Factors affecting long-term survival of dry bdelloid rotifers: a preliminary study

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Abstract

Naturally dried lichens and mushrooms were collected, stored at various relative humidities and temperatures either under air or argon, and extracted in a 0.2 M sucrose solution to determine the long-term survival of resident bdelloid rotifers. Survivorship of rotifers in samples kept at 21 °C for 8 months declined at both <1% and 76% humidities, but remained the same as the starting levels at 23% and 43% humidities. Lowering the temperature to 4 °C improved survival at both <1% and 76% humidities; at −20 °C and <1% humidity, survivorship of rotifers did not decline for up to 18 months. Storage at 21 °C under argon gas improved survival of bdelloids at <1% humidity, but not at 76% humidity. These results suggest that several processes, including oxidation reactions, may be partly responsible for death of anhydrobiotic bdelloids. To facilitate taxonomic work it is recommended that naturally dried samples containing bdelloids be stored over a desiccant at temperatures below 0 °C until they are to be rehydrated.

Introduction

Bdelloid rotifers (Rotifera; Bdelloidea), Tardigrada and Nematoda are the most abundant microscopic invertebrates present in semiterrestrial, freshwater microhabitats: regions subject to frequent drying (Fantham & Porter, 1945; Stubbs, 1989; Wright, 1991). While tolerance of tardigrades and nematodes to desiccation has been well studied (e.g., Crowe & Madin, 1974; Wright et al., 1992), relatively few studies have been done on bdelloids (e.g., Ricci et al., 1987). Previously I have examined survivorship of the eggs of *Adineta vaga* (Davis) at <1% humidity (Örstan, 1995). Here, I report a preliminary study that examines effects of temperature, humidity, and anoxic conditions on survival of naturally dried bdelloids. To do this naturally dried lichens and mushrooms were collected a few days after a rain and stored at various humidities and temperatures either in air or argon atmospheres. After various lengths of storage, portions of these materials were rehydrated, the bdelloids extracted and the survivors enumerated.

Materials and methods

Sample collection and storage

Three sets of samples were collected from a wooded park in Montgomery County, Maryland, USA: Set #1 – Foliose lichens from rocks and tree trunks collected three days after a rain; Set #2 – Polypore mushrooms from a tree trunk collected two days after a rain; Set #3 – Foliose lichens from a tree trunk.

All experiments were begun within 1–3 days after collection of the samples. During this period samples were kept at room temperature (17–22 °C) and humidity (ca. 60%). In each set, the initial sample was cut into small pieces, mixed, and divided into portions. A sample portion and a drying solution (see *Establishing constant humidity*, below) were sealed in separate vials inside either a glass jar (90 ml) with a tight plastic lid or a glass jar (150–500 ml) with a rubber gasket. The latter jars were used for storage under argon. To replace air, a jar was flushed with argon (99.998% pure, $O_2 < 0.0004\%$) from a hose attached to a pressurized argon cylinder for about 2 min before it was sealed. This process was repeated after

every time such a jar was opened to remove portions for extraction. Because argon is heavier than oxygen, I assumed that it would be more effective in replacing oxygen than would a lighter gas, such as nitrogen. I did not attempt to remove the oxygen that may have been dissolved in the salt solutions used to establish constant humidity (see below).

Jars were kept at room temperature (21 ± 2 °C), in a water bath (21 ± 1 °C), in a refrigerator (4 ± 1 °C), or in a freezer (−20 ± 2 °C). Before a sample was placed in the freezer it was dried further over anhydrous calcium sulfate (see below) in a refrigerator for 3–5 days. This drying process was repeated after every time such a jar was opened to remove portions for extraction. To prevent condensation inside the jars and on the samples, jars stored in a refrigerator or freezer were warmed up to room temperature before opening.

Establishing constant humidity

Constant humidity, independent of temperature at 4–21 °C (Rockland, 1960; Rockland & Nishi, 1980), was maintained with saturated solutions of sodium chloride (76% humidity), potassium carbonate (43% humidity) and potassium acetate (23% humidity). Indicating Drierite[R] (anhydrous calcium sulfate, impregnated with cobalt chloride, W.A. Hammond Drierite Co., Xenia, Ohio, U.S.A.) was used to obtain the lowest humidity. I assumed that the relative humidity of air dried with Drierite was temperature independent in the range −20–21 °C and using the water contents of saturated air (Hodgman, 1949) and air dried with calcium sulfate (Skoog & West, 1976), calculated its value to be <1%.

