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Long-term anhydrobiotic survival in semi-terrestrial micrometazoans

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Abstract

This study represents the first systematic investigation of long-term anhydrobiotic survival in tardigrades, rotifers and nematodes inhabiting mosses and lichens. Sixty-three different samples from public and private collections, kept dry for 9–138 years, were examined. Rotifers of the genus *Mniobia* and the eutardigrade *Ramazzottius oberhaeuseri* (hatched from eggs) were found alive from one of the samples (9 years old). These observations represent the longest record for rotifers in the anhydrobiotic state. For tardigrades, our results confirm previous reports on the upper limit of anhydrobiotic survival under atmospheric oxygen conditions. This study suggests the possibility that tardigrade eggs are able to withstand longer periods in anhydrobiosis than animals. Some problems related to the evaluation of long-term anhydrobiotic survival, such as contamination and chemical treatments of samples, are reported. The possible role of the microenvironment in which the anhydrobiotic animals are kept is discussed.

Key words: Tardigrada, Rotifera, Nematoda, anhydrobiosis, cryptobiosis

INTRODUCTION

Substrates subject to frequent periods of drying, including mosses, lichens, leaf litter and small ponds in exposed environments, are often inhabited by micrometazoans such as tardigrades, rotifers and nematodes. To survive periods of desiccation, these animals (usually smaller than 1 mm), possess the capacity to enter a state generally known as anhydrobiosis (Keilin, 1959; Wright, Westh & Ramløv, 1992). Anhydrobiosis is a reversible process in which the animal loses most (> 95%) of its free and bound water and produces substances (e.g. trehalose and glycerol) that protect the cell structures from damage caused by the water loss (Madin & Crowe, 1975; Clegg, 1986; Westh & Ramløv, 1991). In this state the organism's metabolism comes to a complete standstill and is resumed only when conditions for rehydration are restored (Keilin, 1959; Clegg, 1973).

Relatively few studies have been carried out to evaluate how long these organisms may remain in the anhydrobiotic state without lost viability. Several studies on this topic were carried out in the first half of the 20th century (Goodey, 1923; Rahm, 1923; Baumann, 1927; Franceschi, 1948; Fielding, 1951; Lee, 1961).

Most reports on long-term anhydrobiotic survival have been on nematodes of commercial importance. In fact, most studies have focused on plant parasitic nematodes. For instance, Steiner & Albin (1946) reported two species that survived in anhydrobiosis for 39 and 28 years, respectively. Fielding (1951) presented several reports of three species that survived from 20 to 28 years, and Goodey (1923) reported a maximum survival of 9 years. Finally, Lee (1961) reported two species surviving 10 years in anhydrobiosis in dried mud from rock pools.

Data on long-term anhydrobiotic survival in tardigrades and rotifers are much more sparse. In tardigrades, Baumann (1927) revived animals of the genus *Macrobiotus* after nearly 7 years, and Franceschi (1948) found a tardigrade in 120 year-old moss which showed a slight sign of body movement. Although the observation by Francesci has often been referred to as evidence of century-long cryptobiotic survival, this interpretation has recently been criticized (Jönsson & Bertolani, 2001). Finally, Sømme & Meier (1995) reported a high rate of revival in three tardigrade species after 8 years of storage at −22 °C in an anhydrobiotic state.

The only data available on rotifers were reported by Rahm (1923), who found viable animals in moss dried for 2.5 years. Rahm (1923) also reported an active

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rotifer from herbarium moss that had been stored dry for 59 years. However, as noted by Gilbert (1974), 'Rahm, however, was careful to point out that this last observation may be suspect, since powder from a freshly collected moss containing recently dried rotifers might have settled into the old moss.' Consequently, we know relatively little about long-term anhydrobiotic survival and some of the available reports should be treated with caution.

An understanding of how living organisms may protect their cell structures in a dry state over long periods has both biological and biotechnological relevance. From a biotechnological perspective, anhydrobiotic organisms provide an invaluable source of information on how to protect dry material from damage. Moreover from an ecological and evolutionary perspective, more information on long-term anhydrobiotic survival would be welcome. For instance, do species that resist rapid desiccation also have a better chance of surviving long periods in anhydrobiosis?

In order to obtain knowledge on the ability of micrometazoans to recover from long-term anhydrobiosis after storage under atmospheric oxygen conditions, a large number of moss and lichen samples obtained from botanical museums and private collectors were analysed.

