Uniwersytet Adama Mickiewicza w Poznaniu
Wydział Biologii
Instytut Biologii Molekularnej i Biotechnologii
Zakład Bioenergetyki

Rozprawa doktorska

Poszukiwanie markerów starzenia się niesporczaka *Paramacrobiotus experimentalis*

Amit Kumar Nagwani

Promoter: prof. dr. hab. Hanna Kmita
Promoter: prof. UAM dr hab. Łukasz Kaczmarek

Poznań, Poland 2023

Adam Mickiewicz University in Poznan
Faculty of Biology
Institute of Molecular Biology and Biotechnology
Department of Bioenergetics

Doctoral Thesis

Searching on aging markers of the tardigrade *Paramacrobiotus experimentalis*

Amit Kumar Nagwani

Promoter: prof. dr hab. Hanna Kmita
Promoter: prof. UAM dr hab. Łukasz Kaczmarek

Poznań, Poland 2023

ACKNOWLEDGEMENT

It is a genuine pleasure to express my deep sense of thanks and gratitude to my advisor, Professor Hanna Kmita for the continuous support of my Ph.D. study and research, for her understanding, support, patience, motivation, enthusiasm, and immense knowledge. Her guidance helped in all the time of research, writing project grants as well as in writing this thesis. I could not have imagined having a better advisor and mentor for my Ph.D. study.

Prof. Łukasz Kaczmarek for providing me laboratory work space at Department of Animal Taxonomy and Ecology (Faculty of Biology, Adam Mickiewicz University, Poznan, Poland) in the initial years of my Ph.D. and for his constructive discussions, and overall support.

I would like to thank Professor Izabela Poprawa (from University of Silesia in Katowice, Poland) for her significant contribution in planning of my experiments, technical help in analyzing the results, constructive discussion and for overall support.

Prof. Małgorzata Wojtkowska for giving me opportunity to teach the theoretical and practical aspects of Basic Molecular methods to Masters and Bachelors students at the Faculty. I would also like to thank for her invaluable help, encouragement and inspiration.

Wojciech Kwiatkowski for giving me hands-on training with microscopes and for his technical guidance in other instruments-related aspects.

Importantly, I would like to thank all my colleagues, co-workers, professors, administrators and support staff from the Adam Mickiewicz University including special thanks to Dr. Karolina Cerbin and Mrs. Arleta Kucz for their immense administrative support throughout my Ph.D. studies. A very special thanks to all the people from Department of Bioenergetics, specially to Dr. Andonis Karachitos and Dr. Anna Kicińska for their invaluable help, scientific as well as social support, encouragement, and inspiration.

I would also like to thank all members of our collaborator group of Professor Thomas C. Boothby at University of Wyoming, WY, USA.

A very special thanks to my Indian as well as Polish friends in Poznań who all played a pivotal role for my integration into the Polish culture.

With all my heart, I would like to thank my beloved parents, my very close friends and my seniors back in India for their love, care and support.

Dedicated to my mother

Content

Streszczenie

Nieodłączną cechą organizmów żywych jest złożony proces biologicznego starzenia się. Zakłada się, że starzenie się jest nieuniknione, nieodwracalne i postępujące. Nawet w przypadku niesporczaków, znanych jako jedne z najbardziej odpornych zwierząt na Ziemi, można zaobserwować starzenie się, znajdujące odzwierciedlenie w zmianach w ich przeżywalności i sprawności. Dostępne dane wskazują jednak, że starzenie się niesporczaków może zostać opóźnione przez kryptobiozę, która oznacza zdolność do przetrwania ekstremalnych warunków środowiskowych. Anhydrobioza wydaje się być najbardziej znaną formą kryptobiozy i opisywana jest jako zdolność do wznowienia aktywności po całkowitym odwodnieniu (wyschnięciu). Możliwość tę uwzględnia hipoteza Śpiącej Królewny. Weryfikacja tej hipotezy, może mieć istotne konsekwencje aplikacyjne, wymaga jednak zastosowania wielopoziomowych markerów starzenia się niesporczaków. Markery te nie są jednakże znane. Dlatego celem tej pracy doktorskiej było wskazanie potencjalnych markerów starzenia się niesporczaków.