Sample extraction and data collection

Rotifers were extracted from the dried samples with a 0.2 M solution of sucrose in distilled water, a modification of the water extraction method of Peters et al. (1993). Since the bdelloids remain contracted in sucrose, this method allows one to collect as many rotifers as needed, remove any debris, count the rotifers and, then, dilute sucrose with water to allow the animals to resume activity. Also, prehydration at a high osmolarity (or a high humidity) before rehydration in water may improve the recovery of bdelloids, as with dried nematodes (Crowe & Madin, 1975), bacteria (Kosanke et al., 1992) and seeds and pollen (Crowe et al., 1992).

To extract the rotifers, a 0.15–0.4 g portion from a dry sample was placed in a small plastic vial to which 2 ml of 0.2 M sucrose was added. The vial was sealed with Parafilm[R], vigorously shaken for about 25 s, and the extract was poured into a Petri dish. The sample was extracted two more times with 2 ml of 0.2 M sucrose each time. Under a stereomicroscope, the contracted bdelloids (but not their eggs) were removed with a fine-tipped, glass pipet and transferred into a watch glass containing a small amount of 0.2 M sucrose. After a number of rotifers were collected (ca. 25 or more), debris was removed from the sample, and the rotifers were counted. Rotifers remained in 0.2 M sucrose for no longer than about 40 min before it was diluted with distilled water. After a 24-hour recovery period (17–22 °C), the number of live rotifers was determined. When I was unsure if an animal was alive or not, I examined it under a compound microscope; in that case, animals with active flame cells or cloacae were considered alive.

Unless otherwise indicated, each datum in Table 1 and Figures 1 and 2 is the average of two extractions plus or minus the standard deviation. In Sets #1 and #3, at least 40 rotifers were isolated at each extraction, while in Set #2 at least 26 rotifers were isolated at each extraction. The mean percentages of bdelloids alive at the start of the study (i.e., day 0) were obtained from duplicate extractions of each sample set.

Results

List of bdelloids recovered

Set #1: *Adineta barbata* Janson; *Macrotrachela ehrenbergii* (Janson); *Macrotrachela multispinosa* Thompson; *Macrotrachela nana* (Bryce); *Mniobia russeola* (Zelinka); *Mniobia scabrosa* Murray (first U.S.A. record); *Mniobia tetraodon* (Ehrenberg); *Philodina plena* (Bryce). Set #2: *Adineta vaga* (Davis); *Habrotrocha* sp.; *Macrotrachela quadricornifera* Milne; *M. scabrosa*; *Philodina eurystephana* Schulte (first U.S.A. record). Set #3: *Macrotrachela* sp.; *M. scabrosa*; *Philodina* sp.