MATERIAL AND METHODS

Sixty-three samples of mosses and lichens stored dry from 9 to 138 years in botanical museums and private collections (see Table 1) were analysed. Twelve samples came from the herbarium of Swedish Museum of Natural History (SMNH) in Stockholm (Sweden), 34 from the herbarium of Botanical Museum of Lund, Sweden (BML), 15 from a private moss collection (T. Knutsson (TK), Degerhamn, Sweden), and 2 were collected by Dr H. Ramløv (RA), Institute for Biology and Chemistry, Roskilde University, Copenhagen, Denmark.

All samples from the museums and from the private collection had been kept in folded paper containers at room temperature. The sample from H. Ramløv had been kept in plastic bags (not completely closed) at room temperature since collecting.

All samples from the Botanical Museum of Lund and from the Swedish Museum of Natural History had been exposed to at least 1 treatment with fumigation pesticides (mainly methyl bromide).

Each sample was weighed dry and kept for 24 h in a climate room at 65% relative humidity (RH) and 23 °C, then transferred to a climate chamber (FI-totron 600H) with 90% RH and 21 °C for 15 h. The samples were then transferred to a laboratory and sprayed with distilled water, and after 1 h immersed in distilled water. Tardigrades and their eggs, rotifers, and nematodes were extracted by sieves (mesh size: 0.25 mm and 0.04 mm) after 1 h of immersion in distilled water, then collected under a stereomicroscope, and transferred to cups with distilled water. To avoid contamination from laboratory material, sieves, cups, pipettes, etc. were always kept wet when not used, and sterilized in an autoclave at 120 °C for 20 min before each new extraction. The extracted animals were kept in water for at least 5 days (the eggs for at least 1 month). They were frequently observed during daytime (especially during the first day after extraction), at intervals of *c.* 2–3 h, under a stereomicroscope to detect signs of movement. The distilled water was changed every 3 days to restore the oxygen. During daytime, the cups were kept at room temperature (around 20 °C), while during the nights they were kept in a refrigerator at 10 °C.

Two control samples were used to assure that our methods were appropriate for rehydrating anhydrobiotic micrometazoans. These samples had been stored dry (at *c.* 20 °C) and consisted of 1 moss *Orthotrichum cupulatum* Brid. collected in 1999 at the Swedish island Öland, and 1 lichen *Xanthoria parietina* L. collected in September 1998 near Banchory, Scotland (see Table 1).

As a criterion for viability of animals, co-ordinated movements of the animal body were used.

RESULTS

Our control samples verified that the methodology used to rehydrate the samples worked well, and viable tardigrades, rotifer and nematodes were extracted from both the moss and the lichen. It was also verified that animals could survive, and tardigrade eggs could develop, in cups with just distilled water.

From the 61 samples originating from the Swedish Museum of Natural History, the Botanical Museum and the private collection, a total of 1572 tardigrades, 587 tardigrade eggs, 3813 rotifers, and 1409 nematodes was extracted (see Table 1). None of these animals showed any sign of viability (movements) after extraction or in the following 5 days. Moreover, no tardigrade eggs developed or hatched. In most animals from these samples (especially the older ones), the internal organs were often not distinguishable, and in tardigrades they were concentrated into a shapeless mass. In contrast, the cuticle of all animals (tardigrades, rotifers, nematodes) was usually well stretched with the typical shape of a live animal.

From sample no. 63 (9-year-old moss; see Table 1), 195 bdelloid rotifers of the genus *Mniobia* were extracted, 13 of which were found alive with clear signs of movement. The first viable rotifer was observed 20 h after extraction, and the last after 44 h. To verify these results, a second sample (no. 63bis) from the same collection was examined. This time 216 rotifers were extracted and 12 viable rotifers of the same species of *Mniobia* were found. The first viable rotifer was observed 2.5 h after extraction, the last after 28 h. Six more viable large rotifers were found in a second check of the sediment after 8 days in water. These specimens were in better shape than the 12 rotifers collected earlier (yellowish gut and more movements) that were kept in

Table 1. Samples used for investigation of long-term anhydrobiotic survival. The table reports year and site of collection, years of dry storage, kind of substrate, amount of analysed substrate, number of tardigrades (animals and eggs), rotifers, and nematodes collected. smnh = Swedish Museum of Natural History; bml = Botanical Museum of Lund; tk = T. Knutsson's collection; ra = samples collected by H. Ramløv