Aby zrealizować ten cel, przeszukano dostępne publikacje pod względem możliwych markerów, co pozwoliło na wskazanie następujących markerów: przeżywalność, średnia liczba złożonych jaj przez samicę, przeżywalność warunków ekstremalnych (tj. anhydrobioza czy pole hipomagnetyczne – ang. HMF), poziom potencjału błony wewnętrznej mitochondriów ($\Delta\psi$) i poziom wewnątrzkomórkowych reaktywnych form tlenu (ROS). Do doświadczalnej walidacji markerów zastosowano odpowiedni model tj. dwupłciowy gatunek niesporczaka *Paramacrobiotus experimentalis,* charakteryzujący się długą średnią długością życia i dużą zdolnością do anhydrobiozy. Model ten tworzą reprodukujące się samice i samce w różnym wieku, tj. przypisane do pięciu różnych klasach wiekowych. W przeprowadzonych badaniach po raz pierwszy określono wpływ na przeżywalność różnej liczby epizodów anhydrobiozy o różnym czasie trwania dla pięciu klas wiekowych samic i samców, odwadnianych i nawadnianych przy braku lub w obecności innych osobników, tj. w grupach. Ponadto zbadano wpływ ekspozycji na HMF na przeżycie i funkcjonalność mitochondriów u samic i samców w różnym wieku, a także zbadano poziom wewnątrzkomórkowych ROS u samic i samców w różnym wieku.

Weryfikacja przydatności wymienionych markerów starzenia się niesporczaków może przyczynić się do wykorzystania niesporczaków także jako organizmów modelowych w

biologii starzenia się. Wraz z weryfikacją hipotezy Śpiącej Królewny może to przyczynić się do opracowania strategii przeciwstarzeniowych i skutecznych rozwiązań dotyczących konserwacji materiałów biologicznych. Niemniej jednak uzyskane wyniki należy poddać dalszej weryfikacji w odniesieniu do innych dwupłciowych gatunków niesporczaków, co pozwoli na lepsze zrozumienie obserwowanych różnic międzypłciowych.

Abstract

The complex process of biological aging is an intrinsic feature of living organisms. It is assumed that aging is inevitable, irreversible, and progressive. Even for tardigrades, known as one of the toughest animals on Earth, aging can be observed due to changes in their survival rate and fitness. However, available data indicate that tardigrade aging might be delayed by cryptobiosis that denotes ability to withstand environmental extremes. Anhydrobiosis appears to be the most known form of cryptobiosis (also in tardigrades) and is described as the ability to resume activity after complete dehydration (desiccation). The possibility is addressed by the Sleeping Beauty hypothesis. The hypothesis verification, which could have important applicative consequences, requires multilevel markers of tardigrade aging. However, the markers are not available. Therefore, the aim of the thesis was to indicate potential tardigrade aging markers.

To implement the aim available relevant papers were searched for the putative markers which resulted in selection of the vitality rate, average number of laid eggs per female, survival of the extreme conditions (i.e., anhydrobiosis or hypomagnetic field – HMF), the level of the mitochondrial inner membrane potential ($\Delta\psi$) and the level of intracellular reactive oxygen species (ROS). For experimental validation of the markers a suitable model was developed due to application of the dioecious tardigrade *Paramacrobiotus experimentalis* characterized by long average lifespan and high anhydrobiosis capability. The model consists of reproducing females and males of different age, classified to five different age classes. In the studies, for the first time the effect of different number and duration of anhydrobiosis episodes on survival was performed for five age classes of females and males, dehydrated and rehydrated as single individuals or in the presence of other individuals, i.e., in groups. In addition, the effect of exposure to HMF on survival and mitochondria functionality was studied for females and males of different age as well as the level of intracellular ROS for females and males of different age was detected.

Verification of usability of the mentioned tardigrade aging markers could open the opportunity for tardigrades to be also a model organism for aging biology, applicable for testing of aging theories. Together with verification of the Sleeping Beauty hypothesis, this might contribute to developing of anti-aging strategies and efficient approaches for preservation of biological materials. Nevertheless, the obtained results should be further verified for other

dioecious tardigrade species which will allow for a better understanding of the observed differences between the sexes.

