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

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Abstract: 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, occurrence in a group and the combination of the number and duration of anhydrobiosis episodes on the recovery of *Paramacrobiotus experimentalis*. The activity of 1,200 individuals for up to 48 hours after rehydration was evaluated using ANOVA. Age was the main factor influencing return to activity, followed by the number and duration of anhydrobiosis episodes, group influence, 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, more individuals were active after rehydration in groups than individually. 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 *Pam. experimentalis* and provide background for evolutionary, molecular and environmental mechanisms of anhydrobiosis ability.

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].

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., 13–15], 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 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 a group on the return to activity of the individual tardigrade after anhydrobiosis 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) group influence (group *vs.* single individuals) and (4) combination of the number of anhydrobiosis episodes, and their duration, termed here “anhydrobiosis duration”. The term "anhydrobiosis duration" refers to the duration of the tun state. 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 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 and cellular mechanisms and their response to environmental stress.

MATERIALS AND METHODS

Cultures of Paramacrobiotus experimentalis

Females and males of *Pam. 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 ddH_2O 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 (24h), and with relative humidity (RH) of 40%.

On the basis of life history traits, five age classes, defined as growing, young, mature, late and old adults, were distinguished (Supplementary file, Table S1). 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. For the selected age classes an approximate ratio of 2:1 females to males was defined.

Protocol for repeated episodes of anhydrobiosis

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-minute observation under the stereomicroscope (Supplementary file, Figure 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 2h, 6h, 24h, and 48h. 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 *Pam. 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) 48h after rehydration were subjected to another anhydrobiosis episode. The number of active individuals after each anhydrobiosis episode and at a given observation time 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 Figure 1. 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 groups (10 specimens per Petri dish) or singly (one individual in each of 10 Petri dishes). In total of 1,200 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).

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 effect, the age and sex of the individuals, and the interaction between these factors. Details of the data transformation and statistical tests used to assess the ANOVA assumptions are in Supplementary file. 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 (2h-48h), 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 [41]. 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).

RESULTS

General remarks

The number of active individuals of *Pam. experimentalis* 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 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) (Supplementary file, Table S3).

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 (Figure 2; Supplementary file, Table S3). 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).

Effect of age

Based on the post hoc tests, 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 number and duration of anhydrobiosis episodes (Figure 2–3). Taking into account the mean values, the younger age classes had, on average, 37% more active individuals after rehydration than the older age classes (Supplementary file, Table S4).

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 (2h) and mainly for individuals representing the younger age classes (Figure 2 and post hoc tests (not shown)). The differences diminished with the time of observation and became insignificant at 48h after rehydration. Based on the mean values at 48h after rehydration, the young adults (age class 2) returned to activity 52% and 49% faster than the old adults (age class 5) after repeated long and short anhydrobiosis, respectively (Supplementary file, Table S4). The growing adults (age class 1) returned to activity 10% and 19% slower, the mature adults (age group 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 S4).

Effect 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 (Figure 2–3). 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 2h and 6h after rehydration. Although still positive, these correlations became statistically insignificant as rehydration time progressed (observations at 24–48h; Supplementary file, Table S5).

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% to 36% more active individuals were observed after short anhydrobiosis than after long anhydrobiosis, depending on the observation time (Supplementary file, Table S6). Based on post hoc tests, significant differences were found between short and long anhydrobiosis in the number of active individuals after rehydration at each observation time (2–48h; one-way ANOVA, Wilk's Lambda = 0.544, ($F(4,75) = 15.728$, $p<0.001$), Figure 4).

The comparison between consecutive short anhydrobiosis episodes showed no statistically significant differences in the number of active individuals, except a significant difference at 48h after rehydration between the fourth and fifth episode (Supplementary file, Figure S2).

Effect of group influence

Grouping of individuals during anhydrobiosis significantly influenced the number of active individuals after rehydration ($p<0.001$, Supplementary file, Table S3). 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 S7). Irrespective of observation time and duration of anhydrobiosis, more individuals were active in groups than individually after rehydration (Figure 5). 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).