Sample no.	Collection	Year of collection	Collection site	Years of storage	Substrate	Amount of sample (g)	No of tardigrades	No. of tard. eggs	No. of rotifers	No. of nematodes
1	smnh	1921	Sweden	79	*Tortula ruralis*	1.42	45	42	91	109
2	smnh	1928	Sweden	72	*Tortula ruralis*	0.24	7	21	48	11
3	smnh	1922	Denmark	78	*Tortula ruralis*	1.45	34	99	5	72
4	smnh	1921	Sweden	79	*Tortula ruralis*	1.59	73	41	440	145
5	smnh	1878	Sweden	122	*Xanthoria parietina*	1.64	19	9	44	54
6	smnh	1884	Sweden	116	*Xanthoria parietina*	0.11	0	0	7	0
7	smnh	1897	Sweden	103	*Hypogymnia physodes*	0.67	4	1	2	0
8	smnh	1917	Sweden	83	*Thuidium tamariscinum*	0.37	1	0	20	7
9	smnh	1920	Sweden	80	*Tortula ruralis*	0.81	59	7	222	45
10	smnh	1917	Sweden	83	*Tortula ruralis*	0.61	32	35	43	80
11	smnh	1928	Sweden	72	*Rhytidradelphus triquetius*	0.45	1	0	1	0
12	smnh	1925	Sweden	75	*Grimmia hypnotaes*	0.43	2	1	40	3
13	bml	1862	Germany	138	*Tortula ruralis*	0.82	3	0	5	6
14	bml	1870	Germany	130	*Tortula ruralis*	1.27	13	0	14	18
15	bml	1962	France	38	*Tortula ruralis*	1.24	129	0	181	16
16	bml	1956	Sweden	44	*Tortula ruralis*	0.51	5	0	6	19
17	bml	1896	Sweden	104	*Tortula ruralis*	0.53	25	4	8	52
18	bml	1934	Sweden	66	*Tortula ruralis*	0.37	0	2	117	7
19	bml	1968	Yugoslavia	32	*Tortula ruralis*	0.61	0	0	3	12
20	bml	1974	Yugoslavia	26	*Tortula ruralis*	0.39	0	0	1	51
21	bml	1948	Slovenia	52	*Tortula ruralis*	0.39	0	1	0	32
22	bml	1974	France	26	*Tortula ruralis*	0.34	3	1	105	6
23	bml	1978	Ireland	22	*Tortula ruralis*	0.41	0	0	52	79
24	bml	1969	Wales	31	*Tortula ruralis*	0.85	0	0	160	17
25	bml	1953	France	47	*Tortula ruralis*	0.44	0	0	38	17
26	bml	1965	Sweden	35	*Tortula ruralis*	0.51	3	0	5	25
27	bml	1967	U.S.A.	33	*Tortula ruralis*	0.63	0	0	17	54
28	bml	1982	Canada	18	*Tortula ruralis*	0.91	0	2	7	6
29	bml	1987	Canada	13	*Tortula ruralis*	1.01	0	23	44	4
30	bml	1988	Yugoslavia	12	*Tortula ruralis*	0.14	5	0	49	35
31	bml	1987	Bolivia	13	*Tortula ruralis*	0.41	2	3	62	53
32	bml	1987	Bolivia	13	*Tortula leiostoma*	0.11	2	3	20	6
33	bml	1984	Finland	16	*Tortella tortuosa*	0.42	10	16	18	7
34	bml	1944	Sweden	56	*Xanthoria parietina*	0.24	59	4	86	9
35	bml	1940	Sweden	60	*Xanthoria parietina*	0.24	0	0	103	0
36	bml	1987	Sweden	13	*Xanthoria parietina*	0.23	12	0	8	0
37	bml	1964	Faroe Is.	36	*Xanthoria parietina*	0.27	43	8	30	6
38	bml	1962	Sweden	38	*Xanthoria parietina*	0.52	140	23	90	4
39	bml	1989	Finland	11	*Xanthoria parietina*	0.15	5	0	4	0
40	bml	1995	Faroe Is.	5	*Xanthoria parietina*	0.46	3	0	82	0
41	bml	1989	Finland	11	*Xanthoria candelaria*	0.19	9	3	124	8
42	bml	1991	Sweden	9	*Xanthoria candelaria*	0.04	0	0	27	0
43	bml	1987	Slovenia	13	*Orthotrichum speciosum*	0.17	25	8	53	8
44	bml	1966	U.S.A.	34	*Orthotrichum speciosum*	1.26	54	0	133	30
45	bml	1984	Canada	16	*Orthotrichum obtusifolium*	0.42	104	11	121	3
46	bml	1982	Equador	18	*Orthotrichum elongatum*	0.74	6	0	50	2
47	tk	1991	Sweden	9	*Tortula* sp.	0.05	9	0	20	11
48	tk	1991	Sweden	9	*Anomodon* sp.	1.31	5	1	90	10
49	tk	1992	Sweden	8	*Tortula* sp.	0.31	21	2	90	10
50	tk	1982	Sweden	18	*Tortula muralis*	2.25	66	9	55	35
51	tk	1982	Sweden	18	*Plagomnium cuspidatum*	0.14	4	0	5	0
52	tk	1981	Sweden	19	*Tortula ruralis*	0.78	100	35	155	145
53	tk	1991	Sweden	9	*Tortella* sp.	0.05	19	4	34	9
54	tk	1982	Sweden	18	*Tortella tortuosa*	0.92	58	15	76	17
55	tk	1982	Sweden	18	*Orthotricum anomalum*	0.94	86	31	127	19
56	tk	1982	Sweden	18	*Tortula intermedia*	0.78	5	10	37	5
57	tk	1982	Sweden	18	*Orthotrichum anomalum*	1.18	0	0	47	2
58	tk	1992	Sweden	8	*Grimmia* sp.	0.79	123	34	134	0
59	tk	1992	Sweden	8	*Orthotrichum cupulatum*	0.62	24	10	52	1
60	tk	1991	Sweden	9	*Tortella* sp.	0.81	47	3	50	12
61	tk	1991	Sweden	9	*Tortula ruralis*	0.73	68	65	55	15
62	ra	1991	Sweden	9	*Xanthoria parietina*	0.22	123	28	42	4
63	ra	1991	Sweden	9	*Tortula ruralis*	2.02	142	168	195	65
63bis	ra	1991	Sweden	9	*Tortula ruralis*	3.71	89	73	216	0