LIST OF SCIENTIFIC ARTICLES INCLUDED IN THE DISSERTATION

Review article

1. Nagwani AK., Kaczmarek Ł., Kmita H. (2022) Applicable life-history and molecular traits for studying the effects of anhydrobiosis on aging in tardigrades.
   Diversity, 14(8): 664. https://doi.org/10.3390/d14080664
   MEiN (July 2023) points – 70
   Journal Impact factor (2023) – 3.031

Research articles

1. Nagwani AK., Melosik I., Kaczmarek Ł., Kmita H. (2023) Recovery from anhydrobiosis in the tardigrade *Paramacrobiotus experimentalis*: better to be young than old and in a group than alone.
   bioRxiv (Preprint). https://doi.org/10.1101/2023.05.22.541721
   Under review in Heliyon
   MEiN (July 2023) – 40
   Journal Impact factors (2023) – 4.000

2. Nagwani NK., Budka A., Łacka A., Kaczmarek Ł., Kmita H. (2023) The effect of hypomagnetic field on survival and mitochondrial functionality of active *Paramacrobiotus experimentalis* females and males of different age.
   Frontiers in Physiology, 14: 1253483. https://doi.org/10.3389/fphys.2023.1253483
   MEiN (July 2023) – 100
   Journal Impact factor (2023) – 4.000

ABBREVIATIONS

μT	Microtesla
Δψ	Mitochondrial inner membrane potential
SMF	Standard magnetic field
GMF	Geo-magnetic field
HMF	Hypomagnetic field
CIMF	Chamber Isolated from Magnetic Field
TMRM	Tetramethylrhodamine, methyl ester
DAPI	4′,6-diamidino-2-phenylindole
FCCP	Carbonyl-cyanide-p-Trifluoromethoxyphenylhydrazone
FI_{TMRM}	TMRM fluorescence index
ROS	Reactive oxygen species
$DCFH_2$-DA	2′7′-dichlorofluorescein diacetate

IMPORTANT ACHIEVEMENTS DURING Ph.D. STUDIES

Scientific projects:

1. Received the doctoral mini-grant 2021/2022 from the part of "Initiative of Excellence-Research University Programme (ID_UB) at Adam Mickiewicz University (Uniwersytet im. Adama Mickiewicza w Poznaniu, Inicjatywa Doskonalosci – Uczelnia Badawcza 017/02/SNP/0006) for the proposed project: "Is the impact of hypomagnetic conditions on tardigrades dependent on the animal age and sex?"
2. Received the pre-doctoral grant from National Science center in Poland (Narodowe Centrum Nauki – PRELUDIUM 20: 2021/41/N/NZ3/01165) for the proposed project: "Do the storage cell lipids contribute to tardigrade aging in the presence or absence of anhydrobiosis?" in 2021.

Selection for oral presentation of results at scientific meetings:

1. At "15th International Symposium on Tardigrada", Krakow, Poland, (2022).
   Title: Factors affecting survival of repeated anhydrobiosis in dioecious tardigrade *Paramacrobiotus experimentalis* Kaczmarek et al., 2020
2. At "13th Targeting Mitochondria" organized by World Mitochondrial Society, Berlin, Germany, (2022).
   Title: Hypomagnetic field and its impact on mitochondrial functioning in the tardigrade *Paracmacrobiotus experimentalis* Kaczmarek et al., 2020
3. At "Lech Wojtczak (LW) 10th Mitochondrion meeting", organized by Polish Biochemical Society, Warsaw, Poland, (2023).
   Title: Functionality of the tardigrade *Paramacrobiotus experimentalis* mitochondria under a hypomagnetic field
4. At "*Mipschool* 2023" organized by Mitochondrial Physiology Society, Obergurgl, Austria, (2023).
   Title: Mitochondria functionality in tardigrades under the hypomagnetic field.

Training/internship as part of scientific collaborations:

1. One-month internship (August 2023) at Professor Thomas C. Boothby's laboratory at Department of Molecular Biology, University of Wyoming, WY, USA.
   Project: Estimation of level of ROS production in active females and males tardigrade of different age.

Published articles not directly linked with PhD thesis:

1. Tanneru N., Nivya M.A., Adhikari N., Saxena K., Rizvi Z., Sudhakar R., Nagwani A.K., Al-Nihmi F.M., Kumar K.A., Sijwali P.S. (2023) Plasmodium DDI1 is a potential therapeutic target and important chromatin-associated protein.
   International Journal for Parasitology. 53(3):157-75. https://doi.org/10.1016/j.ijpara.2022.11.007
   MEiN (July 2023): 100
   Journal Impact factor (2023) – 4.000

2. Kayastha P., Rzymski P., Gołdyn B., Nagwani A.K., Fiałkowska E., Pajdak-Stós A., Sobkowiak R., Robotnikowski G., Kaczmarek Ł. (2023) Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates.
   Zoological Journal of the Linnean Society. 22: zlad060. https://doi.org/10.1093/zoolinnean/zlad060
   MEiN (July 2023): 140
   Journal Impact factor (2023) – 2.800