Effect of sex

The main effect of sex was statistically significant ($p<0.05$; Supplementary file, Table S3). 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 2h and 6h 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 2h after rehydration (Figure 6).

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 (24–48h) after rehydration. Other interactions where sex was one of the main factors were not significant (Supplementary file, Table S3). Mean numbers of active individuals representing females and males in the context of age, anhydrobiosis duration and observation time after rehydration (2–48h) are presented in Supplementary file, Table S8.

DISCUSSION

The present study demonstrates the potential of the newly-described tardigrade species *Pam. 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 a group or individually 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., ”Sleeping Beauty” or ”The Picture of Dorian Gray” [4, 14, 17, 42].

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, the number and duration of anhydrobiosis episodes and the group influence. These factors should be considered in studies of anhydrobiosis in tardigrades and other anhydrobiotic animals. 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, 43-45]. Accordingly, the most obvious finding to emerge from the analysis performed is that young adults of *Pam. 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). This finding 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 to 191 days old) [31]. However, the ability of embryos to survive anhydrobiosis increased with age in this species [46]. In the population of the *Richtersius coronifer* from Sweden, the body size affected the survival of anhydrobiosis, i.e., larger individuals showed a lower probability of return to activity than medium-sized ones [30]. Assuming a correlation between body size and age [47], younger individuals would recover from anhydrobiosis better than older ones, but this may apply to limited age ranges. Accordingly, in our experiments, young adults (age in days 120–150) showed a better return to activity after rehydration than growing adults (age in days 60–90). Thus, our results are somewhat consistent with data for the bdelloid rotifer *Macrotrachela quadricornifera*, for which adult individuals showed better recovery from anhydrobiosis than juveniles and eggs [48, 49]. Similarly, studies on the nematode *Panagrolaimus rigidus* showed that increasing age had a negative effect on recovery from anhydrobiosis [42].

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, 50]. 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 [51]. 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., 52]. For semi-terrestrial tardigrades, also represented by *Pam. experimentalis*, the ability of anhydrobiosis has been studied, among others, for the eutardigrade *Ramazzottius 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 *Pam. experimentalis*, significant differences in the number of active individuals after repeated anhydrobiosis persisted during the initial observation times after rehydration (2–6h), but became insignificant over time, indicating important differences in the rate of recovery. The observed return to activity at 48h after repeated short and long anhydrobioses was consistent with our previous experiments on *Pam. 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 using a common protocol to detect 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, 53].

We found a significant positive correlation, at least for the initial observation time after rehydration, between return to activity and the number and duration of anhydrobiosis episodes, confirming previous findings in nematodes [54] 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 is generally consistent with that for the tardigrade *Ric. coronifer*, which can survive up to six repeated short anhydrobiosis episodes [35], albeit with a decreasing ability to form proper tuns. The decline was not observed between consecutive anhydrobiosis episodes in the present study, probably because animals were fed

between episodes [35]. 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 anhydrobiosis may be influenced by factors acting during dehydration and rehydration [55], making the distinction between the effects of the number and duration of anhydrobiosis episodes more complex.

The effect of the grouping of individuals (which may be related to aggregations) on recovery from anhydrobiosis has not been widely studied and is not fully understood in tardigrades [e.g., 56], although such a phenomenon is well-known in nematodes [e.g., 57]. It has also been shown in the tardigrade *Ric. coronifer* that aggregations of individuals can improve the survival of anhydrobiosis [56]. 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 groups recovered from anhydrobiosis better than single individuals. Undoubtedly, aggregations of animals 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. However, we did not observe typical aggregations of individuals with minimal distances between them (Supplementary file, Figure S1). Therefore, we can only speculate that the presence of other individuals could be a source of factors enhancing *Pam. experimentalis* recovery from anhydrobiosis, such as chemical signals that could be released in response to dehydration. However, neither the nature of the signals nor their relationship to the age of individuals is known. Nevertheless, research on the mating behaviour of *Isohypsibius dastychi* may suggest the possibility of chemical communication between tardigrades [58].

