



Research article

Recovery from anhydrobiosis in the tardigrade *Paramacrobiotus experimentalis*: Better to be young than old and in a group than alone

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A B S T R A C T

Desiccation-tolerant organisms can survive dehydration in a state of anhydrobiosis. Tardigrades can recover from anhydrobiosis at any life stage and are considered among the toughest animals on Earth. However, the factors that influence recovery from anhydrobiosis are not well understood. The study aimed to evaluate the effect of sex, age, the presence of other individuals and the combination of the number and duration of anhydrobiosis episodes on the recovery of *Paramacrobiotus experimentalis*. The activity of 1200 individuals for up to 48 h after rehydration was evaluated using analysis of variance (ANOVA). Age was the main factor influencing return to activity, followed by the combination of number and duration of anhydrobiosis episodes, influence of the presence of other individuals, and sex. More individuals returned to activity after repeated short than repeated long anhydrobiosis episodes and older individuals were less likely to recover than younger individuals. In addition, when compared to single animals, the presence of other individuals resulted in higher number of active animals after dehydration and rehydration. The effect of sex was significant, but there was no general tendency for one sex to recover from anhydrobiosis better than the other one. The results contribute to a better understanding of the anhydrobiosis ability of *Paramacrobiotus experimentalis* and provide background for full explanation of molecular, cellular and environmental mechanisms of anhydrobiosis.

1. Introduction

The ability of some organisms to survive dehydration, resulting in almost complete loss of body water (desiccation) and entering a state of reversible suspension, is called “anhydrobiosis”, which comes from the Greek for “life without water” and indicates “desiccation tolerance” [1–5]. Anhydrobiosis is extremely important for survival in harsh environments with periodically unavailable water, which can affect growth and reproduction. It also affects lifespan and may therefore slow down the rate of evolution [4,6].

However, it has been shown that the longer the time spent in anhydrobiosis, the longer it takes to return to activity. Exceeding a certain critical period of desiccation can lead to the death of the organism [7–9]. As water availability is one of the most important factors for life, a full understanding of the underlying mechanisms of anhydrobiosis is crucial for the development of technologies based on organism tolerance to desiccation. The discovery and understanding of these mechanisms could have an impact on several areas of research, including DNA protection and repair mechanisms, the preservation of biological materials for clinical applications or food production, and enzymes working in a small amount of water (e.g., [4,10,11]).

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Abbreviations

DNA	Deoxyribonucleic acid
e.g.	Exempli gratia (for example)
i.e.	Id est (in other words)
ca.	Circa
RH	Relative humidity
ddH ₂ O	Double distilled water
h	Hour
RM_ANOVA	Multivariate repeated measures analysis of variance
GLM	General linear models

Tardigrades (commonly called water bears) are an important group of invertebrates due to their place between two major invertebrate model organisms, i.e., *Caenorhabditis elegans* and *Drosophila melanogaster*, and their aquatic-to-terrestrial transition, which may provide insight into the evolution of mechanisms that allow adaptation to stressful conditions [2]. Like other invertebrates, such as nematodes and rotifers, tardigrades show a remarkable ability to survive in an anhydrobiotic state at all life stages (e.g., [12–15]), although not with the same success for all species and life stages [16,17].

Tardigrades can serve as an excellent model in biological research, including for the impact of phenotypic and environmental factors on anhydrobiosis as a survival strategy. Indeed, various aspects of tardigrade biology have already been intensively studied, including reproduction (e.g., [18,19]), dormancy strategies (e.g., [2,20–22]), mechanisms of adaptation to the most extreme environments (e.g., [23,24]), phylogenetic relationships (e.g., [25–27]), metabolic functions (e.g., [28]) and experience of exposure to space conditions (e.g., [11,29]).

Anhydrobiosis in tardigrades is a complex phenomenon. It includes entering, permanent, and leaving steps, which correspond to dehydration (i.e., a tun formation), tun state (i.e., desiccated state) and rehydration, respectively [2]. These steps are fully elucidated at the level of the organism's morphology [3,13,14,20,21], but full access to the underlying mechanisms requires consideration of additional factors. It is known that the survival rate of anhydrobiosis can be affected by the type of environment, feeding behaviour (e.g., diet), environmental/culture conditions (e.g., ambient temperature, water quality, culture substratum) and other factors such as overall body size, conditions of dehydration, as well as, the number, and duration of anhydrobiosis episodes (e.g., [9,17,30–33]).

Up to now, ca. 1400 tardigrade species have been described [34], but fewer than 1.5% (ca. 20 species) have been studied for anhydrobiotic ability. Most of the studies have been mainly performed on parthenogenetic species. However, many bisexual species have been reported in tardigrades (e.g., [19]). In three bisexual species, females were predominantly analysed, or the sex was not specified [9,35,36]. However, males can also occur in parthenogenetic lineages, suggesting a switch in the reproductive mode between parthenogenesis and bisexual reproduction [19,37], which makes interpreting results even more complicated.

Some of the factors we examined that affect anhydrobiosis survival have already been evaluated, while others have not, or knowledge of them is incomplete, but they could potentially increase the risk of anhydrobiosis failure. Although individual age is generally considered to be a factor influencing anhydrobiosis survival (e.g., [30]), the only study addressing this issue found no significant effect of age [31]. The duration of anhydrobiosis episodes is a known factor affecting survival. It is explained by cellular damages, the severity of which correlates with the duration of the tun state (e.g., [25,35,38]). The negative impact of repeated anhydrobiosis episodes on survival has also been shown (e.g., [35]). It has been hypothesized that the repeated entering and leaving steps cause additional cellular damage, which is supposed to be eliminated by feeding before the next episode of anhydrobiosis, thereby enhancing cellular repair mechanisms [30,31,35,39]. However, a comparative analysis of the combined effect of the number and duration of anhydrobiosis episodes has not been performed. In addition, the effect of sex and occurrence in the presence of other individuals on the return to activity has not yet been assessed.

In response to the shortcomings of research on anhydrobiosis in tardigrades, active individuals per test unit were examined after experimentally induced repeated anhydrobiosis episodes for the bisexual species *Paramacrobiotus experimentalis* [40]. The effect of the following factors was assessed [1]: sex [2], age [3], presence of other individuals termed here shortly “group influence” and [4] combination of the number and duration of anhydrobiosis episodes. In addition, the interactions between these factors were analysed. The approach used here differs from previous anhydrobiosis studies in several points because [1] males and females were analysed separately [2]; dehydration and rehydration were performed for single individuals and in the presence of other individuals, i.e., in groups [3]; individuals were divided into five age classes and [4] different number and duration of anhydrobiosis episodes were applied.

Results obtained are important for developing a better understanding of the process of anhydrobiosis, providing the background for describing underlying molecular, cellular and environmental mechanisms and their response to environmental stress.

2. Materials and methods

2.1. Cultures of *Paramacrobiotus experimentalis*

Females and males of *P. experimentalis* [40], were cultured together in covered, vented plastic Petri dishes (55 mm in diameter),

with the bottom scratched with sandpaper to allow the animals to move. Individuals were coated with a thin layer of the culture medium, a mixture of spring water (Żywiec Zdrój S.A., Poland), and ddH₂O in a 1:3 ratio. The culture medium was changed every week, and animals were fed with the rotifer *Lecane inermis* (strain 1.A2.15) provided by Dr Edyta Fiałkowska (Institute of Environmental Sciences, Jagiellonian University, Krakow, Poland). The Petri dishes were kept in the climate chamber POL EKO KK 115 TOP + at 20 °C, in the dark (24 h), and with relative humidity (RH) of 40%. The first oviposition in *P. experimentalis* takes place at 19.3 ± 3.6 days after hatching and the estimated average or mean lifespan is 360 days [41].

