufelandi* within each dehydration group. U equals the Mann-Whitney U-test statistica value. (NS = Non Significant, * = p< 0.05, ** = p<0.01, *** = p<0.001).

	Survival			Trehalose		
% RH	U	p	n	U	P	n
33	32.5	NS	15	2.0	NS	5
45	22	NS	15	0.0	NS	5
58	7.5	NS	12	-	-	-
65	49.5	NS	18	0.5	NS	4
69	48.5	NS	18	0.0	NS	5
75	5.0	*	12	0.0	NS	5
79	37.0	NS	15	0.0	NS	5
85	6.0	NS	12	0.0	NS	5

Hydrated animals of the species *M. cf. hufelandi* contained 0.78% trehalose, while *R. coronifer* contained 0.48% in the hydrated state. Hydrated *E. testudo* were not analysed for trehalose content. Only two dehydrated samples of *E. testudo*, each containing approximately 300 animals, were extracted and analysed in the GC. The sample dehydrated in 45% RH contained 0.64% trehalose and the animals dehydrated in 75% contained 0.72% trehalose.

3.4 Associations between survival and trehalose level

The Spearman rank correlation (r_s) was used to evaluate the associaton between trehalose level and survival. There was no relationship between trehalose level and survival in *M. cf. hufelandi* (r_s = 0.054, p>0.10, n = 7, Fig. 10). In *R. coronifer* a weak positive and marginally significant trend was found, suggesting a possible impact of trehalose on survival (r_s = 0.71, 0.10>p>0.05, n = 8, Fig. 11). Thus higher levels of trehalose were associated with a higher proportion of surviving tardigrades. The letter r is the correlation coefficient measuring the connection between two variables. Note that the x-axis, representing trehalose content, differs between the two figures below, because of lower trehalose levels in *M. cf. hufelandi*.

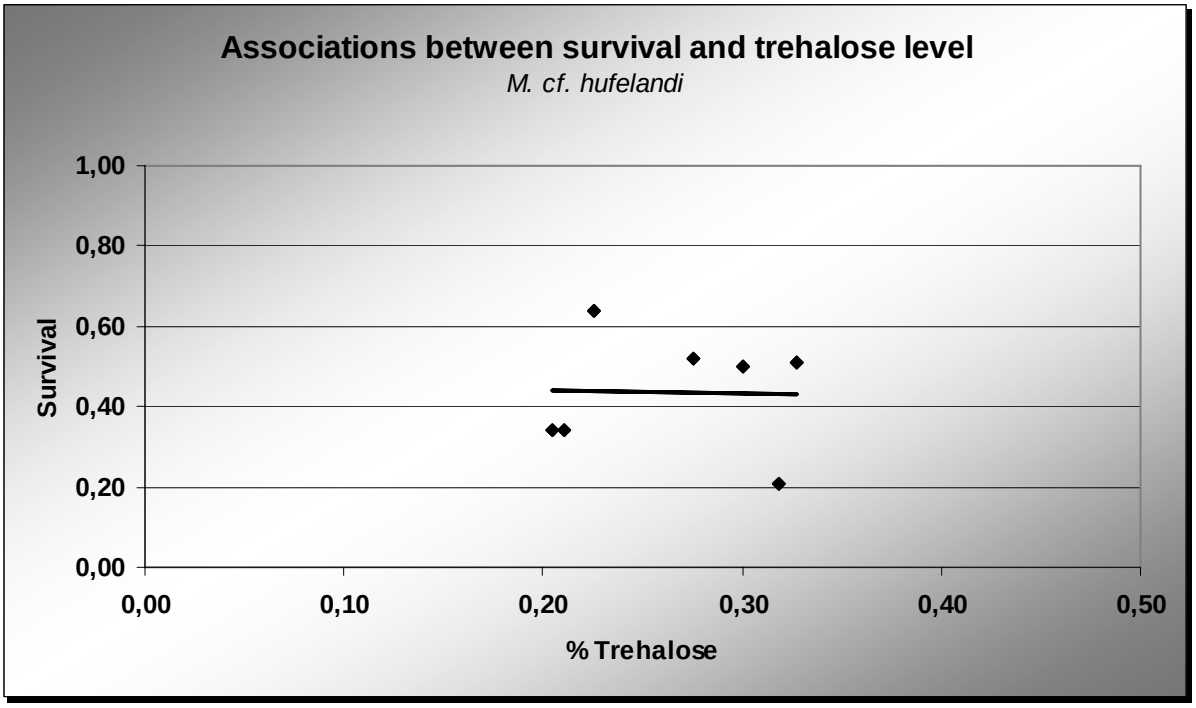


Figure 10. Associations between survival and trehalose level for *M. cf. hufelandi* (r_s = 0.054, p>0.10, n = 7).