A sex ratio close to 1:1 was noted in tardigrades of the genus *Macrobiotus* C.A.S. [59], but 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 [60]. The female-biased sex ratio (2:1), noted in *Pam. experimentalis,* might indicate that males may be less able to return to activity after rehydration than females, particularly in environments with at least occasional high dehydration pressure. 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, therefore, requires further research.

CONCLUSIONS

The most significant predictor of recovery from repeated anhydrobiosis in *Pam. experimentalis* is the age of the individual. The number and duration of anhydrobiosis episodes, and the presence of individuals in groups are secondary predictors. Although there was a little evidence for the effect of sex on the recovery, this factor should be further analyzed in different populations of this species. Further research should also focus on a standardized research protocol for tardigrade anhydrobiosis, which should include components such as culture medium, conditions of dehydration, observation time of recovery, and sample size. Such a protocol will allow for a comprehensive comparison of experimental results, including other bisexual tardigrade species. Greater control over the analyzed predictors and the experimental conditions 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.

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Author contributions

Conceptualization, H.K. and A.K.N.; data curation, A.K.N and I.M; investigation, A.K.N. and I.M.; methodology, Ł.K., H.K. and I.M.; statistical analysis, I.M.; validation, I.M., H.K., and Ł.K.; supervision, H.K. and Ł.K.; writing, I.M., H.K., Ł.K., and A.K.N. All authors accepted the final version of the manuscript.

Data Availability Statement

All relevant data are within the paper and the Supplementary file.

Ethical approval

Samples of *Pam. 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).

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figures and captions

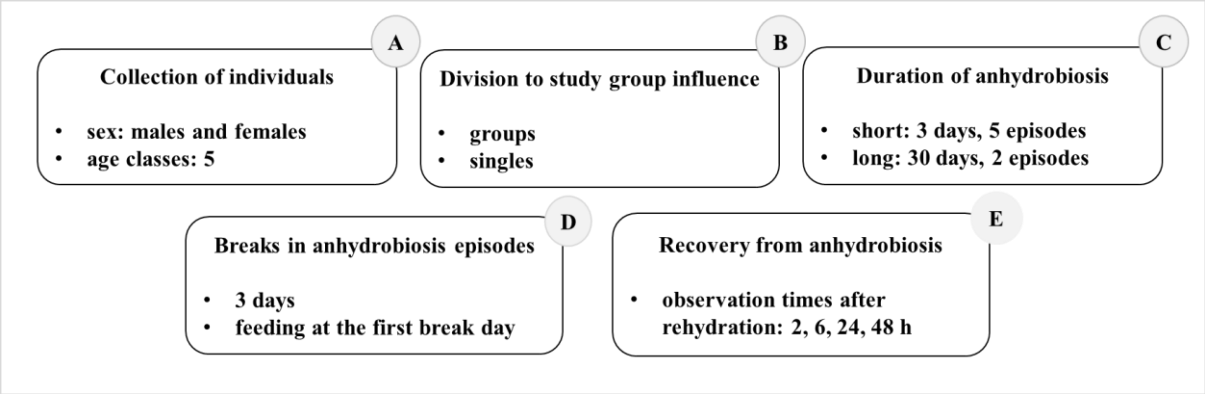

Figure 1. Graphic representation of the repeated anhydrobiosis experiment performed in *Paramacrobiotus experimenatlis.* A-E, the applied steps of the experiments. The term "anhydrobiosis duration" refers to the duration of the tun state.