water without any sediments. A third sample (no. 63tris) from the same collection was examined to test the importance of rehydration under high humidity conditions before immersion in water. This sample was directly immersed in water for 1.5 h before extraction of the animals. This time we collected 154 rotifers, but no viable animals were found.

The 31 extracted live rotifers were all kept in distilled water without any direct source of food (although bacteria were probably present in the water) and 23 of them died after 6–12 days, showing signs of starvation. They became more transparent, and the gut, which at the beginning was yellowish, was completely clear. The other eight rotifers (including the six collected last) were fed with *Escherichia coli* only after 13 days from extraction and stayed alive for > 40 days.

From samples nos. 62, 63 and 63bis, a total of 13 eggs of *Ramazzottius oberhaeuseri* (Doyère, 1840), 105 eggs of *Richtersius coronifer* (Richters, 1903), 145 eggs of a species of the *Macrobiotus hufelandi* group, and an exuvium with six eggs of *Milnesium tardigradum* Doyère, 1840 were extracted. From these eggs, only four eggs of *R. oberhaeuseri* hatched. One egg of sample no. 62 (9-year-old lichen; see Table 1) hatched after 12 days, and a juvenile, for which it was not possible to determine the exact date of birth, was found alive after 25 days. From sample no. 63 (9-year-old moss; see Table 1) one egg hatched after 18 days, and another juvenile was found dead 20 days after extraction. The three juveniles survived in distilled water without any direct food sources for 10–40 days.

DISCUSSION

Survival after long periods of anhydrobiosis

Our investigation of old samples of mosses and lichens did not reveal any evidence for much longer anhydrobiotic survival in tardigrades than earlier published data have indicated (Baumann, 1927; Sømme & Meier, 1995). It is important, however, to remember that the 8 years reported by Sømme & Meier (1995) represent survival under frozen conditions, and that a high proportion of the animals were still viable after 8 years. Storage at temperatures below zero may allow considerably longer periods of anhydrobiosis, due to reduction of the potentially damaging reactions with oxygen in the air (Örstan, 1998).

The four eggs of *R. oberhaeuseri* that hatched after 9 years in anhydrobiosis represent the first report for tardigrades of long-term anhydrobiotic survival of eggs. Although our results refer to only four eggs, our finding suggests a possibly higher capacity of eggs to withstand long anhydrobiotic periods compared to animals. This would be contrary to that observed in rotifers and nematodes, in which the eggs seem to be less resistant than the animals after a relatively short period of anhydrobiosis (up to 30 days; Ricci, Vaghi & Manzini, 1987; Ricci & Abbruzzese, 1989).