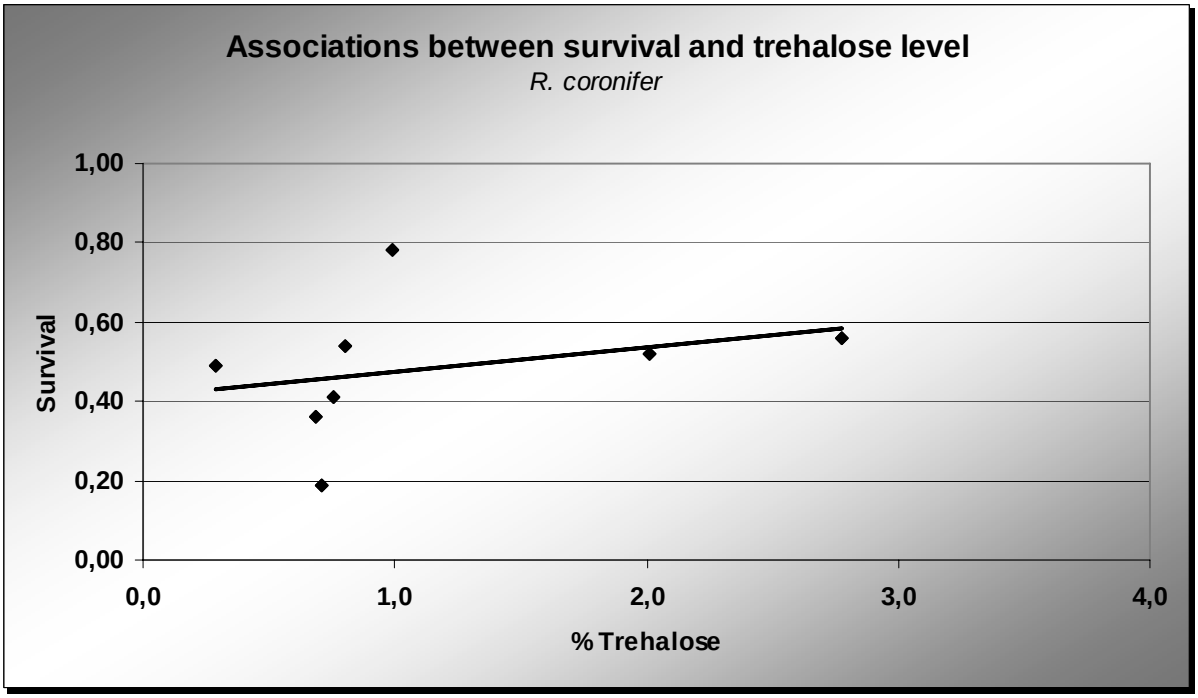


Figure 11. Associations between survival and trehalose level for *R. coronifer* (r_s = 0.71, 0.10>p>0.05, n = 8). The .correlation shows a positive trend, i.e. the survival increases with increasing trehalose level.

4. Discussion

Revival rate

The rate of revival differed between the two tardigrade species used in our study. *R. coronifer* clearly needs a longer time period for recovery after rehydration than *M. cf. hufelandi*. *R. coronifer* however had the highest proportion of active animals in the final survival count after three hours and thus has a better total revival than *M. cf. hufelandi*. But when considering the activity during the early readings *M. cf. hufelandi* showed a remarkably faster revival. During the study it was observed that some animals of *M. cf. hufelandi* started to move quickly after water was added, sometimes within minutes. The difference in rate is probably due to species specific factors. One of which could be the difference in body size between the two species. *R. coronifer* is much bigger and thicker than the slimmer *M. cf. hufelandi*. Also other physical explanations could be possible. One may be the period of time spent in the dry state, though it is well established that there is a positive correlation between recovery and time spent in inactivity (Jönsson 2003). The longer revival time is probably a consequence of DNA repair processes, since the animal needs longer time to repair more damage (Jönsson 2003). *R. coronifer* moves about at a slower speed than *M. cf. hufelandi*, which may affect internal movements and water transport. There may also be a difference in the thickness of the cuticle. A thick cuticle helps the animal to retain its water in the beginning of dehydration and so gives the tardigrade enough time to contract into a tun. When once again exposed to water the thick cuticle would prevent a fast penetration, and that is why a longer time may be needed before the tardigrades of species *R. coronifer* are once again active. These are speculations, and we do not know if the thicknesses of the cuticules in the two species in fact differ. Horikawa & Higashi (2004) looked into the revival time among tardigrades dehydrated in relative humidities between 49% and 63% and their results, like ours, showed that at lower humidities it takes longer time for the animals to revive and once again become active. *M. cf. hufelandi* shows an interesting pattern in revival rate. Between 0% and 69% RH the tardigrades need much longer time to recover than at 75% and higher RH. At higher humidities the maximum number of survivals was reached already after 60 minutes, and at the last count there were small variations in number of survivals. The slower rate at lower humidities is due to larger DNA damages. In *R. coronifer* there was no such pattern. The number of active animals continuously rised until the last count. No statistical analyses were made on our results regarding revival rate due to repeated values on the same specimens.