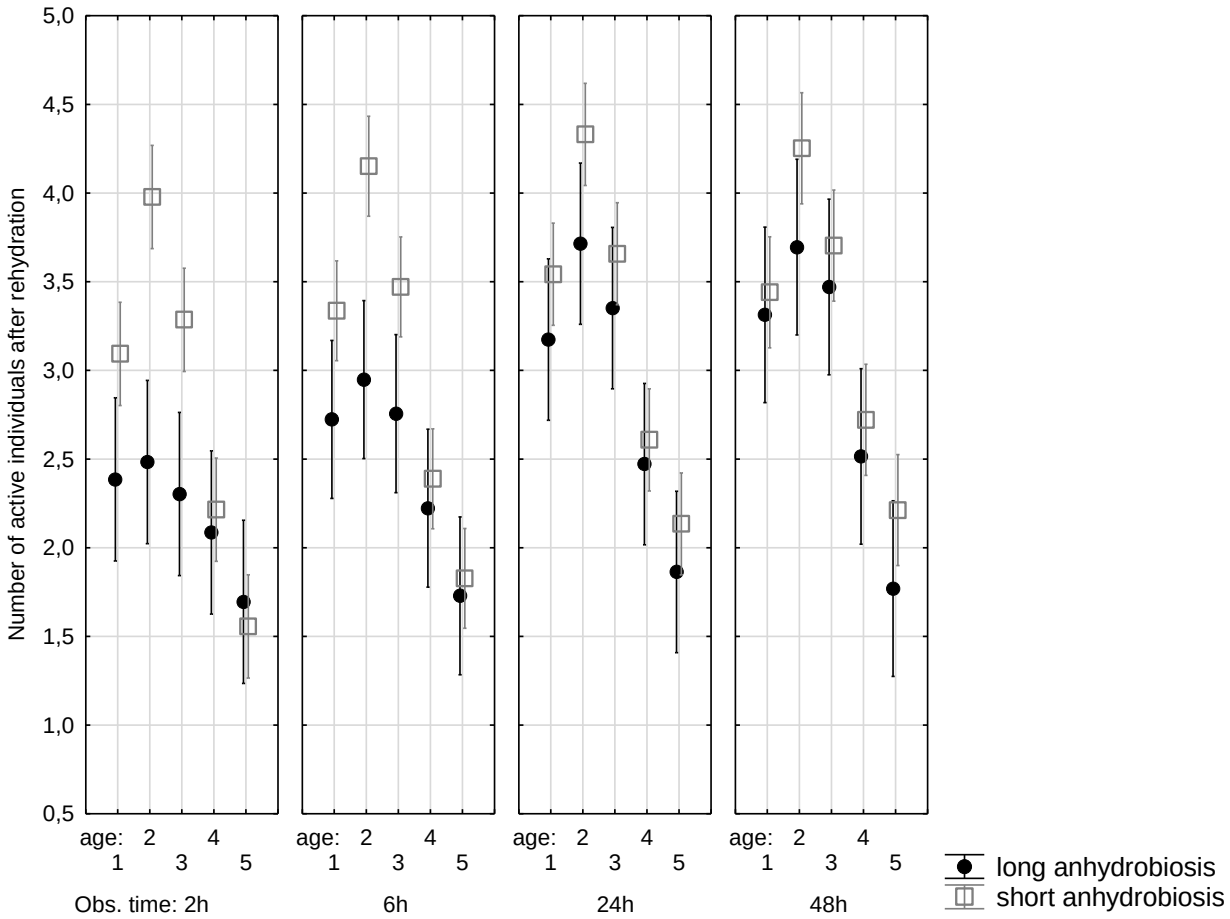

Figure 2. Number of active individuals of *Paramacrobiotus experimentalis* observed at 2h-48h after rehydration, taking into account their age and the combined effect of the number and duration of anhydrobiosis episodes (long and short anhydrobiosis). Age (1-5); the selected age classes; Obs. time - observation time. Expected marginal means and 0.95% confidence intervals are presented.