Survivorship
In Set #1, portions of a lichen sample were stored in air at specific humidities and temperatures indicated in Table 1. After 8 months at 21 °C, the percentages of live rotifers at 23% and 43% humidities were roughly the same as on day 0. In comparison, at 21 °C, 0%
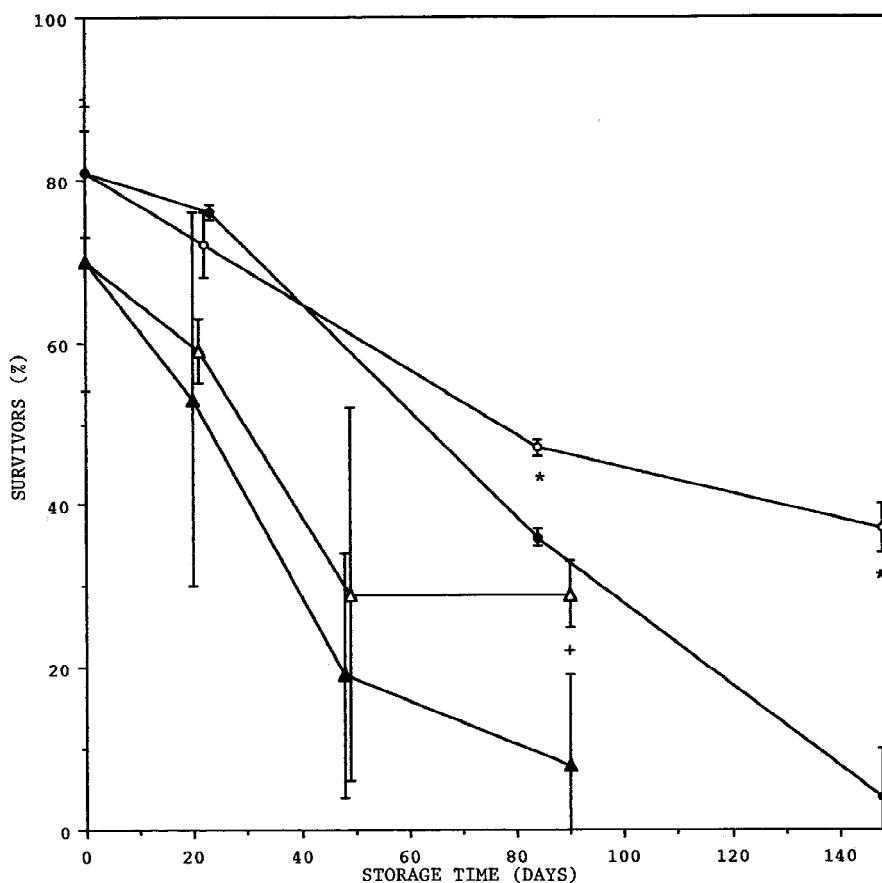
Figure 1. Survival of bdelloids in mushroom (set 2, triangles) and lichen (set 3, circles) stored in air (closed symbols) or argon (open symbols) at 21 °C, <1% humidity. (Overlapping error bars for day 20 for argon in set 2, and at day 22 for air in set 3 were shifted to the right by one day to reveal their position.) Statistical significance obtained from pairwise comparison with Student's *t*-test are indicated as follows: $p < 0.02 =$ *; $0.1 < p < 0.2 = +$.

of rotifers survived at <1% humidity and only $4 \pm 5\%$ survived at 76% humidity for 8 months. However, lowering the temperature to 4 °C increased survival at both <1% and 76% humidity. Further, the percentage of survivors did not decline from day 0 over an 18-month period at −20 °C and <1% humidity (Table 1).

To duplicate some of the conditions in Table 1, portions of the lichen sample in Set #3 were stored in air at 21 °C and 43% humidity and at −20 °C and <1% humidity. After about 8 months, $74 \pm 5\%$ of rotifers at 43% humidity and $72 \pm 1\%$ of rotifers at <1% humidity were alive (Figure 2). In agreement with Table 1, these values were not significantly different (Student's *t*-test, $0.2 < p < 0.4$) than the percentage of live rotifers in Set #3 at day 0 ($81 \pm 8\%$).

The effect of replacing air with argon on survival was evaluated in Sets #2 and #3 at 21 °C. At <1% humidity, in both sets survival in argon was significantly better than that in air after about 3 months (Figure 1). However, at 76% humidity argon had no significant effect on the survival of rotifers (Figure 2).