Survival and trehalose

The survival after dehydration in different humidities shows remarkable similarities between the two species. The outcome was not the continuous increase as anticipated, but the two species showed a similar pattern. It is surprising to see that tardigrades of both species dehydrated in 65% RH had this low survival despite repeated experiments. The dip at 65% RH is hard to explain but could possibly depend on defects in the method. One source of error might be a change in humidity over time since there was no possibility to keep a hygrometer in the desiccators during the study. The humidities were however controlled at the end of the study and only a small change of a few percent was noted, so this might not be the whole explanation. The salt used for controlling the 65% RH was ammonium nitrate which was also used in 69% RH (where different water concentrations were used) and in this humidity the survival response was much higher. A nematode study performed by Higa & Womersley (1993) is similar to our study and interestingly enough they show similarities in having a higher survival response in lower and higher humidities. In contrast to our study they used a different method, which included an exposure to high humidity before desiccation and the

nematodes were moved between different humidities. In their study there was a dip in all experiments at 40% RH. At 40% RH few animals survived, it seemed as though they needed to be fully dehydrated before they once again could resume an active life when exposed to water. The explanation for the drop at 65% RH in our study must for the time being remain an open question.

In our survival study the animals were aggregated in rather large clumps at tun formation and there should not be any big difference in survival between the two species, even though one of them might be more sensitive to dehydration than the other. The curve above 65% RH matches previous studies, where the survival apparently increases at higher humidities, and 75-79% RH seems to be the most favourable for anhydrobiotic survival. Crowe (1972) observed a higher number of survivals when the tardigrades were dried at relative humidities greater than 70% RH. This corresponds quite well with the results of this study where the highest number of survivals was found in 75% RH for *R. coronifer* and 79% RH for *M. cf. hufelandi*. This is probably related to the fact that at high humidities the tardigrades retain large amounts of their body water for quite a long time. At lower humidities they lose water much faster and do not have time to form a tun, and so the animals die instead. Lapinski and Tunnacliffe (2003) studied dehydrated rotifers and noted that they have a poor survival at lower humidities, around 50% RH, and better at higher humidities. This indicates that the animals need time during desiccation to be able to survive. Their results correspond with the results in this study, where a higher proportion of tardigrades survive at higher humidities than at lower ones. The difference between the two species according to highest survival may be due to desiccation rate. Ivarsson and Jönsson (2004) found that aggregation positively affected the survival after desiccation in *R. coronifer*. They argue that larger animals desiccate at a slower rate since they have a smaller relative surface area. Due to body size differences between *R. coronifer* and *M. cf. hufelandi*, the total size of the group of 25 will differ. The larger group has less relative surface area compared to the smaller one. Consequently *M. cf. hufelandi* will suffer a higher desiccation rate than *R. coronifer* at the same humidity, and furthermore this may make a difference at the RH where survival has its maximum. Possibly this is one of the reasons why *M. cf. hufelandi* have the highest survival at 79%, while the corresponding value for *R. coronifer* is 75%. Hence there should probably also be a difference in survival between the two species at lower humidities, but this is not the case. At lower humidities there is a clear decline in survival which does not correspond to previous studies. Horikawa & Higashi (2004) found an increase in survival over the whole range between 49% RH and 63% RH. More humidities and more replicates would probably be needed in order to get a smoother line or a distinct breakpoint.

Wright (1988) studied different tardigrade species dehydrated at nine different humidities, ranging from 50% to 90%. All three species used in our study were included in Wright's study as well, and his results clearly showed the highest number of survivals in the group dehydrated at 90% RH. Wright did not find any surviving tardigrades of species *M. cf. hufelandi* and *R. coronifer* at 70% or lower RH, while in this study we observed survivals at very low humidities, down to 33% RH. A big difference between his work and this study is how long the tardigrades were left in the desiccators. Wright allowed them to dehydrate for only one hour whereas the tardigrades in our study were dehydrated for 48 hours. This suggests that total desiccation is needed for tardigrades to survive the anhydrobiotic state. The same conclusion was drawn by Higa & Womersley (1993) in their nematode study, discussed above.

According to Ramlöv and Westh (2001) *R. coronifer* accumulates a maximum of 2.3% dry weight trehalose when entering anhydrobiosis. Kjellman's (2004) study on the other hand showed a trehalose level of 5.5% dry weight trehalose in dehydrated *R. coronifer* and 2.3% dry weight in *M. cf. hufelandi*. Our study shows an accumulation of 2.8% trehalose in *R. coronifer*, this level was found in tardigrades dehydrated in 85% RH. The trehalose level in *M. cf. hufelandi* in this study was higher in hydrated samples than in the dehydrated, opposite all previous results. The hydrated tardigrades had 0.8% trehalose while the dehydrated had only 0.3% at 69% RH, which was the highest mean value. In this study the trehalose level in *R. coronifer* increased from 0.5% to 2.8%, almost six times higher. Ramlöv and Westh (2001) had a 23 fold increase and Kjellman (2004) approximately a two time rise in the same species. A possible explanation for the different trehalose levels may be differences in the methodology. Kjellman (2004) discussed the differences in the method of extraction between her study and the study of Ramlöv. The method used in our study is a one extraction step shorter version of Kjellman's, and we only used about half as many tardigrades in the preparation.