Thirty-one per cent of the *R. oberhaeuseri* eggs developed successfully, while no eggs from the other tardigrade species developed. This suggests that eggs of *R. oberhaeuseri* may have a higher resistance to a long period of anhydrobiosis compared to the other species examined. In a comparison of several anhydrobiotic tardigrades, *R. oberhaeuseri* exhibited the highest degree of desiccation tolerance, surviving desiccation at 55% relative humidity (Wright, 1989). This suggests a high adaptation of this species to rapidly desiccating conditions, in agreement with its presence in xeric environments (Ramazzotti & Maucci, 1983; Wright, 1991). It also indicates that tolerance to rapid desiccation may be connected to resistance to long-term anhydrobiosis.

The rotifers of *Mniobia* sp. found alive after 9 years in anhydrobiosis represent the longest period recorded for animals of this phylum. Interestingly, the previous reports on long-term anhydrobiosis in rotifers also refer to the genus *Mniobia* (*Mniobia magna* (Plate, 1889) and *Mniobia russeola* (Zelinka, 1891)), revived after 2.5 years in the anhydrobiotic state (Rahm, 1923). This suggests that this genus contains several species with a high capacity for long-term anhydrobiosis. Species of the genus *Mniobia* dominate the rotifer fauna in mosses exposed to desiccation (Ricci, 1987).

The nematodes collected in the 63 samples did not show any viability. If these data are compared to the results obtained for parasitic (Goodey, 1923; Steiner & Albin, 1946; Fielding, 1951) or fresh water species (Lee, 1961), there seem to exist large differences among species within this phylum in long-term anhydrobiotic resistance.

Since organisms in anhydrobiosis are ametabolic, senescence based on metabolic processes should not act. If no other factors affect the dry body, anhydrobionts should be potentially immortal. At least under normal atmospheric oxygen conditions this seems not to be the case, since survival declines with time spent in anhydrobiosis (Clegg, 1967; Crowe, 1975). This is also supported by our results, where only a small portion (6–8%) of our collected rotifers survived. The same factors that led to 100% mortality in tardigrades and nematodes probably also acted on rotifers of *Mniobia* sp., reducing their capability to recover.

Fielding (1951: table 1) reported 90–100% survival in nematodes (based on 100–200 specimens each from six different samples) after 11–28 years of anhydrobiosis. In view of the suggested negative impact of air on anhydrobiotic animals, and compared with our own estimates and those of Goodey (1923) and Lee (1961), some doubts must be raised about the report of Fielding.

The damaging effect of oxidation processes is accepted as one of the causes of death under long-term anhydrobiosis (Clegg, 1967; Crowe, 1975; Örstan, 1998), even if other factors may also be involved (Örstan, 1998). Oxidation could destroy the molecular organization of cellular structures, including DNA, or give rise to toxins responsible for the immediate

death of animals during rehydration (Heckly, 1978; Womersley, 1987). The amount of accumulated damage should increase with time spent in anhydrobiosis, potentially reducing the vitality of the animal. However, the viable rotifers and tardigrades found in this study survived for several days and exhibited normal activities. This suggests that animals capable of recovering after a long time in anhydrobiosis are also able to maintain normal activities for several days, with no signs of accumulated damage. The factor determining whether an animal recovers or not may involve repair of damaged structures during a critical phase of rehydration. If repair is successful, the animal recovers with no or little remaining damage. If repair is unsuccessful, the animal does not recover. Instead, effects of long periods in anhydrobiosis seem to be expressed in terms of a long period of recovery after rehydration. A positive correlation between anhydrobiotic storage time and time for recovery was reported by Crowe & Madin (1975) in nematodes, and by Crowe & Higgins (1967) in tardigrades. Since viable animals were found in only one of our samples, this pattern could not be verified.

Other than the concentration of oxygen, Örstan (1998) pointed out that the level of humidity to which the anhydrobiotic animal is exposed also plays an important role in the prospects for long-term anhydrobiotic survival. The microenvironment may provide more or less exposure of the animals to external factors (e.g. humidity and oxygen). If so, we would expect longer anhydrobiotic survival when animals have been kept protected within the natural shelter of their substrates. The records of anhydrobiotic survival in parasitic nematodes seem to support this prediction. Nematodes that were found alive after > 10 years were stored within plant galls (Bastian, 1865; Goodey, 1923; Steiner & Albin, 1946; Fielding, 1951), plant tissue (Steiner & Albin, 1946; Fielding, 1951) or dry mud (Lee, 1961). Animals stored within these kinds of microenvironments may be better screened from ambient levels of humidity and oxygen than animals stored on the surface of lichen or moss, such as the samples examined in this study. The larvae of the parasitic nematode *Ditylenchus dipsaci* (Kühn, 1857) were claimed to survive within the plant tissue for 23 years (Fielding, 1951, see our remark above on this report). However, isolated from the plant, they survive only for a few days (Ellenby, 1968) or a month (Wallace, 1962). Longer periods of survival of the larvae outside the plant tissue were obtained only by storing the animals at low temperature (2–4 °C), when they can survive for 7 years (see Ellenby, 1969).