Funding

1. The Polish National Science Centre grant, NCN OPUS 2016/21/B/NZ4/00131 (PI Prof. Hanna Kmita).
2. The Polish National Science Centre grant, NCN PRELUDIUM 2021/41/N/NZ3/01165 (PI Amit Kumar Nagwani).
3. The European Union: Passport to the future - Interdisciplinary doctoral studies at the Faculty of Biology, Adam Mickiewicz University. POWR.03.02.00-00-I022/16-17.
4. ID_UB grant number 017/02/SNP/0006. (PI Amit Kumar Nagwani).

DESCIPTION OF THE RESULTS OF DOCTORAL THESIS

1. Scientific profile of the PhD candidate

I started my Bachelor studies in Biotechnology at the MATS University in Raipur, Chhattisgarh, India, in 2010. My Bachelor thesis entitled "Antimicrobial and antifungal activity of ashwagandha (*Withania somnifera*) and amla (*Phyllanthus emblica*)" was performed at niTza Biological, Hyderabad, Telangana, India. In 2014, I started my master studies in Microbiology and Bioinformatics at Bilaspur University (renamed as Atal Bihari Vajpayee University) in Bilaspur, Chhattisgarh, India. I prepared my master thesis in the Computational Biology Laboratory in the same institution. The title of the thesis was "Docking study on *W. somnifera* secondary metabolites on Tetrahydrodipicolinate N-Succinyltransferase protein involved in the lysine biosysnthesis pathway in *Pseudomonas aeruginosa*". The results were published in research article "A Docking Study on Various Secondary Metabolites from *W. somnifera* on Tetrahydrodipicolinate NSuccinyltransferase Protein Involved in The Lysine Biosysnthesis Pathway in *P. aeruginosa*" (2018) in "Molecular Biology: Open access" (DOI: 10.4172/2168-9547.1000214).

After my Master, I joined Centre for Cellular and Molecular Biology (CCMB)- a research institute of Council of Scientific and Industrial research (CSIR) Hyderabad) as junior research fellow and worked on a project related to characterization of malarial proteins (i.e., DNA-damage induced (DDI) and neural precursor cell expressed developmentally down-regulated protein 8 (NEDD 8)). The results on DDI protein were published in research article "Plasmodium DDI1 is a potential therapeutic target and important chromatin-associated protein" (2023) in "International Journal of Parasitology" (https://doi.org/10.1093/zoolinnean/zlad060). Furthermore, after my tenure at CCMB, I joined National Institute of Animal Biotechnology (a research institute of Department of Biotechnology (DBT)) in Hyderabad as junior research fellow and worked on a project related to the "Generation of a potential vaccine candidate via studying the regulation and expression of virulence factors of pathogenic strains of bacterium Leptospira."

In December 2018, I started my PhD studies at the Adam Mickiewicz University in Poznań under the supervision of Prof. dr. hab. Hanna Kmita. I joined a project concerning testing of anhydrobiosis as tardigrade anti-aging strategy, which involved verification of one of the proposed hypothesis termed the "Sleeping beauty" hypothesis. In meantime, I also participated in another project within the activities of BARg (Biodiversity and Astrobiology Research group) at Adam Mickiewicz University in Poznań. Namely, I participated in

investigation of tardigrade tolerance against the magnesium perchlorate (known to be present in Martian regolith). The obtained results were published in research article "Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates" (2023) in "Zoological Journal of the Linnean Society" (https://doi.org/10.1093/zoolinnean/zlad060).

During my PhD studies I also had the opportunity to present my results as posters (seven) and oral presentations (four) at scientific conferences (both national and international). The list of oral presentations is given above (see "Important achievements during Ph.D. studies"), the list of posters presented during scientific meetings is as follows:

1. At "BioLOGIES: Passport to the future" Faculty of Biology, Adam Mickiewicz University in Poznań, Poland (2019).
   Title: Preliminary data on life-history traits of newly described *Paramacrobiotus* sp. (*richtersi* group) from Madagascar.
2. At the "44th Federation of European Biochemical Societies (FEBS) Congress" Krakow, Poland (2019).
   Title: Life history and mitochondrial traits in aging research: Preliminary studies on *Paramacrobiotus* sp. (*richtersi* group).
3. At BioLOGIES: Passport to the future" Faculty of Biology, Adam Mickiewicz University in Poznań, Poland (online conference: 2020).
   Title: Aging research on tardigrades: Description of life history and cellular traits of *Paramacrobiotus experimentalis* Kaczmarek et. al., 2020.
4. At COMPASS: Passport to the future" Faculty of Biology, Adam Mickiewicz University in Poznań, Poland (2021). Title: Repeated anhydrobiosis in bisexual tardigrade species *Paramacrobiotus experimentalis* Kaczmarek et. al., 2020.
5. At on line scientific meeting on Anhydrobiosis – cheating death and telling the tale organized by The Royal Society, UK (2022). Title: Factors affecting survival of repeated anhydrobiosis in bisexual tardigrade *Paramacrobiotus experimentalis* Kaczmarek et. al., 2020.
6. At the "Developmental circuits in Aging" (European Molecular Biology Organization (EMBO) workshop), Heraklion, Greece, (2022).
   Title: Factors affecting survival of repeated anhydrobiosis in bisexual tardigrade *Paramacrobiotus experimentalis* Kaczmarek et. al., 2020.
7. At the annual scientific meeting organized by Gerontological society of America (GSA), Indianapolis, USA (2022).

Title: Factors affecting anhydrobiosis survival in Madagascan eutardigrade *Paramacrobiotus experimentalis* (Macrobiotidae).

2. Aim of thesis

The main aim of the thesis was to determine the multilevel markers of aging of the selected tardigrade species *Paramacrobiotus experimentalis*. These markers are inevitable for verification of the "Sleeping Beauty" hypothesis assuming that animals ignore the time spent in anhydrobiosis that denotes that aging does not occur. The selection of *Pam. experimentalis* resulted from its dioecious reproductive behavior, long average lifespan and remarkable anhydrobiotic capability.

The partial aims of the thesis were as follows:

a) Searching available data for known tardigrade aging markers.
b) Building of a model containing animals of different age and sex.
c) Testing of selected aging markers, including cellular ones.

3. Introduction

Tardigrades (commonly known as water bears) are cosmopolitan and important group of invertebrates particularly due to their taxonomic position between two well-known invertebrate model organisms i.e., *Caenorhabditis elegans* and *Drosophila melanogaster*. Tardigrade taxonomic and phylogenetic position remains debated, but both morphological and molecular analyses usually find them closely related to the arthropods [1], although there are also studies suggesting a closer association with nematodes [2]. Currently, for the phylum 33 families, 159 genera, 1464 species and 21 additional subspecies have been identified and described [3]. The phylum is generally divided into two classes, Eutardigrada and Heterotardigrada, distinguished mainly on the basis of claws, dorsal cuticle, body appendages, and reproductive organs [4]. The reproductive modes of tardigrades are as follows; dioecious, parthenogenetic, or hermaphroditic. Morphologically, tardigrades are hydrophilous micrometazoans with a bilaterally symmetrical body and four pairs of lobopodous legs, usually terminating in claws and/or, in some marine species, digits. Their average body size ranges from 40 µm (juveniles) up to 1200 µm (adults) and the mature adults average size ranges between 90 and 500 µm [5]. Tardigrade genome size is small and shows variability between species within the same families but whole genome of only four tardigrade species, namely, *Hypsibius expempleris* [6] *Ramazzottius varieornatus* [7] *Milnesium tardigradum* [8] and *Paramacrobiotus* sp., (later described as *Paramacrobiotus metropolitanus*) [9], have been fully

sequenced till now. However, transcriptomes of several species, e.g., *Echiniscoides cf. sigismundi*, *Richtersius cf. coronifer* [10] and *M. tardigradum* [11] are also available.

Tardigrades are commonly known as the most stress-tolerant animals on Earth because of the ability of some taxa within the phylum to survive some of the most challenging environmental stress conditions for living organisms, such as desiccation, freezing, and radiation [12]. They ability to withstand such environmental extremes is provided by cryptobiosis. Specifically, five major types of cryptobiosis is distinguished, namely anhydrobiosis (caused by desiccation), anoxybiosis (caused by oxygen depletion), chemobiosis (caused by high level of toxicant concentration), cryobiosis (caused by extremely low temperature) and osmobiosis (caused by high solute concentration) [13]. Anhydrobiosis is the most known form of cryptobiosis in tardigrades as well as in other organisms. In this particular state, organisms do not show any signs of life but retain the ability to resume activity after complete dehydration [14]. In anhydrobiotic state, tardigrades contract longitudinally, while withdrawing their legs, forming a "tun" to reduce their evaporation surface which indicates a morphological and behavioral adaption to desiccation [13,15]. Although, number of studies have been performed concerning anhydrobiosis in tardigrades, the mechanism of their survival is not completely understood at molecular and cellular levels. The same applies to multilevel factors associated with anhydrobiosis success. Some of these factors have already been evaluated (e.g. overall body size, dehydration conditions, duration of desiccation, air humidity, temperature and strength of surrounding magnetic field) [16,17,18,19] whereas others are not or their knowledge is rather incomplete although the factors could potentially influence tardigrade anhydrobiosis. Age, sex of individuals and the presence of other individuals (i.e., group influence or social effect) could belong to those important factors. A few reports concerning the influence of age on anhydrobiosis are available, but the importance of sex and group influence were rather not investigated till the date.