In the trehalose study only two replicates of *R. coronifer* were analysed since we did not have enough animals for three replicates, and this gives the large standard deviations. The survival curve does not completely correspond to the trehalose curve as the survival changes over the different humidities. One can see an overall increase in the trehalose level at the different humidities in *R. coronifer*. The trehalose curve follows the survival curve as we move up from lower to higher humidities, as expected. This supports the idea of functional role of trehalose in anhydrobiotic survival. There is however a clear absence of trend in *M. cf. hufelandi*. Perhaps trehalose is not that important in *M. cf. hufelandi* as this species has lower general levels. If that is not the case there is at the moment no obvious explanation to the low levels. More research is needed before we can be sure how the trehalose level and survival in tardigrades relate to each other in anhydrobiosis.

Wright (1988) documented that *E. testudo* does survive dehydration but no analysis has been made according to trehalose content. Regarding the question about whether or not *E. testudo* contains trehalose the answer is yes; they do contain trehalose even though they belong to a completely different order than *M. cf. hufelandi* and *R. coronifer*. Our result of *E. testudo* is the first evidence of trehalose in heterotardigrades. In our study no hydrated animals were analysed for trehalose content, consequently supplementary research needs to be done on *E. testudo* to actually prove that trehalose takes part in the desiccation process.

Associations between survival response and trehalose level

If equal amounts of trehalose would be synthesized in all animals at the different humidities, this would be a strong indication of a fast production of trehalose and that trehalose is not the main factor determining survival under different desiccation conditions. If the level of trehalose should be lower after rapid dehydration in low humidities, this disaccharide is probably an important substance for the anhydrobiotes. The experiment on nematodes by Higa & Womersley (1993) suggests that trehalose is important as a preparation for anhydrobiotic survival but it is not alone in ensuring the survival in nematodes. Instead, slow rates of drying are necessary. This allows initiation of other adaptations that are responsible for the stabilization of cellular structures in the anhydrobiotic state. Westh & Ramlöv (1991) showed a correlation between trehalose accumulation and anhydrobiosis, thus trehalose had a positive influence on anhydrobiotic survival. In our study the survival response on the trehalose level differed between species. There was a weak positive correlation for *R. coronifer*, while in *M. cf. hufelandi* no correlation was found. A suggestion might be made

that the trehalose is complemented by other mechanisms or substances. One such mechanism could be DNA repair. There are currently no studies on DNA repair in tardigrades (K. I. Jönsson, personal communication), but it would be interesting to examine DNA damage gained during for example a too rapid dehydration. Even though there seems to be a positive correlation in *R. coronifer* the same conclusion as before must be made. Trehalose is probably the dominating substance formed in the dehydration process, but most likely other substances are also involved.

5. Summary

The results of this study are based on a rather small material, but they are however statistically significant. It seems as though the desiccation rate does affect revival time for both species, and there is a tendency towards faster revival at higher humidities. The survival shows a weak increase when the RH rises, with the exception of desiccation at 65% RH which seems to be a bad humidity for tardigrades to survive anhydrobiosis. It is obvious that the proportion of surviving *M. cf. hufelandi* in the different dehydration groups and the trehalose level in them do not correlate. This may indicate that *M. cf. hufelandi* is depending on some other substance for its success in anhydrobios. Trehalose seems to be a more important substance in *R. coronifer*, since animals dehydrated in high humidities produce more trehalose than the ones in lower humidities. The levels of trehalose are peculiarly low in *M. cf. hufelandi*. In *R. coronifer* there is an inclination suggesting that the more trehalose the tardigrades synthesize the better the survival after anhydrobiosis. Since *E. testudo* did in fact contain trehalose when dehydrated, it is likely that other organisms not yet tested also utilize this substance. More research is definitely needed both on trehalose content in different organisms and the relationship between trehalose and survival of tardigrades dehydrated in different humidities. If other substances in fact have a big part in protecting these animals, it certainly would be interesting to find out what these are and how they work.

Acknowledgements
Ola Persson and K. Ingemar Jönsson for all your guidance and help.
Pär Hallgren for helping us with the GC.
Petra Svensson for the excellent front page drawing.
Britt-Marie Svensson for letting us borrow the ultra sonic bath numerous times.
The staff on level three, house 20 Kristianstad University, for opening locked doors for us.

Johanna Lundström Linda Svensson

References

Andersson, S. (2005). Urval på alvar. [http://www.planteco.lu.se/people/sa/urval.html] [2006-04-21].

Behm, C. (1997). The role of trehalose in the physiology of nematodes. *International Journal for Parasitology* 27: 215-229.

Bertolani, R., Guidetti, R., Jönsson, K.I., Altiero, T., Boschini, D. & Rebecchi, L. (2004). Experiences with dormancy in tardigrades. *Journal of Limnology* 63 (Suppl. 1): 16-25.

Budd, G. (2005). Introduktion till leddjur, sid. 50-52. Nationalnyckeln till Sveriges flora och fauna. Mångfotingar. Myriapoda. ArtDatabanken, SLU, Uppsala.

Bradbury, J. (2001). Of tardigrades, trehalose, and tissue engineering. *The Lancet* 358 (Issue 9279): 392.

Crowe, J.H. (1971). Anhydrobiosis: An unsolved problem. *The American Naturalist* 105: 563-573.