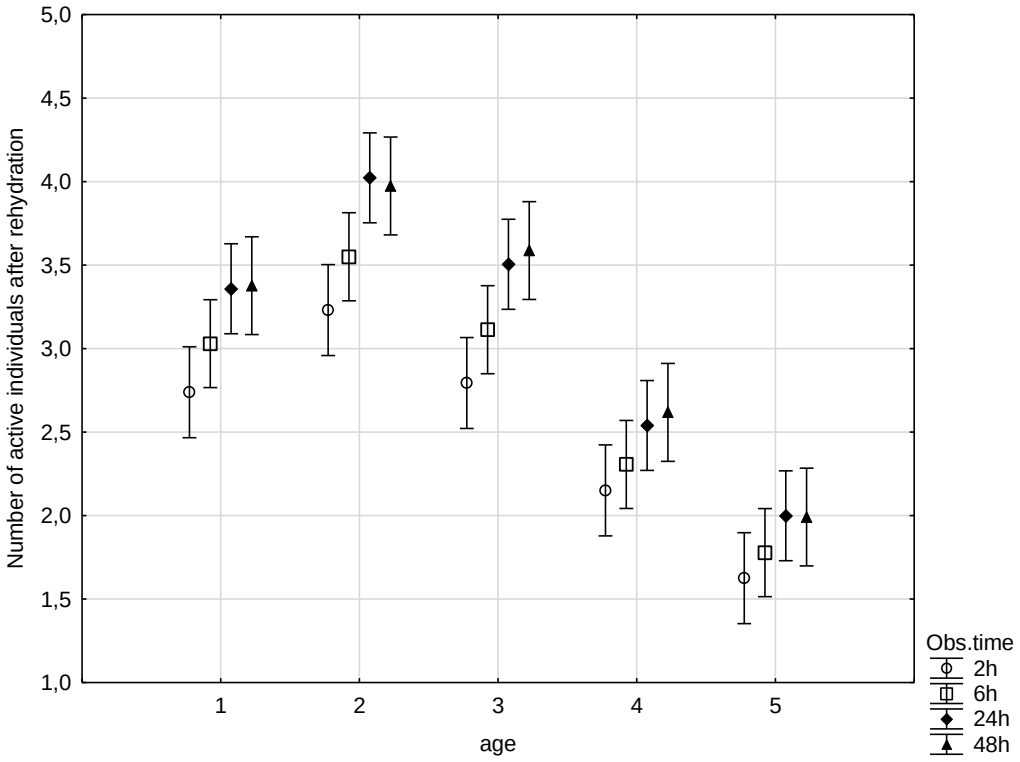

Figure 3. Number of active individuals of *Paramacrobiotus experimentalis* observed at 2h-48h after rehydration in age classes. Long and short anhydrobiosis were considered together. Age (1-5); the selected age classes; Obs. time - observation time. Expected marginal means and 95% confidence intervals are presented.