Studies related to tardigrade aging are scarce although available data constitute exciting point for further studies. For example, research on *Acutuncus antarcticus* showed the age-specific reproductive performance that suggests a minimal decline in fertility with age in this species [20] whereas in *Hys. exempleris*, a gradual increase in carbonyl accumulation was detected when anhydrobiotic tardigrade aged [21]. The most interestingly, the available data indicate at functional relationship between aging and cryptobiosis. Namely, studies on *Mil. tardigradum* proved that anhydrobiosis could result in a relevant time shift in the age of tardigrades which denotes that the time spent in anhydrobiosis was ignored which ultimately supports the "Sleeping Beauty" hypothesis assuming that aging does not occur during

anhydrobiosis [22]. Moreover, recent studies on *Mil. inceptum* showed that animals did not age in frozen state [23] which also seems to support the prediction of the "Sleeping Beauty" hypothesis i.e. the lack of aging during anhydrobiosis. There is also another hypothesis proposed to explain effect of anhydrobiosis on aging, i.e., the "Picture of Dorian Gray" hypothesis. This hypothesis predicts that during anhydrobiosis an organism ages, at least at its initial stage and this phenomenon seems to apply in several species of nematodes [24, 25]. Although, these aging hypotheses appear to be crucial to test the impact of anhydrobiosis on aging, their predictions have rarely been tested in few invertebrate organisms which requires further verification.

Altogether, in available studies age impact on fitness and survival was described, but they neither show a clear aging pattern nor any potential aging markers in tardigrades including different sexes. At the same time, the markers are crucial for verification of the mentioned aging hypothesis. Therefore, indication and experimental corroboration of the markers of tardigrade aging should be undertaken. For the studies, newly identified, limno-terrestrial dioecious species *Pam. experimentalis* was used [26]. The developed laboratory culture of the species allowed for selection of females and males of different age. Moreover, the species is characterized by long average lifespan and high anhydrobiosis capability [19].

4. Main thesis and achievement of the work

An important step contributing to possible application of anhydrobiosis as an anti-aging strategy is the verification of the hypotheses concerning its impact on tardigrade aging. This, in turn, requires, convincing multilevel markers of tardigrade aging. Therefore, based on published data including other animals, several potential aging markers were proposed. In addition, based on performed research, a few multilevel markers were proposed. Verification of usability of the mentioned markers could open the opportunity for tardigrades to be also a model organism for aging biology.

4.1 Indication of applicable life-history and cellular markers of tardigrade aging

1. **Nagwani AK**., Kaczmarek Ł., Kmita, H. (2022) Applicable life-history and molecular traits for studying the effects of anhydrobiosis on aging in tardigrades. *Diversity*, 14(8): 664. https://doi.org/10.3390/d14080664

Aging is largely associated with the decline in behavioral, morphological and physiological traits, and can be considered at different levels of organism organization. It is

known that aging is not dependent on a single gene and is a consequence of multiple processes that may interact and operate at different levels of functional organization which has been explained by various aging theories (**for the theories short review see publication 1**). Several models such as invertebrates *Caenorhabditis elegans* and *Drosophila melanogaster* have been used to prove these theories and they allowed for identification of different intra- and extracellular markers of aging. However, in tardigrades, information concerning the possible markers of aging is very limited. Available data indicate that the markers could be found between life-history traits such as lifespan and fecundity (**see Table 1 in publication 1**). For the lifespan two terms are applied, i.e., maximum or total lifespan and mean or average lifespan.