The groups of animals that differed in age by a month were kept in separate Petri dishes and characterized by selected life history traits including vitality rate, average total body length, and fertility (Supplementary file, “Cultures of *Paramacrobiotus experimentalis*” and Table S1). On the basis of the life history traits, five age classes of adult animals were distinguished that represented different adult life stages defined as growing, young, mature, late and old adults. They represent the following age ranges in days: 60–90, 120–150, 150–180, 240–270 and > 300, and correspond to age classes 1–5, respectively. It has been already shown [41] that the first oviposition in *P. experimentalis* takes place at 19.3 ± 3.6 days after hatching. This denotes that distinguishing between females and males can be made for animals in the age over 30 days. Therefore, animals in the age cannot be considered as juveniles. The age class 60–90 days was described as the youngest one in the study because they are bigger than animals in the age of 30–60 days and distinguishing between females and males is a little easier. For the selected age classes an approximate ratio of 2:1 females to males was defined.

2.2. Protocol for repeated episodes of anhydrobiosis

The protocol includes dehydration and rehydration procedure optimized for *P. experimentalis* [33]. All experiments were performed in covered, vented plastic Petri dishes of 35 mm diameter lined at the bottom with white filter paper (grammage 85–87, Chemland Company, Poland). Females and males representing the distinguished age classes (Supplementary file, Table S1) were transferred using an automatic pipette into dishes filled with 450 µl of the culture medium. The dishes were placed into the climate chamber PolLab Q-Cell 140, and the individuals were allowed to dry slowly at 20 °C, with 40–50% RH, and in the dark for 72 h. The tun formation was checked once every 24 h by a brief 1-min observation under the stereomicroscope (Supplementary file, Fig. S1). After the tun formation, these conditions were maintained for 3 days or 30 days. Next the individuals were rehydrated and their return to activity was observed after 2 h, 6 h, 24 h, and 48 h. The rehydration was achieved by adding 3 ml of the culture medium to each Petri dish. Tuns were transferred using an automatic pipette to small glass cubes and kept at 20 °C and 40–50% RH, with light conditions regulated by seasonal changes in the day/night cycle (according to our observations, the photoperiod does not affect the return of *P. experimentalis* tuns to the active state).

Those individuals that returned to activity (defined here as coordinated movements of the body and legs, i.e., the onset of crawling) 48 h after rehydration were subjected to another anhydrobiosis episode. The number of active individuals after each anhydrobiosis episode and at a given observation time (Supplementary file, Table S2) was used to calculate the activity score defined as the number of active individuals per test unit (3×10 individuals). All variants of anhydrobiosis applied in this study are summarised in Fig. 1 (A–E). A break of three days was allowed between the consecutive anhydrobiosis episodes, and the animals were fed. The feeding took place three days before the start of the next anhydrobiosis episode. Females and males of different ages were subjected to repeated anhydrobiosis in the presence of other individuals, i.e., in groups (10 specimens per Petri dish) or singly (one individual in each of 10 Petri dishes). In total of 1200 individuals were analysed, 600 each in short and long anhydrobiosis episodes with the same duration of dehydration and rehydration steps (see Supplementary file, Table S2 for more details).

2.3. Statistical analysis

A multivariate repeated measures analysis of variance (RM_ANOVA) was performed using the GLM procedure to compare differences in the activity of individuals after rehydration according to the levels of the main order factors considered, i.e. the combined effect of the number and duration of anhydrobiosis episodes, the group influence, the age and sex of the individuals, and the interaction between these factors. Data were tested for normal distribution using Kolmogorov-Smirnov (KS) tests [42] and Levene’s tests for homogeneity of group variances [43]. Left skewed distributions and non-homogeneity of variances were observed for some variables. Although, in general, the Type I error and the power of the F-statistic are not altered by violations of normality, as predicted by the

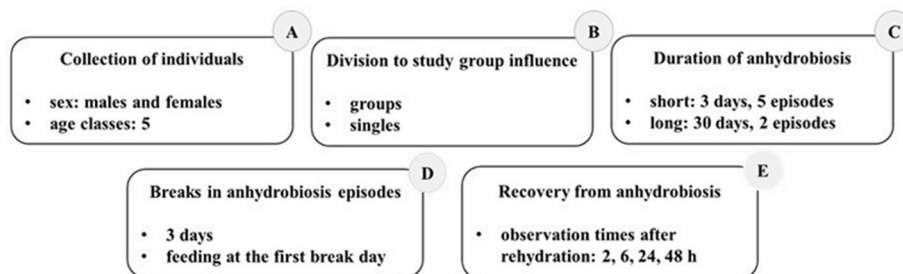


Fig. 1. Graphic representation of the repeated anhydrobiosis experiment performed in *Paramacrobiotus experimentalis*. A–E, the applied steps of the experiments. The term “anhydrobiosis duration” refers to combination of the number and duration of anhydrobiosis episodes.

central limit theorem, especially for large sample sizes (e.g., [44]), ANOVAs were performed using the square root transformed reflected variable, x (transf.) = $\sqrt{1 + \max(x)-x}$. This data transformation gave the best results in the context of the tests. One variable, the number of active individuals 2 h after rehydration, was not normally distributed, $d = 0.134$, $p < 0.05$. Eta squared, an integral part of ANOVA, was used to assess the size of one or more effects (i.e., the proportion of variance accounted for by the effects). After each anhydrobiosis episode for a given observation time (2 h–48 h), the number of active individuals in each sample unit was treated as a repeated measure. Pairwise comparisons were evaluated using Tukey’s post hoc test when the F ratio was statistically significant (at $\alpha < 0.05$) for the main determinants and their interactions [45]. The effect of the number of episodes applied for long and short anhydrobioses in the context of active individuals after rehydration was evaluated using the Student’s t-test or the one-way ANOVA. All statistical analyses were performed in Statistica version 13.0 (StatSoft, Poland).

3. Results

3.1. General remarks

The number of active individuals of *P. experimentalis* (see Supplementary file, Table S2 for raw data) depended most strongly on the observation time after rehydration and the age of the individuals (Eta-squared = 0.633 and 0.553, $p = 0.001$, respectively). The effect of the combination of the number and duration of anhydrobiosis episodes, and the presence of other individuals (i.e., group influence) appeared to be of medium size (Eta-squared = 0.133 and = 0.103, respectively, $p = 0.001$). In contrast, the proportion of variance accounted for by sex was small but significant (Eta-squared = 0.043, $p = 0.05$) (Table 1).

The most significant interactions were between observation time, age of individuals, and the combination of the number and duration of anhydrobiosis episodes (Eta-squared = 0.260, $p = 0.001$, or interactions of two of these variables (Table 1). Some of the other interactions were also significant. However, Eta-square indicates that the proportion of variance a given interaction can explain was relatively small (Eta-square from 0.02 to 0.09).

Table 1

Multivariate repeated measures ANOVA (RM_ANOVA) factor analysis for combination of the number and duration of anhydrobiosis episodes (anhydrobiosis duration), the presence of other individuals (i.e., group influence), age and sex of individuals (as independent variables) on the number of active individuals of *Paramacrobrotus. experimentalis* observed at 2 h–48 h after rehydration. ANOVA was performed using the square root transformed reflected variable, x (transf.) = $\sqrt{1 + \max(x)-x}$; the test unit was 30 individuals (3 replicates x 10 individuals). in red - statistically significant results. SS - Sum of Squares, MS - Mean Squares, F - F ratio and P - p values are given. In total of 1200 individuals were analysed, 600 (300 females and 300 males) each in short and long anhydrobiosis episodes with the same duration of dehydration and rehydration steps.