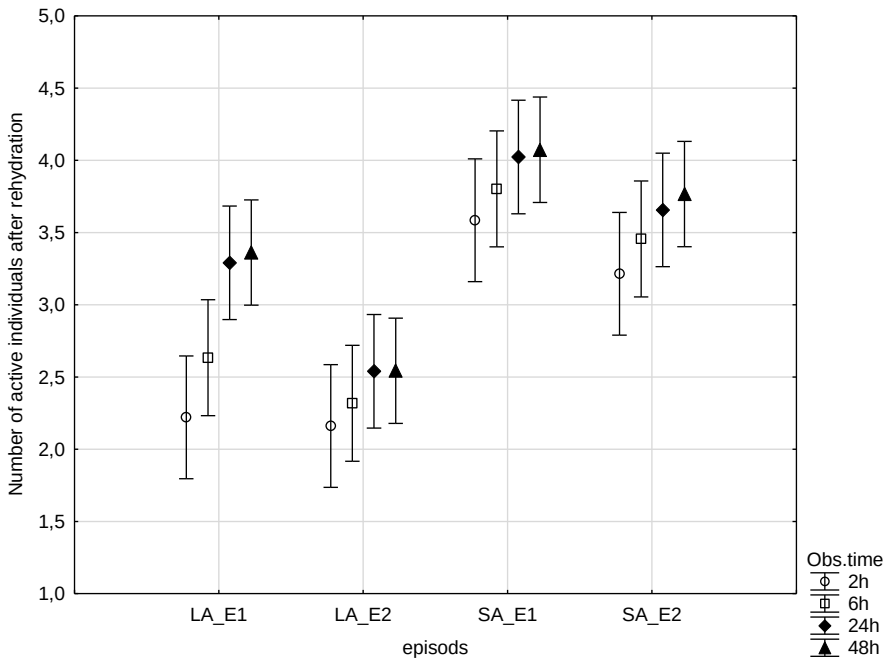

Figure 4. Number of active individuals of *Paramacrobiotus experimentalis* observed at 2h-48h after rehydration following the first two episodes of short and long anhydrobiosis. LA_E1 and LA_E2, the first and second episode of long anhydrobiosis; SA_E1 and SA_E2, the first and second episode of short anhydrobiosis; Obs. time - observation time.

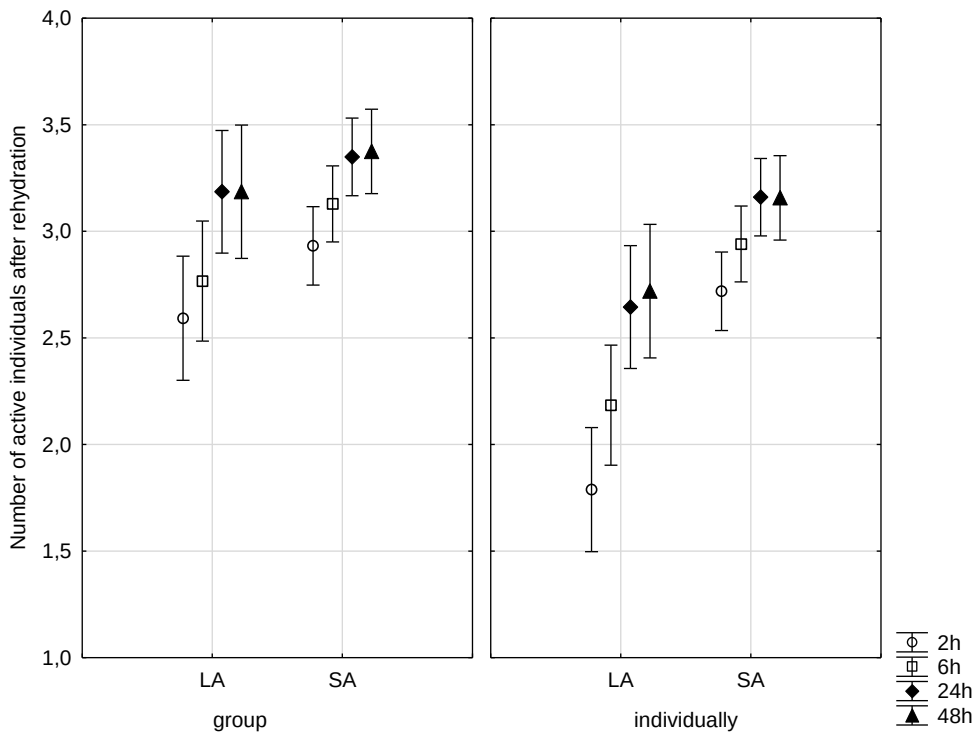

Figure 5. Number of active individuals of *Paramacrobiotus experimentalis* according to the combined effect of number and duration of anhydrobiosis episodes, and observation times after rehydration (2h-48h; F(3, 300)=2.687, *p*=0.047). LA and SA, long and short anhydrobiosis, respectively; Obs. time - observation time. Expected marginal means and 95% confidence intervals are shown.