Maximum lifespan refers to the age at which the oldest member of the species or experimental group died whereas mean lifespan is a statistical measure of the average time an organism is expected to live and corresponds to life expectancy. The mean (19-360 days) and maximum lifespan (1-24 months) vary between known tardigrade species. Our unpublished data indicate that *Pam. experimentalis* is one of the known tardigrade species with the longest maximum and average lifespan, i.e., 420 days and 360 days, respectively. Fecundity reflects the physiological state of individuals, usually corresponding to their age and refers to the total number of offspring in a particular time period. Certain parameters are related to fecundity, e.g., number of eggs, number of reproductive days, reproductive effort, and age at first and last reproduction. In tardigrades, the parthenogenesis (a self-fertilization strategy) is the most common reproduction strategy, but some of the species are known to be dioecious (both females and males) or hermaphroditic (have both types of reproductive organs) [27]. The first appearance of eggs in female ovary is considered to be an indication of sexual maturity. Several factors are known to directly affect the egg production in tardigrades, such as available food source, temperature and number of animals in the surrounding environment. Because *Pam. experimentalis* belong to dioecious species it could be assumed that the maximum and mean lifespan might differ between females and males as well as the number of eggs might also change with age. Therefore, in the review the importance of these life-history traits was mentioned and the issue was further studied (**see publication 2**). Nevertheless, the length of maximum and mean lifespan for *Pam. experimentalis* (**see above**) make the traits difficult to apply because of the required time. However, the trait might be replaced by the vitality rate defined as the ratio of active individuals in the given age class and the total number of individuals in that class (i.e., active and inactive). Moreover, the average number of laid eggs per female could serve as the marker (**see Table S1 in publication 2**).

On the basis of available reports concerning other organisms, it is possible to indicate putative cellular markers of aging to be verified in tardigrades (**the markers are reviewed in publication 1**). These are mainly level of ROS, mitochondria functioning, as well as several epigenetic markers such as DNA methylation and histone modification. It is well known that mitochondria functioning is coupled with ROS formation and excessive formation of ROS due to limited anti-oxidative defenses results in oxidative stress (i.e., an imbalance between prooxidant and antioxidant molecules) which co-occurs with aging. The oxidative stress can be detected at the level of oxidative modifications of lipids, proteins and DNA as well as activity of antioxidative defense including ROS-scavenging enzymes and appearance of epigenetic markers [28-31].

Among the markers, mitochondria functioning appears to be very promising because of well-known contribution of mitochondria to aging [32]. As mentioned in the review, the markers may include mitochondrial DNA copy number, metabolome changes important also for epigenetic mechanisms, the mitochondrial inner membrane potential, and selected mitochondrial proteins. Importantly, methods enabling monitoring of mitochondria functioning from the level of isolated mitochondria to the level of intact organism are commonly available **(see publication 3**). Similarly, many methods are available for ROS detection including spectrophotometry methods and application of fluorescence probes (**see Appendix 1**).

4.2 Tardigrade age is decisive for survival of extreme conditions

2. **Nagwani AK**., Melosik I., Kaczmarek Ł., Kmita H. (2023) Recovery from anhydrobiosis in the tardigrade *Paramacrobiotus experimentalis*: better to be young than old and in a group than alone. *bioRxiv* (Preprint). https://doi.org/10.1101/2023.05.22.541721

3. **Nagwani NK**., Budka A., Łacka A., Kaczmarek Ł., Kmita H. (2023) The effect of hypomagnetic field on survival and mitochondrial functionality of active *Paramacrobiotus experimentalis* females and males of different age. *Frontiers in Physiology*, 14: 1253483. https://doi.org/10.3389/fphys.2023.1253483

Since the regular studies of age effect on extreme condition tolerance are missing, the studies were performed for anhydrobiosis and hypomagnetic conditions (hypomagnetic field, HMF). Namely, survival of anhydrobiosis or hypomagnetic conditions by tardigrades of different age was estimated. The survival denoted the number of active individuals (i.e., displaying coordinated movements of the body and legs) after rehydration for anhydrobiosis or