Effect	SS	Degree of freedom	MS	F	P
Intercept	3750,842	1	3750,842	2361,121	0,0000000
{1} anhydrobiosis duration	24,362	1	24,362	15,336	0,0001646
{2} group influence	18,285	1	18,285	11,511	0,0009928
{3} sex	7159	1	7159	4507	0,0362302
{4} age	196,546	4	49,136	30,931	0,0000000
anhydrobiosis duration*group influence	4488	1	4488	2825	0,0959270
anhydrobiosis duration*sex	2631	1	2631	1656	0,2010482
group influence*sex	2736	1	2736	1722	0,1924293
anhydrobiosis duration*age	10,134	4	2533	1595	0,1815664
group influence*age	0,791	4	0,198	0,124	0,9733427
sex*age	0,920	4	0,230	0,145	0,9648783
anhydrobiosis duration*group influence*sex	2514	1	2514	1582	0,2113643
anhydrobiosis duration*group influence*age	4241	4	1060	0,667	0,6161241
anhydrobiosis duration*sex*age	3140	4	0,785	0,494	0,7400715
group influence*sex*age	2060	4	0,515	0,324	0,8611674
anhydrobiosis duration*group influence*sex*age	1080	4	0,270	0,170	0,9532145
Error	158,859	100	1589		
{5}OBS.TIME	28,290	3	9430	172,376	0,0000000
OBS.TIME*anhydrobiosis duration	2182	3	0,727	13,298	0,0000000
OBS.TIME*group influence	0,473	3	0,158	2883	0,0360823
OBS.TIME*sex	0,209	3	0,070	1271	0,2844629
OBS.TIME*age	2305	12	0,192	3511	0,0000698
OBS.TIME*anhydrobiosis duration*group influence	0,441	3	0,147	2687	0,0466582
OBS.TIME*anhydrobiosis duration*sex	0,329	3	0,110	2004	0,1134477
OBS.TIME*group influence*sex	0,107	3	0,036	0,650	0,5833932
OBS.TIME*anhydrobiosis duration*age	5761	12	0,480	8776	0,0000000
OBS.TIME*group influence*age	1067	12	0,089	1625	0,0837031
OBS.TIME*sex*age	1665	12	0,139	2537	0,0033537
5*1*2*3	0,466	3	0,155	2837	0,0383341
5*1*2*4	0,765	12	0,064	1166	0,3072984
5*1*3*4	1712	12	0,143	2608	0,0025540
5*2*3*4	0,682	12	0,057	1039	0,4131341
5*1*2*3*4	0,964	12	0,080	1468	0,1351782
Error	16,412	300	0,055		

3.2. Effect of age

Based on Tukey's post hoc test, more individuals were active after rehydration in the younger age classes (age classes 1–3) than in the older age classes (age classes 4–5), regardless of the combination of number and duration of anhydrobiosis episodes (Fig. 2). Taking into account the mean values, the younger age classes [1–3] had, on average, 37% more active individuals after rehydration than the older age classes [4,5] (Supplementary file, Table S3).

Statistically significant differences in the number of active individuals after repeated long and short anhydrobiosis were observed especially at the initial observation time after rehydration (2 h) and mainly for individuals representing the younger age classes (Fig. 2 and Tukey's post hoc test results (not shown)). The differences diminished with the time of observation and became insignificant at 48 h after rehydration. Based on the mean values at 48 h after rehydration, the young adults (age class 2) returned to activity 52% and 49% more successfully than the old adults (age class 5) after repeated long and short anhydrobiosis, respectively (Supplementary file, Table S3). The growing adults (age class 1) returned to activity 10% and 19% slower, the mature adults (age class 3) 7% and 14% slower, and the late adults (age class 4) 32% and 37% slower than individuals representing the young adults (age class 2) (Supplementary file, Table S3).

3.3. Effect of combination of the number and duration of anhydrobiosis episodes

The number of active individuals decreased significantly with the increasing number and duration of anhydrobiosis episodes but increased with the observation time after rehydration (Fig. 2). In general, significant positive correlations ($p < 0.05$) were found between the combined effect of the number and duration of anhydrobiosis episodes and observation at 2 h and 6 h after rehydration. Although still positive, these correlations became statistically insignificant as rehydration time progressed (observations at 24–48 h; Supplementary file, Table S4).

In the subsequent analysis, two episodes of long anhydrobiosis were compared with the first two episodes of short anhydrobiosis to distinguish the effect of the duration of anhydrobiosis from the number of anhydrobiosis episodes (2 vs. 5). With a 90% difference in the duration of the tun state (i.e., 6 vs. 60 days), 25%–36% more active individuals were observed after short anhydrobiosis than after long anhydrobiosis, depending on the observation time (Supplementary file, Table S5). Based on Tukey's post hoc test, significant differences were found between short and long anhydrobiosis in the number of active individuals after rehydration at each observation time (2–48 h; one-way ANOVA, Wilk's Lambda = 0.544, $F(4,75) = 15.728$, $p < 0.001$), Fig. 3).

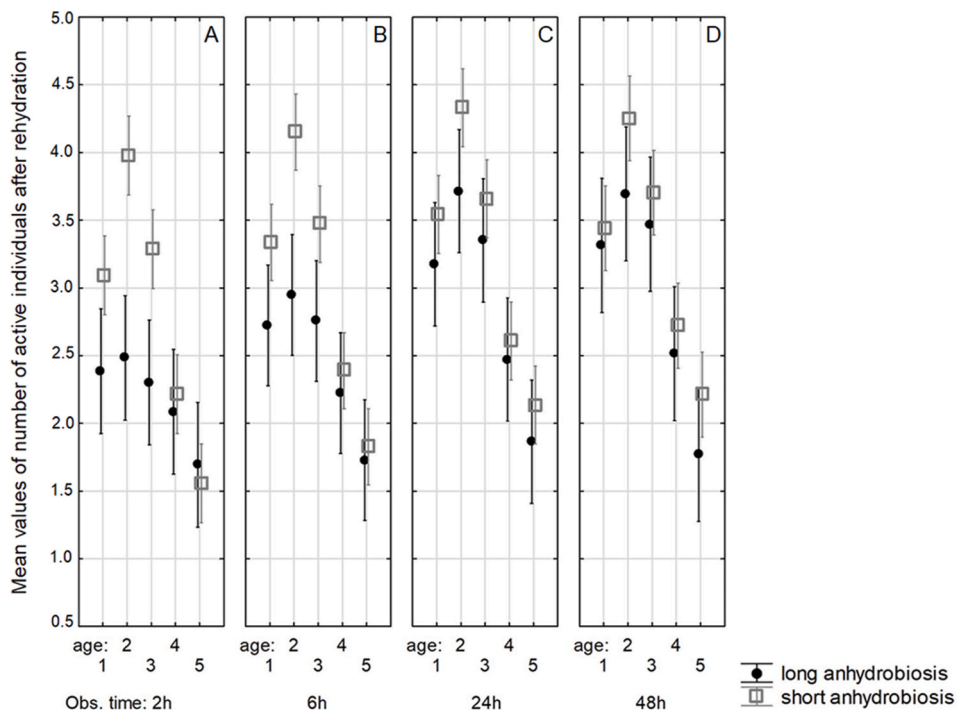


Fig. 2. Mean values of number of active individuals of *Paramacrobiotus experimentalis* observed at 2 h–48 h after rehydration (panels in the figure represents the different observation time i.e., 2 h (A), 6 h (B), 24 h (C) and 48 h (D), taking into account their age and the combined effect of the number and duration of anhydrobiosis episodes (long and short anhydrobiosis). Age [1–5]; the selected age classes; Obs. time - observation time. Expected marginal means and 0.95% confidence intervals are presented. In total of 1200 individuals were analysed, 600 (300 females and 300 males) each in short and long anhydrobiosis episodes with the same duration of dehydration and rehydration steps (see Table S2 for raw data). Results of Multivariate repeated measures analysis of variance (RM_ANOVA) are presented in Tables S3 and S4.

The comparison between consecutive short anhydrobiosis episodes showed no statistically significant differences in the number of active individuals, except a significant difference at 48 h after rehydration between the fourth and fifth episode.