In agreement with earlier observations (Jacobs, 1909; Hickernell, 1917), I have noticed that bdelloids in recently dried samples or in dry samples kept under conditions that do not decrease survivorship quickly become active after rehydration. For example, some bdelloids extracted from the lichen in Set #3 that had been kept at 21 °C, 43% humidity for 8 months started feeding as soon as sucrose was diluted with distilled water. Whereas, bdelloids from samples kept dry under less favorable conditions took several hours to fully revive after rehydration. It is easiest to extract the
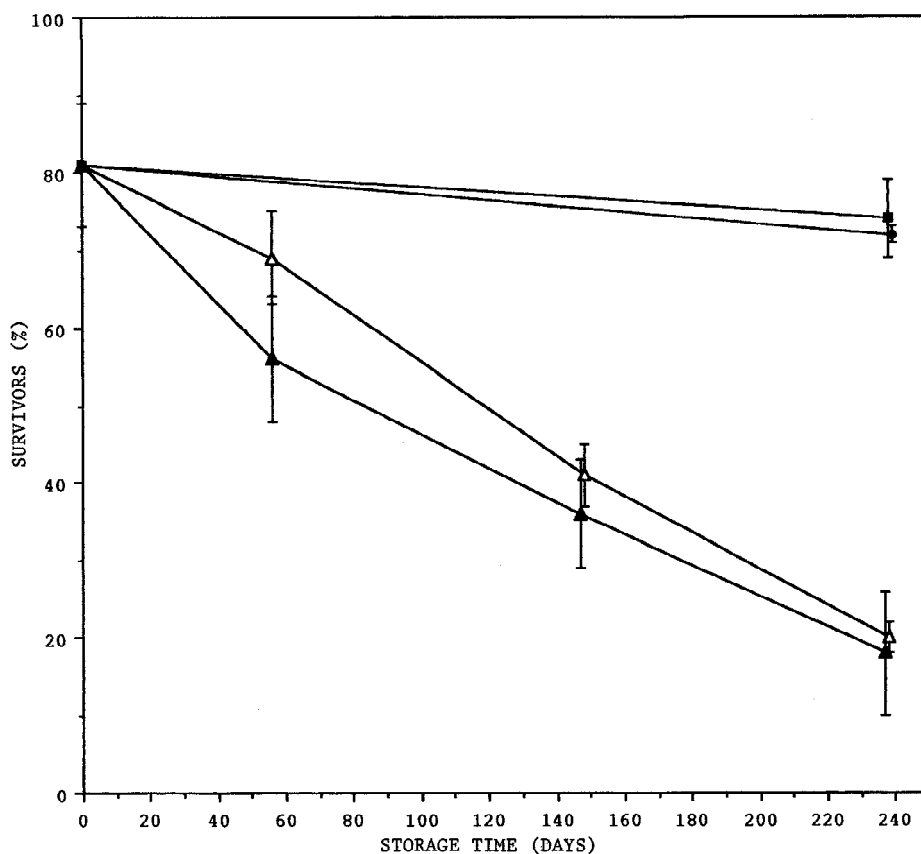
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Figure 2. Survival of bdelloids in lichen (set 3) stored in air (closed symbols) or argon (open symbols) at 21 °C, 76% humidity (triangles) and at 21 °C, 43% humidity (square) and at −20 °C, <1% humidity (circle). (Overlapping error bars for argon at days 147 and 237 were shifted to the right by one day to reveal their position.)

Table 1. Percentage survivorship of bdelloids at different relative humidities and temperatures from Set #1 (sample of lichen)

Conditions [a]		Month number [b]			
Humidity (%)	°C	5	8	12	18
<1	21	5 ± 4	0	–	–
23		72 ± 11	67 ± 19	–	–
43		68 ± 8	68 ± 5	–	–
76		28 ± 1	4 ± 5	–	–
<1	4	53 ± 4	46 ± 18	51 ± 15	28 ± 15
76		77 ± 2	70 ± 16	–	–
<1	−20	73 ± 1	72 ± 4	–	75 ± 2

[a] Conditions of humidity and temperature under which the samples were held.
[b] At the start of the procedure (day 0), a mean of 69 ± 8% of bdelloids were scored as being alive in Set #1. Extractions under a given month (column) were actually done over a 1–3-day period. Number of months are approximations calculated by dividing the number of days by 30. (All values are averages of two extractions, except for the values at 23% and 43% humidity at 5 months, which are averages of 3 extractions.)

former samples with 0.2 M sucrose, while the latter may be extracted with water. To check the effect of the extraction medium on post-extraction survival of bdelloids, I extracted two portions of a lichen sample kept for 86 days under adverse conditions (i.e., 21°C, <1% humidity) with 0.2 M sucrose and two additional portions with water. In this case, there was no significant difference between the percentages of survivors 24 h after the extraction with sucrose (46 ± 1%) and water (39 ± 12%) as analyzed using the *t*-test.

Discussion

What processes are responsible for the gradual decline in the number of live rotifers in dry samples? I suggest that drying itself is not lethal to the rotifers for two reasons. (1) If removal of water killed the rotifers (perhaps by damaging their cells and organs), most would be expected to die immediately upon or soon

after drying, assuming that their internal water content quickly equilibrated with the ambient humidity. However, at 76% and <1% humidities, the death rate was relatively slow at 21 °C and it could be decreased further by lowering the temperature (Table 1) or by replacing air with argon at <1% humidity (Figure 1). (2) At the intermediate humidities of 23% and 43%, the percentages of live rotifers did not decline during the 8-month period (Table 1, Figure 2).