Crowe, J.H. (1972). Evaporative water loss by tardigrades under controlled relative humidities. *Biological Bulletin* 142: 407-416.

Crowe, L.M. & Crowe, J.H. (1992). Anhydrobiosis: A strategy for survival. *Advances in Space Research* 12: 239-247.

Crowe, J.H., Hoekstra, F.A. & Crowe, L.M. (1992). Anhydrobiosis. Annual Review of Physiology 54: 579-599.

Crowe, L.M. (2002). Lessons from nature: the role of sugars in anhydrobiosis. *Comparative Biochemistry and Physiology* Part A 131: 505-513.

Goldstein, B. & Blaxter, M. (2002). Tardigrades. *Current Biology* 12: R475.

Greenspan, L. (1977). Humidity fixed points of binary saturated aqueous solutions. *Journal of Research of the National Bureau of Standards-A* 81: 89-96.

Guidetti, R., Gandolfi, A., Rossi, V. & Bertolani, R. (2005). Phylogenetic analysis of Macrobiotidae (Eutardigrada, Parachela): a combined morphological and molecular approach. *Zoologica Scripta* 34: 235-244.

Hallgren, P., Westbom, R., Nilsson, T., Sporring, S. & Björklund, E. (In press). Measuring bioavailability of polychlorinated biphenyls in soil to earthworms using selective supercritical fluid extraction. *Chemosphere*.

Higa, L.M. & Womersley, C.Z. (1993). New insights into the anhydrobiotic phenomenon: The effects of trehalose content and different rates of evaporative water loss on the survival of *Aphelenchus avenae*. *The Journal of Experimental Zoology* 267: 120-129.

Hoekstra, F.A., Wolkers, W.F., Buitink, J., Golovina, E.A., Crowe, J.H. & Crowe, L.M. (1997). Membrane stabilization in the dry state. *Comparative Biochemistry and Physiology* 117A: 335-341.

Horikawa, D.D. & Higashi, S. (2004). Desiccation tolerance of the tardigrade *Milnesium tardigradum* collected in Sapporo, Japan, and Bogor, Indonesia. *Zoological Science* 21: 813-816.

Ivarsson, H. & Jönsson, K.I. (2004). Aggregation effects on anhydrobiotic survival in the tardigrade *Richtersius coronifer*. *Journal of Experimental Zoology* 301A: 195-199.

Jones, L. & Atkins. P. (2002). Chemistry; molecules, matter and change. Fourth edition. W.H. Freeman and Company, New York, USA.

Jönsson, K.I. (2001). The nature of selection on anhydrobiotic capacity in tardigrades. *Zoologischer Anzeiger* 240: 409-417.

Jönsson, K.I. (2003). Causes and consequences of excess resistance in cryptobiotic metazoans. *Physiological and Biochemical Zoology* 76: 429-435.

Jönsson, K.I. (2005). The evolution of life histories in holo-anhydrobiotic animals: a first approach. *Integrative and Comparative Biology* 45: 764-770.

Jönsson, K.I. & Bertolani, R. (2001). Facts and fiction about long-term survival in tardigrades. *The Zoological Society of London* 255:121-123.

Jönsson K.I., Borsari, S. & Rebecchi, L. (2001). Anhydrobiotic survival in populations of the tardigrades *Richtersius coronifer* and *Ramazzottius oberhaeuseri* from Italy and Sweden. *Zoologischer Anzeiger* 240: 419-423.

Jönsson, K.I., Harms-Ringdahl, M. & Torudd, J. (2005). Radiation tolerance in the eutardigrade *Richtersius coronifer*. *International Journal of Radiation Biology* 81: 649-656.

Jönsson, K.I. & Järemo, J. (2003). A model on the evolution of cryptobiosis. *Annales Zoologici Fennici* 40: 331-340.

Jönsson, K.I. & Rebecchi, L. (2002). Experimentally induced anhydrobiosis in the tardigrade *Richtersius coronifer*: Phenotypic factors affecting survival. *Journal of Experimental Zoology* 293: 578-584.

Keilin, D. (1959). The problem of anabiosis or latent life: history and current concept. *Proceedings of the Royal Society of London B. Biological Sciences* 150: 149-191.

Kinchin, I.M. (1994). The biology of tardigrades. Portland Press, London, UK.

Kjellman, M. (2004). A study of trehalose levels in anhydrobiotic tardigrades. Master thesis in biology, Kristianstad University. Department of Mathematics and Science, Kristianstad University.

Lapinski, J. & Tunnacliffe, A. (2003). Anhydrobiosis without trehalose in bdelloid rotifers. *FEBS Letters* 553: 387-390.

Leeuwenhoek, A. van. (1702). On certain animalcules found in the sediment in gutters of the roofs of houses. The Select Works of Antony van Leeuwenhoek. Letter 144 translated by Samuel Hoole (1798, 1807), *London* 2: 207-213.

Luzardo, M.del C., Amalfa, F., Nuñez, A.M., Díaz, S., Biondi de Lopez, A.C. & Disalvo, E.A. (2000). Effect of trehalose and sucrose on the hydration and dipole potential of lipid bilayers. *Biophysical Journal* 78: 2452-2458.