3.4. Effect of the presence of other individuals

The presence of other individuals (i.e., group influence) during anhydrobiosis significantly influenced the number of active individuals after rehydration ($p < 0.001$, Table 1). Furthermore, interactions between observation time after rehydration, the combination of the number and duration of anhydrobiosis episodes, and the group influence or interactions of two of these specified factors were significant (Supplementary file, Table S6). Irrespective of observation time and combination of the number and duration of anhydrobiosis episodes, more individuals were active in groups after rehydration when compared to single animals (Fig. 4). Based on the mean values, the number of active individuals experiencing repeated long anhydrobiosis individually was 20% lower than in groups, and for short anhydrobiosis, the difference between groups and individuals was 6%. However, the Eta-squared indicates that the proportion of variance explained by the interaction was low (Eta-squared equal to 0.03).

3.5. Effect of sex

The main effect of sex was statistically significant ($p < 0.05$; Table 1). However, there was no general trend to conclude that one sex had a higher number of active individuals after rehydration than the other one. Significant differences between females and males in the number of active individuals were only observed at 2 h and 6 h after rehydration for the repeated short and long anhydrobiosis, respectively. We also found no significant differences between the sexes when analysing the different age classes, with one exception concerning short anhydrobiosis for the oldest age class at 2 h after rehydration (Fig. 5).

The interaction between observation time, age and sex of individuals appeared to be significant ($F(12,300) = 2.537$, $p < 0.05$), meaning that both females and males differed in the number of active individuals between older and younger age classes, particularly at the later observation time (24–48 h) after rehydration. Other interactions where sex was one of the main factors were not significant (Table 1). Mean numbers of active individuals representing females and males in the context of age, combination of the number and duration of anhydrobiosis episodes and observation time after rehydration (2–48 h) are presented in Supplementary file, Table S7.

4. Discussion

The present study demonstrates the potential of the newly-described tardigrade species *P. experimentalis* to return to activity after repeated short and long anhydrobiosis. This potential was investigated by the determination of the activity of individuals after rehydration (defined here as coordinated movements of a body and legs, i.e., the onset of crawling) concerning their age, sex, and whether they occurred in the presence of other individuals (i.e., in a group) or not (i.e., single) during anhydrobiosis steps. The study also focuses on the combined effect of the number and duration of anhydrobiosis episodes. Some of these factors have not been studied before (e.g., the effect of sex) or only limited knowledge of them was previously available. The impact of these factors was evaluated to guide future research on tardigrades anhydrobiosis and to provide a more comprehensive characterisation of the species under study. In a broader sense, this study may also contribute to the validation of two hypotheses proposed to explain anhydrobiosis effect on aging, i.e., "the Sleeping Beauty" or "the Picture of Dorian Gray" [4,14,17,46].

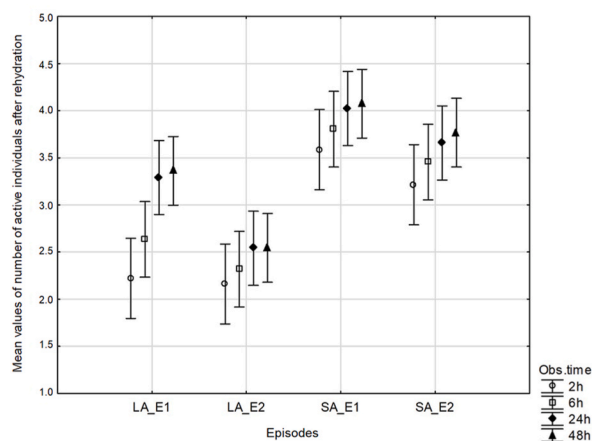


Fig. 3. Mean values of number of active individuals of *Paramacrobiotus experimentalis* observed at 2 h–48 h after rehydration following the first two episodes of short and long anhydrobiosis. LA_E1 and LA_E2, the first and second episode of long anhydrobiosis; SA_E1 and SA_E2, the first and second episode of short anhydrobiosis; Obs. time - observation time. Expected marginal means and 0.95% confidence intervals are presented. In total of 1200 individuals were analysed, 600 (300 females and 300 males) each in short and long anhydrobiosis episodes with the same duration of dehydration and rehydration steps (see Table S2 raw data). Results of One-way ANOVA analysis are presented in Table S5.

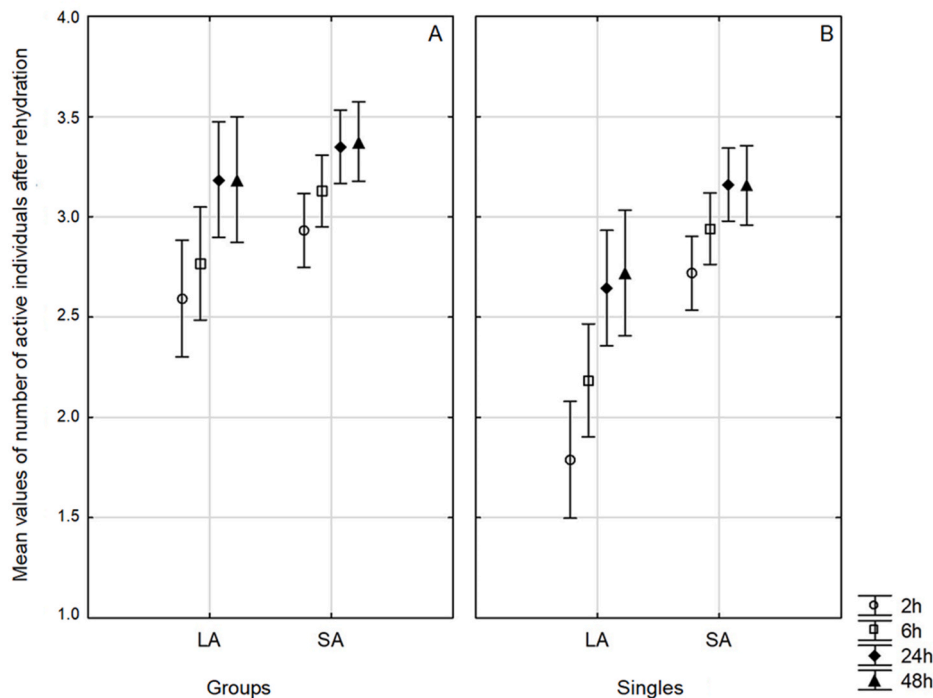


Fig. 4. Mean values of number of active individuals of *Paramacrobiotus experimentalis* according to the combined effect of number and duration of anhydrobiosis episodes, and observation times after rehydration (2 h–48 h; $F(3,300) = 2.687, p = 0.047$). LA and SA, long and short anhydrobiosis, respectively; Obs. time - observation time, panels in the figure represents individuals kept in groups (A) and as singles (B). Expected marginal means and 95% confidence intervals are shown. In total of 1200 individuals were analysed, 600 (300 females and 300 males) each in short and long anhydrobiosis episodes with the same duration of dehydration and rehydration steps (see [Table S2](#) for raw data). Results of Multivariate repeated measures analysis of variance (RM_ANOVA) are presented in [Table S6](#).

The main findings of the study can be summarised as follows [1]: recovery from anhydrobiosis declined with age [2]; regardless of age, the number of active individuals decreased significantly with increasing number and duration of anhydrobiosis episodes, but increased with observation time after rehydration [3]; some individuals were able to return to activity after five short or two long anhydrobiosis episodes [4]; individuals in groups returned to activity after rehydration more efficiently than those treated individually [5]; sex appeared to be a significant predictor of the number of active individuals after rehydration, but the effect size was very small. The results indicate that three of the most important factors influencing return to activity after anhydrobiosis are age, combination of the number and duration of anhydrobiosis episodes and the group influence (i.e., the influence of other individuals' presence). These factors could be considered in studies of anhydrobiosis in tardigrades. The impact of sex on return to activity after rehydration requires further research.