Thus, I argue that certain humidity-controlled chemical reactions, which may use oxygen, seem to be involved in the death of anhydrobiotic bdelloids. Oxidation damage to cellular components, including lipids, has been implicated in the reduced viability of dried prokaryotes (Zentner, 1966; Potts, 1994) and dried invertebrates (Crowe & Madin, 1974). Moreover, the rate of lipid oxidation in dry foods is slowest at around the water activity of 0.2 (20% humidity) (Labuza, 1975), near the range (i.e., 23–44% humidity) where the reactions that kill the dried bdelloids also seem to be the slowest at ordinary temperatures. Therefore, I suggest that lipid oxidation may be involved in the effects of humidity on the survival of dried bdelloids. Nevertheless, bdelloids continue to die even in the presence of argon (Figure 1). If argon completely replaced the air in the jars and no air subsequently leaked back into them, the results indicate that oxidation is not the only process responsible for the death of anhydrobiotic bdelloids. So at present we do not know the mechanism(s) responsible for mortality of anhydrobiotic bdelloids.

Bdelloids are difficult to fix and preserve for study using the conventional techniques that are otherwise adequate for monogonont rotifers. This difficulty often results in the lack of useful specimens for taxonomic work. Thus, I recommend that naturally dried samples be subjected to additional care. These extra steps would include drying over a desiccant for several days in a refrigerator and long-term storage at <0 °C in the absence of oxygen.

Acknowledgements

References

Crowe, J. H. & K. A. Madin, 1974. Anhydrobiosis in tardigrades and nematodes. Trans. am. Mic. Soc. 93: 513–524.

Crowe, J. H. & K. A. Madin, 1975. Anhydrobiosis in nematodes: evaporative water loss and survival. J. exp. Zool. 193: 323–334.

Crowe, J. H., F. A. Hoekstra & L. M. Crowe, 1992. Anhydrobiosis. Ann. Rev. Physiol. 54: 579–599.

Fantham, H. B. & A. Porter, 1945. The microfauna, especially the protozoa found in some Canadian mosses. Proc. zool. Soc. Lond. 115: 97–174.

Hickernell, L. M., 1917. A Study of desiccation in the rotifer, Philodina roseola, with special reference to cytological changes accompanying desiccation. Biol. Bull. 32: 343–407.

Hodgman, C. D. (ed.), 1949. Handbook of Chemistry and Physics, 31st edn. Chemical Rubber Publishing Co., Cleveland, 1938.

Jacobs, M. H., 1909. The effects of desiccation on the rotifer Philodina roseola. J. exp. Zool. 6: 207–263.

Kosanke, J. W., R. M. Osburn, G. I. Shuppe & R. S. Smith, 1992. Slow rehydration improves the recovery of dried bacterial populations. Can. J. Microbiol. 38: 520–525.

Labuza, T. P., 1975. Oxidative changes in foods at low and intermediate moisture levels. In R. B. Duckworth (ed.), Water Relations of Foods. Academic Press, New York: 455–473.

Örstan, A., 1995. Desiccation survival of the eggs of the rotifer Adineta vaga (Davis, 1873). Hydrobiologia 313/314: 373–375.

Peters, U., W. Koste & W. Westheide, 1993. A quantitative method to extract moss-dwelling rotifers. Hydrobiologia 255/256: 339–341.

Potts, M., 1994. Desiccation tolerance of prokaryotes. Microb. Rev. 58: 755–805.

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

Rockland, L. B., 1960. Saturated salt solutions for static control of relative humidity between 5 and 40 °C. Anal. Chem. 32: 1375–1376.

Rockland, L. B. & S. K. Nishi, 1980. Influence of water activity on food product quality and stability. Food Technol. 34(4): 42–51.

Skoog, D. A. & D. M. West, 1976. Fundamentals of Analytical Chemistry. Holt, Rinehart & Winston, New York, 804 pp.

Stubbs, C. S., 1989. Patterns of distribution and abundance of corticolous lichens and their invertebrate associates on Quercus rubra in Maine. Bryologist 92: 453–460.

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., P. Westh & H. Ramløv, 1992. Cryptobiosis in tardigrada. Biol. Rev. 67: 1–29.

Zentner, R. J., 1966. Physical and chemical stresses of aerosolization. Bact. Rev. 30: 551–557.