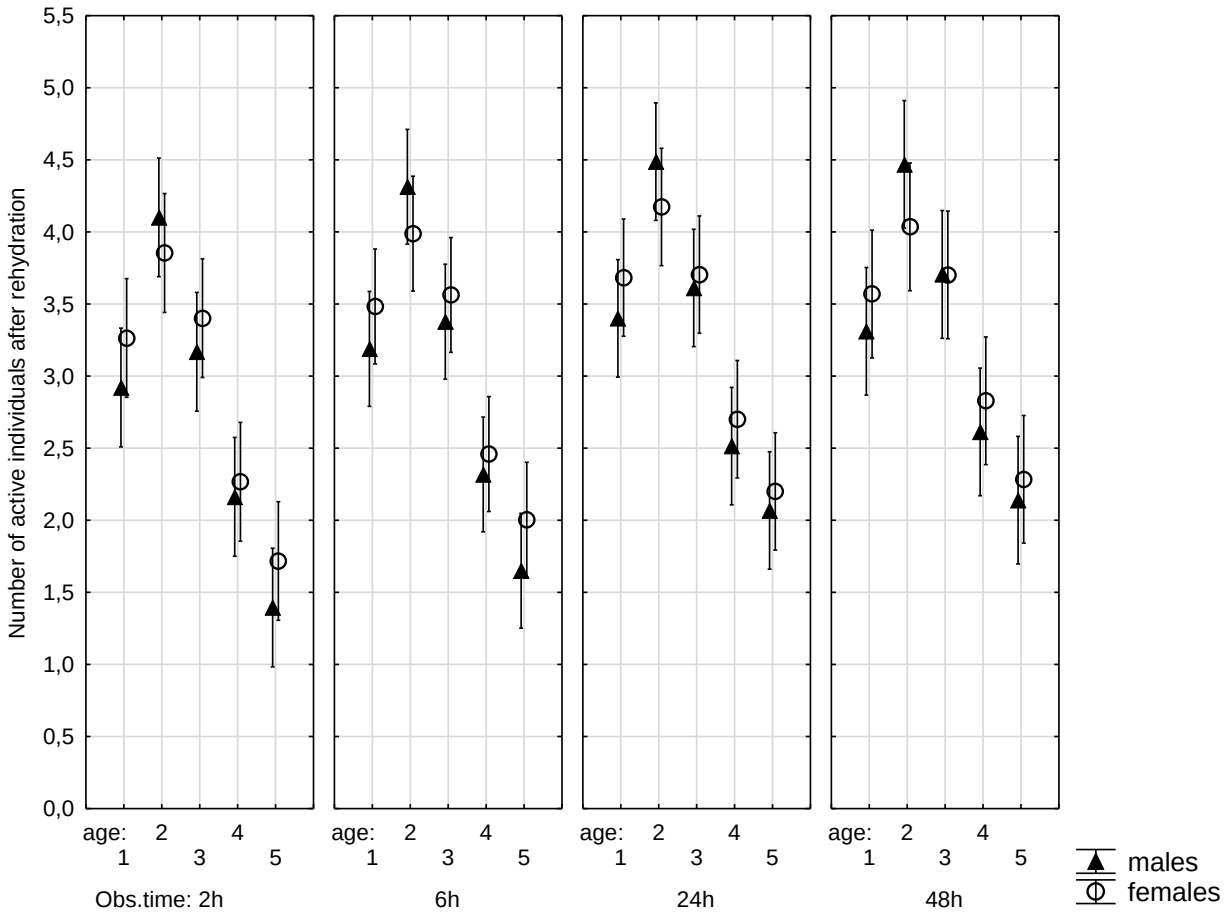

Figure 6. Number of active individuals of *Paramacrobiotus experimentalis* in the context of their sex and age, as well as, observation time after rehydration. Age (1-5); the selected age classes; Obs. time; observation times. Expected marginal means and 95% confidence intervals are shown; see the Materials and methods section for details. Obs. time - observation time.