The lifespan of tardigrades varies between species, populations and individuals, and ranges from a few weeks to two years, not counting the time spent in the dormant state [17,47–49]. Accordingly, the most obvious finding to emerge from the analysis performed is that young adults of *P. experimentalis* (age in days 120–150) showed the best recovery from repeated anhydrobiosis. In contrast, the recovery was the worst for the oldest individuals (age in days >300). Undoubtedly, the lower vitality rate determined for older animals (Supplementary file, [Table S1](#)) increases the chance of death that could happen also during each of the anhydrobiosis steps, i.e., dehydration, tun state and rehydration. However, the data in [Table S2](#) (Supplementary file) indicate that following rehydration the number of active animals did not decrease for consecutive observation times that allows for conclusion that the effect of sudden death during rehydration step is not significant also for older animals. The finding that younger animals recover better from anhydrobiosis than older ones supports the results of other studies linking the age of individuals with their ability to recover from anhydrobiosis. In *Milnesium tardigradum*, higher recovery rates after anhydrobiosis were observed for younger individuals (from 37 to 149 days old) than for older ones (163–191 days old) [31]. Similarly, studies on the nematode *Panagrolaimus rigidus* showed that increasing age had a negative effect on recovery from anhydrobiosis [46]. However, the ability of *M. tardigradum* embryos to survive anhydrobiosis increased with age [50] and for *Ramazzottius varieornatus*, no difference in the recovery was observed between eggs, juvenile animals and adult animals. As far as body size is concerned, the recovery from anhydrobiosis was shown to increase in *Richtersius coronifer* with an increase of the body size and to decrease in *Ramazzottius oberhaeuseri* although these species live in the same moss and have high anhydrobiosis capability [51]. However, for *R. coronifer*, the capability was also shown to increase till medium-size of the body and decrease at large body size ranges [30]. Assuming a correlation between body size and age [30,48], younger individuals would recover from anhydrobiosis better than older ones, but this may apply to limited age ranges. Accordingly, in our experiments, young adults

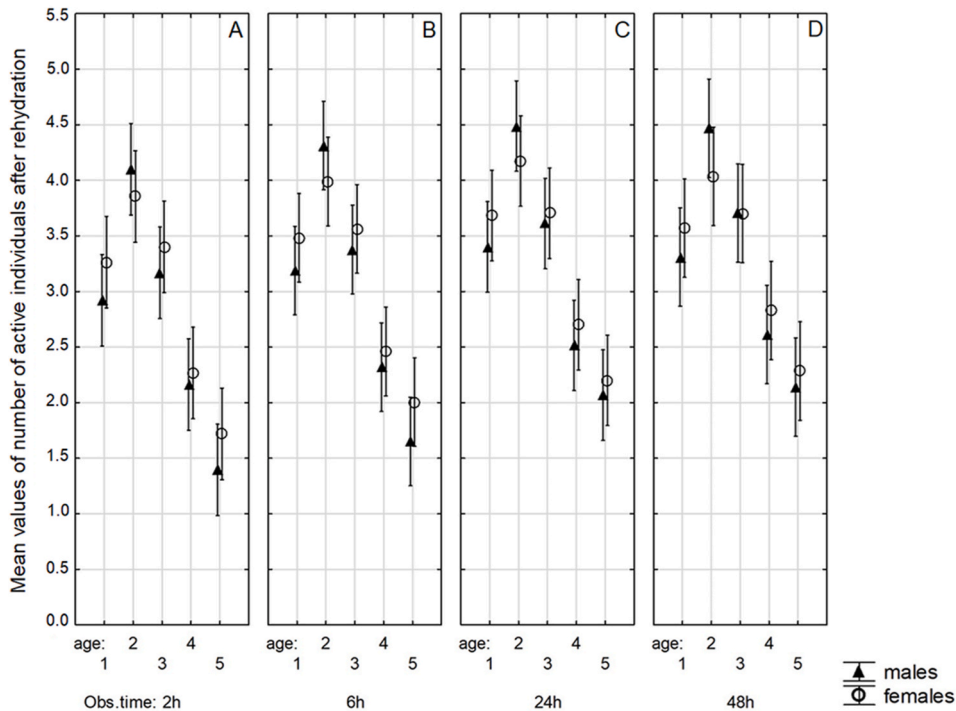


Fig. 5. Mean values of number of active individuals of *Paramacrobriotes experimentalis* in the context of their sex and age, as well as observation time after rehydration. Age [1–5]; the selected age classes; Obs. time; observation times. Panels in the figure represents the different observation time i.e., 2 h (A), 6 h (B), 24 h (C) and 48 h (D). Expected marginal means and 95% confidence intervals are shown; see the Materials and methods section for details. Obs. time - observation time. In total of 1200 individuals were analysed, 600 (300 females and 300 males) each in short and long anhydrobiosis episodes with the same duration of dehydration and rehydration steps (see Table S2 for raw data). Results of Multivariate repeated measures analysis of variance (RM_ANOVA) are presented in Table S7.

(age in days 120–150), bigger than growing adults (age in days 60–90), showed a better return to activity after rehydration than the latter. However, in the study, the difference in the recovery after anhydrobiosis was also observed between animals of comparable size but representing different age classes. Moreover, animals representing growing adults that differed in size (age in days 30–60 and 60–90) did not differ in the recovery after anhydrobiosis (our unpublished results). This observation concerned both females and males. Therefore, we did not analyse the body size effect on the recovery and its combination with sex.

Among the tardigrades tested, ability of anhydrobiosis varies over a wide range [9,14,17,33]. This may be related to abiotic factors, such as the moisture content in the natural environment, the innate ability of the animal to recover and specific conditions for entry into anhydrobiosis and rehydration [7]. Species living in constantly moist habitats tend to have a lower ability to tolerate drought by anhydrobiosis than those living in dry environments [11,52]. It has been suggested that the upper limit of recovery from anhydrobiosis by tardigrades can be counted in years but does not exceed ten years [53]. However, other available data indicate that some tardigrade species can return to activity when the tun (desiccated) state lasts for up to 15–22 years (e.g., [54]). For semi-terrestrial tardigrades, also represented by *P. experimentalis*, the ability of anhydrobiosis has been studied, among others, for the eutardigrade *Ram. oberhaeuseri* and the heterotardigrade *Echiniscus* spp. It was shown that under natural conditions *Ram. oberhaeuseri* recovered from the tun state lasting 1192 days with an average revival of 21.7% but could survive in this state for up to 1604 days. In *Echiniscus* spp., after 706 days of anhydrobiosis, the average revival was 9.9%, but the species could tolerate the tun state lasting for up to 1085 days [8].

Because of the different species tested and the methods used, our study cannot be directly compared with previous studies. Differences include, among others, the number and duration of anhydrobiosis episodes. In the case of *P. experimentalis*, significant differences in the number of active individuals after repeated anhydrobiosis persisted during the initial observation times after rehydration (2–6 h), but became insignificant over time, indicating important differences in the rate of recovery. The observed return to activity at 48 h after repeated short and long anhydrobioses was consistent with our previous experiments on *P. experimentalis* showing that this species has a high capacity for anhydrobiosis, as the average recovery rate of individuals after 240 days of tun state was 43% [33]. However, representatives of other populations of the species would need to be studied to verify correlations between the conditions of the natural environment and anhydrobiosis ability for this species. Previous studies are contradictory regarding population differences in tardigrades' recovery from anhydrobiosis, which is explained by the intraspecific variation of physiology and/or habitat properties [6,33,55].