Ma, X., Jamil, K., MacRae, T.H., Clegg, J.S., Russell, J.M., Villeneuve, T.S., Euloth, M., Sun, Y., Crowe, J.H., Tablin, F. & Oliver, A.E. (2005). A small stress protein acts synergistically with trehalose to confer desiccation tolerance on mammalian cells. *Cryobiology* 51: 15-28.

Mallat, J.M., Garey, J.R. & Shultz, J.W. (2004). Ecdysozoan phylogeny and Bayesian inference: first use of nearly complete 28S and 18S rRNA gene sequences to classify the arthropods and their kin. *Molecular Phylogenetics and Evolution* 31: 178-191.

Nelson, D. (2002). Current status of the tardigrada: evolution and ecology. *Integrative and Comparative Biology* 42: 652-659.

Ohtake, S., Schebor, C., Palecek, S.P. & de Pablo, J.J. (2004). Effect of sugar-phosphate mixtures on the stability of DPPC membranes in dehydrated systems. *Cryobiology* 48: 81-89.

Oliver, A.E., Hincha, D.K. & Crowe, J.H. (2002). Looking beyond sugars: the role of amphiphilic solutes in preventing adventitious reactions in anhydrobiotes at low water contents. *Comparative Biochemistry and Physiology* Part A 131: 515-525.

Oliver, A.E., Leprince, O., Wolkers, W.F., Hincha, D.K., Heyer, A.G. & Crowe, J.H. (2001). Non-disaccharide-based mechanisms of protection during drying. *Cryobiology* 43: 151-167.

Potts, M. (2001). Desiccation tolerance: a simple process? *TRENDS in Microbiology* 9: 553-559.

Ramazzotti, G. & Maucci, W. (1983). Il Phylum Tardigrada. Third edition. Memorie dell'Istituto Italiano di Idrobiologia Dott, Volume 41.

Ramlöv, H. & Westh, P. (2001). Cryptobiosis in the eutardigrade *Adorybiotus (Richtersius) coronifer*: Tolerance to alcohols, temperature and *de novo* protein synthesis. *Zoologischer Anzeiger* 240: 517-523.

Schmidt-Rhaesa, A. (2001). Tardigrades – Are they really miniaturized dwarfs? *Zoologischer Anzeiger* 240: 549-555.

Sohlenius, B. (2006) Tardigrades found in Sweden. [Internet: http://www.nrm.se/forskningochsamlingar/djur/evertebratzoologi/forskning/forskningsprojekt/bjornsohlenius/tardigradlista.4.169b66e107313259f18000156.html] [2006-04-21].

Sun, W.Q. & Leopold, A.C. (1997). Cytoplasmic vitrification and survival of anhydrobiotic organisms. *Comparative Biochemistry and Physiology* 117A: 327-333.

Suzuki, A.C. (2003). Life history of *Milnesium tardigradum* Doyère (Tardigrada) under a rearing environment. *Zoological Science* 20: 49-57.

The Merck Index. An Encyclopedia of chemicals, drugs, and biologicals. (2001). Thirteenth edition. Published by Merck Research Laboratories Division of Merck & CO., INC. Whitehouse Station, NJ, USA.

Watanabe, M., Kikawada, T., Fujita, A., Forczek, E., Adati, T. & Okuda, T. (2004). Physiological traits of invertebrates entering cryptobiosis in a post-embryonic stage. *European Journal of Entomology* 101: 439-444.

Westh, P. & Ramlöv, H. (1988). Cryptobiosis in arctic tadigrades with special attention to the appearance of trehalose. In: G. Höpner Petersen (ed.), Greenland Excursion pp. 227-245. Institute of Polar Ecology, Kiel University.

Westh, P. & Ramlöv, H. (1991). Trehalose accumulation in the tardigrade *Adorybiotus coronifer* during anhydrobiosis. *Journal of Experimental Zoology* 258: 303-311.

Wikipedia, The free encyclopedia. (2006). Cryptobiosis. [Internet: http://en.wikipedia.org/wiki/Anhydrobiosis#Osmobiosis] [2006-05-03].

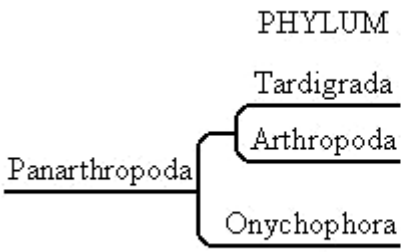
Womersley, C. (1981). Review: Biochemical and physiological aspects of anhydrobiosis. *Comparative Biochemistry and Physiology* 70B: 669-678.

Wright, J.C. (1988). Desiccation tolerance and water-retentive mechanisms in tardigrades. *Journal of Experimental Biology* 142: 267-292.

Wright, J.C. (2001). Cryptobiosis 300 years on from van Leuwenhoek: What have we learned about tardigrades? *Zoologischer Anzeiger* 240: 563-582.