Problems have been reported with leakage of cell contents if anhydrobiotic animals are rehydrated directly in water without previous exposure to high humidity conditions (Crowe & Madin, 1975; Crowe, O'Dell & Armstrong, 1979; Womersley, 1981). This problem may be an effect of keeping the anhydrobiotic animals under unnaturally low humidity conditions, since in nature the onset of rehydration would often start after direct contact with rain. Damage caused by a sudden increase of humidity was also suggested in our studies, where direct immersion of a sample into water prevented the recovery of rotifers, while we found several recovering specimens after exposure to high humidity before immersion.

Still, in some previous studies on long-term anhydrobiosis where viable animals were found, animals were probably not kept in high humidity conditions before immersion in water (Goodey, 1923; Rahm, 1923; Baumann, 1927; Franceschi, 1948; Fielding, 1951; Lee, 1961), although the methods used were not explained in detail.

Problems related to long-term studies on anhydrobiosis

Studies on long-term anhydrobiotic survival in micrometazoans need to consider several problems relating to contamination and the history of the samples. These include contamination from other samples by inappropriate storage methods (e.g. poorly closed storage bags), from laboratory material (e.g. sieves, cups, pipettes, etc.), and from water used for extraction (e.g. in the tap water). At the beginning of our experiment, some of these problems became obvious, and we therefore put considerable effort into eliminating contamination within the laboratory environment. Contamination present already in the original samples can be avoided only by choosing samples with a documented and controlled history of storage. This is rarely obtained for samples from museum collections, including the ones that we used. Unfortunately, previous papers on long-term survival usually do not present enough information about methodology to evaluate the problems of contamination. This gives a high degree of uncertainty in the interpretation of earlier records of long-term anhydrobiotic survival.

The probability of finding viable anhydrobiotic animals after long-term dry storage is not only influenced by the inherent capacity of the animals to maintain viability over long periods. Both the conditions under which the animal enters anhydrobiosis and those under which it is rehydrated may have an impact. Thus, an animal found dead after a number of years may have died at dehydration, e.g. because of desiccation that was too rapid (Wright, 1989) or from a lack of energy resources for the metabolic preparation for anhydrobiosis (see Crowe & Higgins, 1967; Kinchin, 1993). Alternatively, it may have entered anhydrobiosis successfully and remained viable (structurally) over the whole period of anhydrobiosis but died at rehydration. The rate of desiccation is one of the most important limiting factors for successful anhydrobiosis in micrometazoans, as documented in both tardigrades (Wright, 1989) and nematodes (Ellenby, 1969; Womersley & Ching, 1989; Menti, Wright & Perry, 1997). Unfortunately, details on the conditions under which plant materials, obtained from museums or private collectors, were dehydrated are often unknown. Most probably, materials that were collected dry at the natural growing site avoid the problem of lethal post-collection dehydration.

When material for studies of long-term anhydrobiosis are obtained from museums (as in our study), the effects of chemical treatments may influence the results. Fumigation against insects has frequently been used to preserve museum collections and methyl bromide has been one of the most common fumigants. Conventional fumigation with methyl bromide does not influence revival from anhydrobiosis in recently dried tardigrades, but seems to have some negative effects on animals kept dry for longer periods (Jönsson & Guidetti, 2001). If this proves to be a general effect, plant materials from museums treated with fumigants should be avoided in studies of anhydrobiotic survival in animals. Therefore, our data from mosses and lichens stored in the museum collections (all of which had been treated with fumigants) do not alone disprove the possibility that anhydrobiotic micrometazoans may survive for several decades in air at room temperature. However, the absence of viable animals (and eggs) also in samples of private collections (9–19 years old, not treated with chemicals; see Table 1) suggests that the limit of anhydrobiotic survival was exceeded for the species in those substrates.

Another important question that should be considered in this kind of study concerns the criteria to use for considering whether an animal is alive after a period of anhydrobiosis. Previous studies have used different criteria. For instance, Franceschi (1948) reported a distention followed by a short retraction of the first pair of legs in a tardigrade as a sign of life (see Jönsson & Bertolani, 2001), and Fielding (1951) considered alive those nematodes whose body contents 'gushed' from the body due to internal pressure after cutting. The methods used in those studies is not discussed here, but certainly they involve different meanings of 'live specimens', which may give rise to different interpretations of obtained results. In our study, evident signs of coordinated movement were used as the criterion, because for tardigrades this is probably the best indication of a normally functioning body.