We found a significant positive correlation, at least for the initial observation time after rehydration, between return to activity and combination of the number and duration of anhydrobiosis episodes, confirming previous findings in nematodes [56] and various

tardigrade species (e.g., [7,14,25,33,35,38]). Namely, the longer the tun state, the more time the animals need to return to activity. We also showed that some individuals could recover after five repeated short or two repeated long anhydrobiosis episodes. This finding for *P. experimentalis* is generally consistent with that for the tardigrade *Mil. tardigradum* and *Ric. coronifer*, which can survive up to six and even nine consecutive short anhydrobiosis episodes, respectively [31,35]. For the three species, decreasing ability to form proper tuns was only observed for *Ric. coronifer* and the authors suggest that the decrease in anhydrobiotic performance could be explained by the lack of animal feeding between episodes [35]. Accordingly, the feeding was applied in the studies of *Mil. tardigradum* [31] and in our study of *P. experimentalis*. However, it cannot be excluded that other factors may contribute to the difference, including the time between anhydrobiosis episodes, their duration, studied species or the source of specimens, i.e. laboratory culture or environment. Nevertheless, the significant difference in the number of active individuals after rehydration between two long anhydrobiosis episodes and the lack of the difference between two short episodes supports the crucial influence of the duration of the tun state on recovery from repeated anhydrobiosis. Accordingly, it is generally accepted that the duration of the dry state is decisive for recovery from anhydrobiosis (e.g., [14]), but it has also been proposed that recovery from cryptobiosis may be influenced by factors acting during dehydration and rehydration [57], making the distinction between the effects of the number and duration of anhydrobiosis episodes more complex.

The effect of the presence of individuals in a group on recovery from anhydrobiosis has not been widely studied although it has been shown to be related to aggregations [58]. Namely, it has been shown in the tardigrade *Ric. coronifer* that aggregations of individuals can improve the survival of anhydrobiosis because may contribute to a reduction in the body surface area exposed to desiccation and, thus, to a reduction in the rate of water evaporation, thereby increasing the chance of return to activity. This is assumed to be of a crucial meaning for survival of rapid desiccation [58]. From an ecological point of view, the positive consequences of individuals aggregation and its role in animal recovery was highlighted by Jönsson [6], who noted that population density could promote aggregation and *vice versa*. This might explain, for example, the distribution and abundance of tardigrade species in xerothermic habitats [6].

The performed studies showed that tardigrades in the presence of other individuals (i.e., in groups) recovered from anhydrobiosis better than single individuals. However, we did not observe typical aggregations of individuals (Supplementary file, Fig. S1). Therefore, it appears that under certain conditions, formation of the aggregates is not indispensable for successful anhydrobiosis. The decisive factor maybe be the amount of water applied during tun formation and/or the rate of dehydration [52] as well as the size of dehydration area and the amount of treated animals. We can also speculate that the presence of other individuals could be a source of chemical signals that could be released in response to dehydration and/or rehydration, and enhance *P. experimentalis* recovery from anhydrobiosis. Neither the nature of the signals nor their relationship to the age of individuals is known. Nevertheless, research on the mating behaviour of tardigrades illustrate the role of chemical communication between tardigrades [59,60].

On the other hand, the lack of the aggregates appears not to decrease the return to activity after the first episode of short anhydrobiosis when the age class displaying the highest level of the recovery is concerned (for details, see, Supplementary file, Table S2). For the age class 120–150 days (young adults), the return to activity after the first episode of short anhydrobiosis (3 days) was comparable with data on the return from single short anhydrobiosis episode observed for the species [33] as well as for other species (e.g., [61]) including another *Paramacrobiotus* species [62]. Importantly, in the reports the applied dehydration procedures differed from that applied in our study. Moreover, females and males belonging to different age classes were not studied, and the issue of aggregation was not considered. However, for long anhydrobiosis (30 days) the differences appeared to occur, also for the age class showing the highest recovery rate (for details, see, Supplementary file, Table S2). Namely, the recovery after the first episode of long anhydrobiosis (about 65%) was lower than reported for the same species single anhydrobiosis episode of the same duration (close to 90%) [33]. Because, the difference observed for females and males from the age class 120–150 days was not pronounced and the issue of aggregation was not considered in Ref. [33], the difference requires further study to be explained.

Although the effect of sex was statistically significant, a general trend for females to recover better from anhydrobiosis than males was not observed. This aspect requires further research because cannot be simply correlated with the calculated 2:1 female-biased sex ratio. Similar value of the female-biased sex ratio (2:1) was reported for *Paramacrobiotus* sp. TYO [59]. In both species the ratio was determined for animals not undergoing anhydrobiosis and in the case of *P. experimentalis* in all age classes. Additionally, we observed only a marginally lower maximum lifespan for *P. experimentalis* males (400 days) when compared to females (420 days). The female-biased sex ratio is an inevitable issue for considerations on the evolution of sexual reproduction that still remains a fascinating enigma in biology. It is usually assumed that males are costly, and their cost can be reduced by decreasing the ratio of males to females [63]. This appears to be a rule in tardigrades. Accordingly, although sex ratio close to 1:1 was noted in tardigrades of the genus *Macrobiotus* C.A.S. [64], in *Ramazzottius* sp. the equal sex ratio was only found in a limited number of samples, and generally, a female-biased sex ratio was observed [65].

5. Conclusions

The most significant predictor of recovery from repeated anhydrobiosis in *P. experimentalis* is the age of the individual. The combination of number and duration of anhydrobiosis episodes, and the presence of other individuals are secondary predictors. Although there was a little evidence for the effect of sex on the recovery, this factor should be further analysed in different populations of this species and other bisexual tardigrade species. The analysed predictors may help to understand the molecular and cellular mechanisms governing tardigrade anhydrobiosis and the response of these mechanisms to environmental stress. This research may also be helpful in the context of evolutionary adaptations and responses to droughts caused by climate change and water shortage.

Data availability statement

All the data generated within this manuscript are available within the paper and the Supplementary file. They are also available within bioRxiv, an open access preprint repository (doi.org/10.1101/2023.05.22.541721). Nevertheless, any data will be made available upon request from the corresponding author.

Ethical approval

Samples of *P. experimentalis* were collected according to research permission from the Direction Generale des Forests, Direction de la Valorisation des Ressources Forestieres, Antananarivo, Madagascar (autorisations de recherche: No: 260/15-MEEMF/SG/DGF/DCAP/SCBT and Service de la Gestion Faune et Flore No: 056N-EA03/MG18).

CRediT authorship contribution statement

Amit Kumar Nagwani: Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Data curation, Conceptualization. **Iwona Melosik:** Writing – review & editing, Writing – original draft, Validation, Methodology, Data curation. **Łukasz Kaczmarek:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology. **Hanna Kmita:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.heliyon.2024.e26807>.