Appendix 1

(A)



(B)

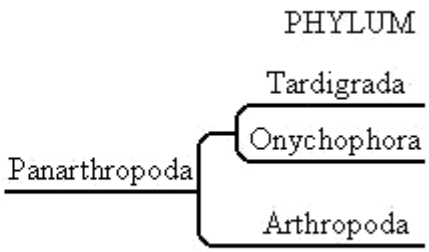


Figure A and B. The relationship of tardigrades to other taxa has been uncertain for a long time. Onychophorans, tardigrades and arthropods are together called panarthropods, based on shared characters like for example a segmental body. The relations among these three phyla are however uncertain (Mallat et al. 2003). There are basically two suggestions as to how they relate to each other; both are based on molecular studies. One suggestion indicates that tardigrades are a monophyletic sister group of the arthropods and that they are more close to the arthropods than the onychophora are (Nelson 2002, Budd 2005), as shown in Figure A. Studies by Mallat et al. (2003) on the other hand, showed a stronger relationship between onychophorans and tardigrades, see Figure B.

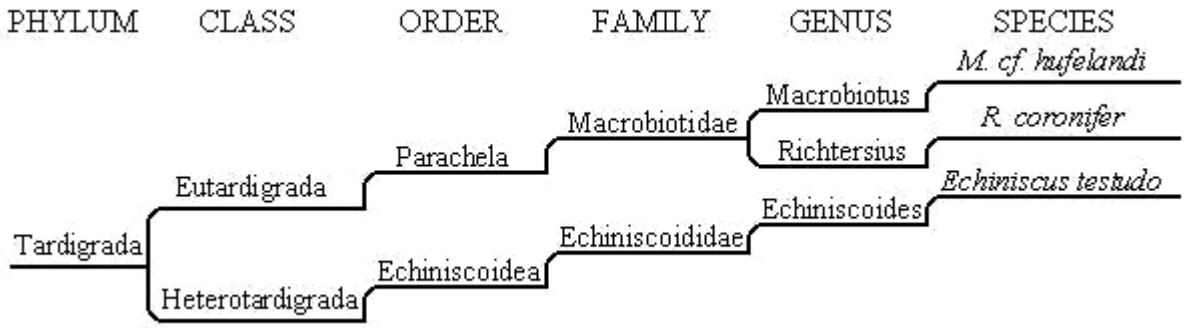


Figure C. A rough scheme of the systematic position of the species used in this study, naming the most important terms in each level and giving an easy view of the systematic. For further details, see Ramazzotti & Maucci (1983).

Appendix 2

The following two tables show the proportion revived tardigrades for *M. cf. hufelandi* and *R. coronifer*. Column two to six show the revival numbers at different humidities. Column six also represents the overall survival since it was the last count occasion. Column seven shows the trehalose content at different humidities. The last row shows the trehalose level in hydrated samples.

Table A. The proportion revivals and standard deviations of the survival and trehalose studies in *M. cf. hufelandi*, corresponding to the Figures 8 and 10.

M. cf. hufelandi						
% RH	**Active after 15 min**	**Active after 30 min**	**Active after 45 min**	**Active after 60 min**	**Active after 180 min/ Overall survival**	Trehalose %
0%	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	**-**
33%	0.07 (±0.05)	0.19 (±0.12)	0.26 (±0.15)	0.33 (±0.16)	0.52 (±0.27)	**0.275 (±0.15)**
45%	0.04 (±0.06)	0.12 (±0.09)	0.18 (±0.14)	0.18 (±0.16)	0.34 (±0.20)	**0.211 (±0.06)**
58%	0.07 (±0.08)	0.16 (±0.17)	0.20 (±0.19)	0.28 (±0.26)	0.33 (±0.23)	**-**
65%	0.03 (±0.03)	0.06 (±0.05)	0.08 (±0.03)	0.12 (±0.07)	0.21 (±0.08)	**0.318 (±0.08)**
69%	0.06 (±0.09)	0.23 (±0.16)	0.30 (±0.18)	0.37 (±0.20)	0.51 (±0.23	**0.327 (±0.07)**
75%	0.15 (±0.06)	0.28 (±0.11)	0.35 (±0.14)	0.49 (±0.14)	0.50 (±0.15)	**0.300 (±0.20)**
79%	0.34 (±0.23)	0.42 (±0.25)	0.54 (±0.26)	0.64 (±0.24)	0.64 (±0.22)	**0.226 (±0.17)**
85%	0.08 (±0.06)	0.18 (±0.11)	0.26 (±0.05)	0.31 (±0.06)	0.34 (±0.08)	**0.205 (±0.06)**
Hydrated						**0.780 (±0.28)**

Table B. proportion revivals and standard deviations of the survival and trehalose studies in *R. coronifer*, corresponding to the Figures 9 and 11.