CONCLUSION

Our study is the first systematic investigation of long-term anhydrobiotic survival in tardigrades, rotifers and free-living nematodes inhabiting mosses and lichens. For tardigrades, our observations confirm those of an earlier report (Baumann, 1927) that the upper limit of anhydrobiotic survival under atmospheric oxygen conditions may not exceed about 10 years. We also documented the first case of long-term anhydrobiotic survival in tardigrade eggs and the longest record of anhydrobiotic survival for rotifers, which were found to be viable after 9 years. More studies are needed to verify the possibility that tardigrade eggs are able to withstand longer periods in anhydrobiosis than animals, as suggested by this study.

Our study also made evident some of the problems of evaluating long-term anhydrobiotic survival, including contamination and the chemical treatment of samples. We have also speculated on the possible role of the microenvironment in which the anhydrobiotic animal is kept, in protecting the animals against high dehydration rates and effects of oxidation.

The factors that allow some anhydrobiotic species to remain viable over nearly a decade, while specimens of other species are killed, remain unknown. Moreover, although our current evidence is limited to the eggs of only one tardigrade species (*Ramazzottius oberhaeuseri*), it is tempting to suggest that a high capacity for rapid desiccation also brings a high capability to survive long periods in anhydrobiosis. Why such a connection should exist is, however, unclear. The selective pressures that have led to differences in anhydrobiotic capacities among species, and the impressive ability of some organisms to survive in a dry state, remain an important and unresolved area of investigation.

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REFERENCES

Bastian, H. C. (1865). II. Monograph on the Anguillulidae, or free nematoids, marine, land and freshwater; with descriptions of 100 new species. *Trans. Linn. Soc. Lond.* **25**: 73–184.

Baumann, H. (1927). Bemerkungen zur Anabiose von Tardigraden. *Zool. Anz.* **72**: 175–179.

Clegg, J. S. (1967). Metabolic studies of cryptobiosis in ecysted embryos of *Artemia salina*. *Comp. Biochem. Physiol.* **20**: 801–809.

Clegg, J. S. (1973). Do dried cryptobiotes have a metabolism? In *Anhydrobiosis*: 141–146. Crowe, J. H. & Clegg, J. S. (Eds). Pennsylvania: Dowden, Hutchinson & Ross.

Clegg, J. S. (1986). The physiological properties and metabolic status of *Artemia* cysts at low water content: the 'water replacement hypothesis'. In *Membranes, metabolism and dry organisms*: 169–187. Leopold, C. A. (Ed.). Ithaca, NY: Comstock.

Crowe, J. H. (1975). The physiology of cryptobiosis in tardigrades. *Mem. Ist. Ital. Idrobiol. Dott. Marco Marchi (Suppl.)* **32**: 37–59.

Crowe, J. H. & Higgins, R. P. (1967). The revival of *Macrobiotus areolatus* (Tardigrada) from the cryptobiotic state. *Trans. Am. Microsc. Soc.* **86**: 286–294.

Crowe, J. H. & Madin, K. A. C. (1975). Anhydrobiosis in nematodes: evaporative water loss and lipid and carbohydrate metabolism. *J. exp. Zool.* **193**: 323–342.

Crowe, J. H., O'Dell, S. & Armstrong D. A. (1979). Anhydrobiosis in nematodes: permeability during rehydratation. *J. exp. Zool.* **207**: 431–438.

Ellenby, C. (1968). Desiccation survival in the plant parasitic nematodes, *Heterodera rostochiensis* Wollenweber and *Ditylenchus dipsaci* (Kuhn) Filipjev. *Proc. R. Soc. Lond. B Biol. Sci.* **169**: 203–213.

Ellenby, C. (1969). Dormancy and survival in nematodes. *Soc. Exp. Biol. Symp.* **23**: 83–97.

Fielding, M. J. (1951). Observations on the length of dormancy in certain plant infecting nematodes. *Proc. Helminthol. Soc. Wash. DC* **18**: 110–112.

Franceschi, T. (1948). Anabiosi nei tardigradi. *Boll. Mus. Ist. Biol. Univ. Genova* **22**: 47–49.

Gilbert, J. J. (1974). Dormancy in rotifers. *Trans. Am. Micros. Soc*. **93**: 490–513.

Goodey, T. (1923). Quiescence and reviviscence in nematodes, with special reference to *Tylenchus tritici* and *Tylenchus dipsaci*. *J. Helminthol*. **1**: 47–52.