References

- [1] M. Watanabe, Anhydrobiosis in invertebrates, *Appl. Entomol. Zool.* 41 (2006) 15–31, <https://doi.org/10.1303/aez.2006.15>.
- [2] L. Rebecchi, T. Altiero, R. Anhydrobiosis Guidetti, The extreme limit of desiccation tolerance, *Invertebr. Surviv. J.* 4 (2007) 65–81.
- [3] L. Rebecchi, Dry up and survive: the role of antioxidant defences in anhydrobiotic organisms, *J. Limnol.* 72 (2013) 62–72, <https://doi.org/10.4081/jlimnol.2013.s1.e8>.
- [4] Ł. Kaczmarek, et al., Staying young and fit? Ontogenetic and phylogenetic consequences of animal anhydrobiosis, *J. Zool.* 309 (2019) 1–11, <https://doi.org/10.1111/jzo.12677>.
- [5] K. Arakawa, Examples of extreme survival: tardigrade genomics and molecular anhydrobiology, *Annu. Rev. Anim. Biosci.* 10 (2022) 17–37, <https://doi.org/10.1146/annurev-animal-021419-083711>.
- [6] K.I. Jönsson, The nature of selection on anhydrobiotic capacity in tardigrade, *Zool. Anz.* 240 (2001) 409–417, <https://doi.org/10.1078/0044-5231-00049>.
- [7] J.C. Wright, Desiccation tolerance and water-retentive mechanisms in tardigrades, *J. Exp. Biol.* 142 (1989) 267–292, <https://doi.org/10.1242/jeb.142.1.267>.
- [8] L. Rebecchi, R. Guidetti, S. Borsari, T. Altiero, R. Bertolani, Dynamics of long-term anhydrobiotic survival of lichen-dwelling tardigrades, *Hydrobiologia* 558 (2006) 23–30, <https://doi.org/10.1007/s10750-005-1415-7>.
- [9] M. Roszkowska, et al., Tolerance to anhydrobiotic conditions among two coexisting tardigrade species differing in life strategies, *Zool. Stud.* 60 (2021) 74, <https://doi.org/10.6620/ZS.2021.60-74>.
- [10] R.O. Schill, et al., Molecular mechanisms of tolerance in tardigrades: new perspectives for preservation and stabilization of biological material, *Biotechnol. Adv.* 27 (2009) 348–352, <https://doi.org/10.1016/j.biotechadv.2009.01.011>.
- [11] R. Guidetti, A.M. Rizzo, T. Altiero, T. Rebecchi, What can we learn from the toughest animals of the Earth? Water bears (tardigrades) as multicellular model organisms in order to perform scientific preparations for lunar exploration, *Planet. Space Sci.* 74 (2012) 97–102, <https://doi.org/10.1016/j.pss.2012.05.021>.
- [12] M. May, L'évolution des tardigrades de la vie aquatique à la vie Terrestre, *Bull. Fr. Peche Piscic.* 168 (1953) 93–100.
- [13] D.R. Nelson, R. Guidetti, L. Rebecchi, in: H. James, D. Thorp, C. Rogers (Eds.), *Phylum Tardigrada in Thorp and Covich's Freshwater Invertebrates*, Academic Press, Cambridge, 2015, pp. 347–380, <https://doi.org/10.1016/B978-0-12-385026-3.00017-6>.
- [14] R.O. Schill, S. Hengherr, in: R. Schill (Ed.), *Environmental Adaptations: Desiccation Tolerance in Water Bears: the Biology of Tardigrades*, Zoological Monographs, Springer, Switzerland, 2018, pp. 273–293, https://doi.org/10.1007/978-3-319-95702-9_10.
- [15] J.D. Hibshman, J.S. Clegg, B. Goldstein, Mechanisms of desiccation tolerance: themes and variations in brine shrimp, roundworms, and tardigrades, *Front. Physiol.* 11 (2020) 592016, <https://doi.org/10.3389/fphys.2020.592016>.
- [16] L. Soemme, Anhydrobiosis and cold tolerance in tardigrades, *Eur. J. Entomol.* 93 (1996) 349–358.