R. coronifer						
% RH	**Active after 15 min**	**Active after 30 min**	**Active after 45 min**	**Active after 60 min**	**Active after 180 min/ Overall survival**	Trehalose %
0%	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	-
33%	0.00 (±0.00)	0.03 (±0.03)	0.12 (±0.08)	0.22 (±0.14)	0.49 (±0.13)	**0.286 (±0.20)**
45%	0.00 (±0.00)	0.02 (±0.03)	0.05 (±0.06)	0.09 (±0.06)	0.36 (±0.14)	**0.684 (±0.36)**
58%	0.00 (±0.00)	0.07 (±0.09)	0.13 (±0.12)	0.20 (±0.14)	0.54 (±0.19)	**0.807 (±0.35)**
65%	0.00 (±0.00)	0.00 (±0.01)	0.04 (±0.07)	0.07 (±0.09)	0.19 (±0.19)	**0.707 (±0.47)**
69%	0.01 (±0.03)	0.09 (±0.09)	0.14 (±0.11)	0.20 (±0.16)	0.41 (±0.24)	**0.753 (±0.10)**
75%	0.06 (±0.06)	0.20 (±0.17)	0.36 (±0.21)	0.51 (±0.23)	0.78 (±0.20)	**0.988 (±0.29)**
79%	0.00 (±0.00)	0.09 (±0.12)	0.14 (±0.13)	0.25 (±0.20)	0.52 (±0.26)	**2.009 (±1.08)**
85%	0.00 (±0.00)	0.05 (±0.08)	0.1 (±0.11)	0.26 (±0.17)	0.56 (±0.23)	**2.772 (±1.12)**
Hydrated						**0.483 (±0.02)**

Appendix 3

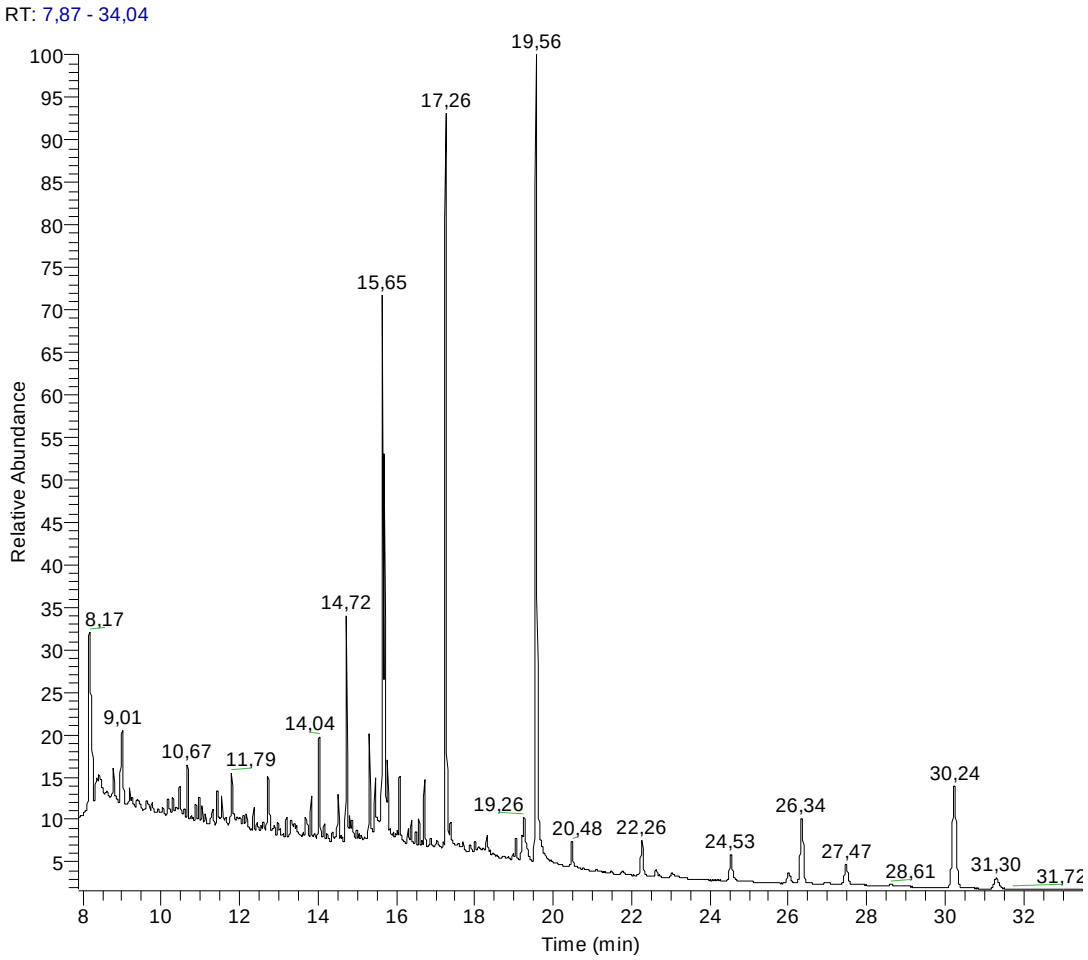


Figure D. An example of a chromatogram, in this case from a sample of dehydrated *R. coronifer*. Both the standard sorbitol and trehalose are detected in this sample. The peak at retention time 15.65 corresponds to the sorbitol standard, while the peaks at 17.26 and 19.56 originate from carboxylic acids, and the trehalose peak is shown at retention time 30.24. The carboxylic acids probably originate from cell membranes (Kjellman 2004).