Heckly, R. J. (1978). Effects of oxygen on dried organisms. In *Dry biological systems*: 257–278. Crowe, J. H. & Clegg, J. (Eds). New York: Academic Press.

Jönsson, K. I. & Bertolani R. (2001). Fact and fiction about long-term survival in tardigrades. *J. Zool. (Lond.)* **255**: 121–123.

Jönsson, K. I. & Guidetti, R. (2001). Effects of methyl bromide fumigation on anhydrobiotic micrometazoans. *Ecotoxicol. Environ. Saf.* **50**: 72–75.

Keilin, F. R. S. (1959). The problem of anabiosis or latent life: history and current concepts. *Proc. R. Soc. Lond. B Biol. Sci.* **150**: 149–191.

Kinchin, I. M. (1993). An observation on the body cavity cells of *Ramazzottius* (Hypsibiidae, Eutardigrada). *Quekett J. Microsc.* **37**: 52–55.

Lee, D. L. (1961). Two new species of cryptobiotic (anabiotic) freshwater nematodes, *Actinolaimus hintoni* sp. nov. and *Dorylaimus keilini* sp. nov. (Dorylaimidae). *Parasitology* **51**: 237–240.

Madin, K. A. C. & Crowe, J. H. (1975). Anhydrobiosis in nematodes: carbohydrate and lipid metabolism during dehydratation. *J. exp. Zool.* **193**: 335–342.

Menti, H., Wright, D. J. & Perry, R. N. (1997). Desiccation survival of populations of the entomopathogenic nematodes *Steinernema feltiae* and *Heterorhabditis megidis* from Greece and the UK. *J. Helminthol.* **71**: 41–46.

Örstan, A. (1998). Factors affecting long-term survival of dry bdelloid rotifers: a preliminary study. *Hydrobiologia* **387/388**: 327–331.

Rahm, P. G. (1923). Biologische und physiologische Beiträge zur Kenntnis der Moosfauna. *Z. Allg. Physiol.* **20**: 1–34.

Ramazzotti, G. & Maucci, W. (1983). Il Phylum Tardigrada. Terza edizione riveduta e corretta. *Mem. Ist. Ital. Idrobiol. Dott. Marco Marchi* **41**: 1–1012.

Ricci, C. N. (1987). Ecology of bdelloids: how to be successful. *Hydrobiologia* **147**: 117–127.

Ricci, C. & Abbruzzese, G. (1989). Il costo dell'anidrobiosi in nematodi e rotiferi bdelloidei. *S.IT.E. Atti* **7**: 297–302.

Ricci, C., Vaghi, L. & Manzini, M. L. (1987). Desiccation of rotifers (*Macrotrachela quadricornifera*): survival and reproduction. *Ecology* **68**: 1488–1494.

Sømme, L. & Meier, T. (1995). Cold tolerance of Tardigrada from Dronning Maud Land, Antarctica. *Polar Biol.* **15**: 221–224.

Steiner, G. & Albin, F. E. (1946). Resuscitation of the nematode *Tylenchus polyhypnus* n. sp. after almost 39 years' dormancy. *J. Wash. Acad. Sci*. **36**: 97–99.

Wallace, H. R. (1962). Observations on the behavior of *Datylenchus dispaci* in soil. *Nematologica* **7**: 91–101.

Westh, P. & Ramløv, H. (1991). Trehalose accumulation in the tardigrade *Adorybiotus coronifer* during anhydrobiosis. *J. exp. Zool.* **258**: 303–311.

Womersley, C. (1981). Biochemical and physiological aspects of anhydrobiosis. *Comp. Biochem. Physiol.* **70**: 669–678.

Womersley, C. (1987). A reevaluation of strategies employed by nematode anhydrobiotes in relation to their natural environment. In *Vistas on nematology*: 165–173. Veech, J. & Dickson, D. W. (Eds). USA: Society of Nematology.

Womersley, C. & Ching, C. (1989). Natural dehydration regimes as a prerequisite for the successful induction of anhydrobiosis in the nematode *Rotylenchulus reniformis*. *J. exp. Biol.* **143**: 359–372.

Wright, J. C. (1989). Desiccation tolerance and water-retentive mechanisms in tardigrades. *J. exp. Biol.* **142**: 267–292.

Wright, J. C. (1991). The significance of four xeric parameters in the ecology of terrestrial Tardigrada. *J. Zool. (Lond.)* **224**: 59–77.

Wright, J. C., Westh, P. & Ramløv, H. (1992). Cryptobiosis in Tardigrada. *Biol. Rev.* **67**: 1–29.