- [17] A.K. Nagwani, Ł. Kaczmarek, H. Kmita, Applicable life-history and molecular traits for studying the effects of anhydrobiosis on aging in tardigrades, *Diversity* 14 (2022) 664, <https://doi.org/10.3390/d14080664>.
- [18] R. Bertolani, Evolution of the reproductive mechanisms in tardigrades—a review, *Zool. Anz.* 240 (2001) 247–252, <https://doi.org/10.1078/0044-5231-00032>.
- [19] K. Sugiura, M. Matsumoto, Sexual reproductive behaviours of tardigrades: a review, *Invertebr. Reprod. Dev.* 65 (2021) 279–287, <https://doi.org/10.1080/07924259.2021.1990142>.
- [20] J.H. Crowe, The physiology of cryptobiosis in tardigrades, *Mem. Ist. Ital. Idrobiol.* 32 (1975) 37–59.
- [21] J.C. Wright, P. Westh, H. Ramløv, Cryptobiosis in tardigrada, *Biol. Rev.* 67 (1992) 1–29, <https://doi.org/10.1111/j.1469-185X.2013.tb01657.x>.
- [22] R. Guidetti, T. Altiero, R. Bertolani, P. Grazioso, L. Rebecchi, Survival of freezing by hydrated tardigrades inhabiting terrestrial and freshwater habitats, *Zool.* 114 (2011) 123–128, <https://doi.org/10.1016/j.zool.2010.11.005>.
- [23] D.D. Horikawa, in: A. Altenbach, J. Bernhard, J. Seckbach (Eds.), *Survival of Tardigrades in Extreme Environments: A Model Animal for Astrobiology in Anoxia: Evidence for Eukaryote Survival and Paleontological Strategies*, vols. 205–217, Springer Netherlands, Dordrecht, 2012, https://doi.org/10.1007/978-94-007-1896-8_12.
- [24] I. Giovannini, T. Altiero, R. Guidetti, L. Rebecchi, Will the Antarctic tardigrade *Acutuncus antarcticus* be able to withstand environmental stresses related to global climate change? *J. Exp. Biol.* 221 (2018) jeb160622, <https://doi.org/10.1242/jeb.160622>.
- [25] R. Guidetti, R.O. Schill, R. Bertolani, T. Dandekar, M. Wolf, New molecular data for tardigrade phylogeny, with the erection of *Paramacrobotus* gen. nov., *J. Zool. Syst. Evol.* 47 (2009) 315–321, <https://doi.org/10.1111/j.1439-0469.2009.00526.x>.
- [26] Y. Yoshida, et al., Comparative genomics of the tardigrades *Hypsibius dujardini* and *Ramazzottius varieornatus*, *PLoS Biol.* 15 (2017) e2002266, <https://doi.org/10.1371/journal.pbio.2002266>.
- [27] A. Jørgensen, R.M. Kristensen, N. Møbjerg, in: R. Schill (Ed.), *Phylogeny and Integrative Taxonomy of Tardigrada in Water Bears: the Biology of Tardigrades*, vols. 95–114, Zoological Monographs, Springer, Switzerland, 2018, https://doi.org/10.1007/978-3-319-95702-9_3.
- [28] C. Hesgrove, T.C. Boothby, The biology of tardigrade disordered proteins in extreme stress tolerance, *Cell Commun. Signal.* 18 (2020) 1–15, <https://doi.org/10.1186/s12964-020-00670-2>.
- [29] K.I. Jönsson, Tardigrades as a potential model organism in space research, *Astrobiology* 7 (2007) 757–766, <https://doi.org/10.1089/ast.2006.0088>.
- [30] K.I. Jönsson, L. Rebecchi, Experimentally induced anhydrobiosis in the tardigrade *Richtersius coronifer*: phenotypic factors affecting survival, *J. Exp. Zool.* 293 (2002) 578–584, <https://doi.org/10.1002/jez.10186>.
- [31] S. Hengherr, F. Brümmer, R.O. Schill, Anhydrobiosis in tardigrades and its effects on longevity traits, *J. Zool.* 275 (2008) 216–220, <https://doi.org/10.1111/j.1469-7998.2008.00427.x>.
- [32] N. Møbjerg, R.C. Neves, New insights into survival strategies of tardigrades, *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 254 (2021) 110890, <https://doi.org/10.1016/j.cbpa.2020.110890>.
- [33] M. Roszkowska, et al., How long can tardigrades survive in anhydrobiotic state? Searching on tardigrade anhydrobiosis patterns, *PLoS One* 18 (2023) e0270386, <https://doi.org/10.1371/journal.pone.0270386>.
- [34] P. Degma, R. Guidetti, Actual Checklist of Tardigrada Species, 09-01-2023) (2009, 42th Edition, 2023, <https://iris.unimore.it/retrieve/bf8e14a4-625f-4cdd-8100-347e5cb5f63/Actual%20checklist%20of%20Tardigrada%2042th%20Edition%2009-01-23.pdf>. (Accessed 15 October 2022).
- [35] M. Czernekova, K.I. Jönsson, Experimentally induced repeated anhydrobiosis in the eutardigrade *Richtersius coronifer*, *PLoS One* 11 (2016) e0164062, <https://doi.org/10.1371/journal.pone.0164062>.
- [36] I. Giovannini, T.C. Boothby, M. Cesari, B. Goldstein, G. Guidetti, L. Rebecchi, Production of reactive oxygen species and involvement of bioprotectants during anhydrobiosis in the tardigrade *Paramacrobotus spatialis*, *Sci. Rep.* 12 (1938) (2022), <https://doi.org/10.1038/s41598-022-05734-6>.
- [37] A.C. Suzuki, Appearance of males in a thelytokous strain of *Milnesium cf. tardigradum* (Tardigrada), *Zool. Sci.* 25 (2008) 849–853, <https://doi.org/10.2108/zsj.25.849>.
- [38] S. Neumann, A. Reuner, F. Brümmer, R.O. Schill, DNA damage in storage cells of anhydrobiotic tardigrades, *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 153 (2009) 425–429, <https://doi.org/10.1016/j.cbpa.2009.04.611>.
- [39] I.M. Kinchin, *The Biology of Tardigrades*, Portland Press, London, 1994, <https://doi.org/10.1017/s002531540001540x>, 1–186.
- [40] Ł. Kaczmarek, et al., Integrative description of bisexual *Paramacrobotus experimentalis* sp. nov. (Macrobiotidae) from republic of Madagascar (Africa) with microbiome analysis, *Mol. Phylogenet. Evol.* 145 (2020) 106730, <https://doi.org/10.1016/j.ympev.2019.106730>.
- [41] A.K. Nagwani, A. Budka, A. Łacka, Ł. Kaczmarek, H. Kmita, The effect of hypomagnetic field on survival and mitochondrial functionality of active *Paramacrobotus experimentalis* females and males of different age, *Front. Physiol.* 14 (2023) 1253483, <https://doi.org/10.3389/fphys.2023.1253483>.
- [42] H.W. Lilliefors, On the Kolmogorov-Smirnov Test for normality with mean and variance unknown, *J. Am. Stat. Assoc.* 62 (1967) 399–402, <https://doi.org/10.1080/01621459.1967.10482916>.
- [43] H. Levene, in: I. Olkin, S.G. Ghurye, W. Hoeffding, W.G. Madow, H.B. Mann (Eds.), *Robust Tests for Equality of Variances in Contributions to Probability and Statistics*, vols. 278–292, Stanford University Press, Stanford, 1960.
- [44] J.C. Keselman, L.M. Lix, H.J. Keselman, The analysis of repeated measurements: a quantitative research synthesis, *Br. J. Math. Stat. Psychol.* 49 (1996) 275–298, <https://doi.org/10.1111/j.2044-8317.1996.tb01089>.
- [45] R. Bender, S. Lange, Adjusting for multiple testing - when and how? *J. Clin. Epidemiol.* 54 (2001) 343–349, [https://doi.org/10.1016/S0895-4356\(00\)00314-0](https://doi.org/10.1016/S0895-4356(00)00314-0).
- [46] C. Ricci, M. Pagani, Desiccation of *Panagrolaimus rigidus* (Nematoda): survival, reproduction and the influence on the internal clock, *Hydrobiologia* 347 (1997) 1–13, <https://doi.org/10.1023/a:1002979522816>.
- [47] A.C. Suzuki, Life history of *Milnesium tardigradum* Doyère (Tardigrada) under a rearing environment, *Zool. Sci.* 20 (2003) 49–57, <https://doi.org/10.2108/zsj.20.49>.
- [48] R.O. Schill, Life-history traits in the tardigrade species *Paramacrobotus kenianus* and *Paramacrobotus palaui*, *J. Limnol.* 72 (2013) 160–165, <https://doi.org/10.4081/jlimnol.2013.s1.e20>.
- [49] K.I. Jönsson, R. Bertolani, Facts and fiction about long-term survival in tardigrades, *J. Zool.* 255 (2001) 121–123, <https://doi.org/10.1017/s0952836901001169>.
- [50] R.O. Schill, G.B. Fritz, Desiccation tolerance in embryonic stages of the tardigrade, *J. Zool.* 276 (2008) 103–107, <https://doi.org/10.1111/j.1469-7998.2008.00474.x>.
- [51] L. Rebecchi, C. Boschetti, D.R. Nelson, Extreme-tolerance mechanisms in meiofaunal organisms: a case study with tardigrades, rotifers and nematodes, *Hydrobiologia* 847 (2020) 2779–2799, <https://doi.org/10.1007/s10750-019-04144-6>.
- [52] I. Poprawa, et al., Verification of *Hypsibius exemplaris* Gąsiorek et al., 2018 (Eutardigrada; Hypsibiidae) application in anhydrobiosis research, *PLoS One* 17 (2022) e0261485, <https://doi.org/10.1371/journal.pone.0261485>.
- [53] R. Guidetti, K.I. Jönsson, Long-term anhydrobiotic survival in semi-terrestrial micrometazoans, *J. Zool.* 257 (2002) 181–187, <https://doi.org/10.1017/s095283690200078x>.
- [54] M. Roszkowska, H. Kmita, Ł. Kaczmarek, Long-term anhydrobiosis in two taxa of moss dwelling Eutardigrada (Tardigrada) desiccated for 12 and 15 years, respectively, *Eur. Zool. J.* 87 (2020) 642–647, <https://doi.org/10.1080/24750263.2020.1829110>.
- [55] D.D. Horikawa, S. Higashi, Desiccation tolerance of the tardigrade *Milnesium tardigradum* collected in Sapporo, Japan, and Bogor, Indonesia, *Zool. Sci.* 21 (2004) 813–816, <https://doi.org/10.2108/zsj.21.813>.
- [56] K.A.C. Madin, J.H. Crowe, Anhydrobiosis in nematodes: carbohydrate and lipid metabolism during dehydration, *J. Exp. Zool.* 193 (1975) 335–342, <https://doi.org/10.1002/jez.1401930309>.
- [57] J. Sieger, F. Brümmer, H. Ahn, G. Lee, S. Kim, R.O. Schill, Reduced ageing in the frozen state in the tardigrade *Milnesium inceptum* (Eutardigrada: apochela), *J. Zool.* 318 (2022) 253–259, <https://doi.org/10.1111/jzo.13018>.
- [58] H. Ivarsson, K.I. Jönsson, Aggregation effects on anhydrobiotic survival in the tardigrade *Richtersius coronifer*, *J. Exp. Zool. A Ecol. Integr. Physiol.* 301 (2004) 195–199, <https://doi.org/10.1002/jez.a.20018>.

- [59] K. Sugiura, H. Minato, A.C. Suzuki, K. Arakawa, T. Kunieda, M. Matsumoto, Comparison of sexual reproductive behaviors in two species of Macrobiotidae (Tardigrada: eutardigrada), *Zool. Sci.* 36 (2) (2019) 120–127, <https://doi.org/10.2108/zs180103>.
- [60] S. Bartel, K. Hohberg, Experimental investigations on the partner-finding behaviour of *Isohypsibius dastychi* (Isohypsibiidae: tardigrada), *Zool. J. Linn. Soc.* 188 (3) (2020) 878–886, <https://doi.org/10.1093/zoolinnean/ziz076>.
- [61] T.C. Boothby, et al., Tardigrades use intrinsically disordered proteins to survive desiccation, *Mol. Cell.* 65 (6) (2017) 975–984, <https://doi.org/10.1016/j.molcel.2017.02.018>.
- [62] Y. Hara, et al., Parallel evolution of trehalose production machinery in anhydrobiotic animals via recurrent gene loss and horizontal transfer, *Open Biol* 11 (7) (2021) 11200413, <https://doi.org/10.1098/rsob.200413>.
- [63] K. Kobayashi, E. Hasegawa, A female-biased sex ratio reduces the twofold cost of sex, *Sci. Rep.* 6 (1) (2016) 23982, <https://doi.org/10.1038/srep23982>.
- [64] R. Bertolani, Sex ratio and geographic parthenogenesis in *Macrobiotus* (Tardigrada), *Experientia* 28 (1972) 94–95, <https://doi.org/10.1007/bf01928285>.
- [65] R. Bertolani, L. Rebecchi, G. Beccaccioli, Dispersal of *Ramazzottius* and other tardigrades in relation to type of reproduction, *Invertebr. Reprod. Dev.* 18 (1990) 153–157, <https://doi.org/10.1080/07924259.1990.9672137>.