following the treatment for exposure to HMF (**see publication 2 and 3**). The experiments required development of a relevant model comprising females and males of different age. Females and males were distinguished based on morphological characteristics, e.g., average body length (males are smaller than females), average body width (males are slimmer than females), body shape (females have a barrel shape, especially in the late stages of oogenesis, and the posterior part of the body not hooked whereas males do not have the barrel shape but sometimes the posterior part of their body is slightly hooked) and the presence of eggs in the females' ovary. The accuracy of the applied approach of identification of sexes was confirmed by detecting of spermatozoa movements in the gonad using a stereomicroscope (OLYMPUS SZ61) and observing gonads using a transmission electron microscope (Hitachi H500). The number of active animals and eggs as well as their body length, body width and body shape were assessed using the stereomicroscope. The calibrated grid of the stereomicroscope was used to measure the body length and body width to an accuracy of 10 μm. Females and males were reared together and the laid eggs (the first oviposition took place 19.3 ± 3.6 days after hatching) were collected to prepare, and maintain a living assemblage of animals. The distinguishing of age classes was based on life history traits including vitality rate (calculated as the ratio of active individuals and the total number of individuals, i.e., active and inactive), average total body length, and fertility (measured by the average number of eggs laid per female). The following five age classed were distinguished: growing, young, mature, late and old adults, (**see Table S1 in publication 2**). They represent the following age ranges in days: 60–90, 120–150, 150–180, 240–270 and over 300 days (**further details on the model can be found in publication 2 and 3**).

Anhydrobiosis in tardigrades is known to be affected by the type of environment, feeding behavior (e.g., diet), environmental/culture conditions (e.g., ambient temperature, water quality, culture substratum) and other factors such as overall body size, conditions of desiccation as well as the number and duration of anhydrobiosis episodes [19]. However, as mentioned above, knowledge concerning impact of several other factors such as age, sex and group influence is rather limited. Additionally, most of the studies related to anhydrobiosis were performed for parthenogenetic tardigrade species. Therefore, the effect of age, sex and group influence as well as combination of number and durations of anhydrobiosis episodes were tested for *Pam. exerimentalis*. In order to study these factors, females and males of five different age classes were selected and kept in groups (10 individuals per Petri dish) and singly (one individual per Petri dish) and subjected to five short (three days) or two long (30 days) repeated episodes of anhydrobiosis. The individuals were rehydrated after each episode and

their activity was observed at four different time-points, i.e., 2, 6, 24 and 48 hours, following rehydration. Subsequent anhydrobiosis episodes were performed for active animals at the latest observation time point. Importantly, a three days break was allowed between the consecutive anhydrobiosis episodes during which animals were fed **(the scheme of the experiment in shown in Figure 1 in publication 2).**

Obtained results indicated that age was the main factor influencing return to activity. It was followed, in the descending order, by the combination of number and duration of anhydrobiosis episodes, influence of the presence of other individuals, and sex. Accordingly, young adults showed the highest survival of short and long repeated anhydrobiosis, followed by mature adults, growing adults, late adults and old adults. In addition, regardless of number and duration of anhydrobiosis episodes, younger individuals (growing adults, young adults and mature adults) returned to activity more efficiently than older individuals (late and old adults). Furthermore, when analyzing the effect of sex on survival of repeated short and long anhydrobiosis, a significant difference between females and males in the number of active individuals was observed at 2h and 6h following rehydration, but not for later observation times following rehydration. Also, higher number of individuals returned to activity when anhydrobiosis was performed in the presence of other individuals (i.e. in groups) when compared to singles. Because in the presence of other specimens, individuals survived repeated anhydrobiosis better than the individuals who were treated as singles, the phenomena of "social effect" was proposed. The social effect was more pronounced for the repeated long anhydrobiosis when compared with the repeated short one. Available data on another tardigrade species indicated that the influence of other individuals on anhydrobiosis survival correlated with aggregation [33]. The aggregation was suggested to 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 [33]. In the performed study, the aggregation was not observed (**see Figure S1 in publication 2**). Therefore, it appears that under certain conditions, formation of aggregates is not indispensable for successful anhydrobiosis. It could be also speculated 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 *Pam. experimentalis* recovery from anhydrobiosis. Neither the nature of the signals nor their relationship to the age of individuals is known. On the other hand, the absence of the aggregates may explain the lower return to activity after the first episode of long anhydrobiosis (30 days) when compared with the relevant data available for *P. experimentalis* [19], although the

deterioration was not observed for the first episode of short anhydrobiosis (3 days). However, the age and sex were not considered in that study, and well as the shortest duration of the tun state was 7 days, that does not allow for deeper comparison.

Beside the observation on "social effect" requiring further research as well as the potential importance of the results for understanding of molecular and cellular mechanisms governing tardigrade anhydrobiosis, the results presented in **publication 2** also provide interesting new aging marker for tardigrades being the anhydrobiosis survival dependent on age and influenced by other factors such as combination of number and duration of anhydrobiosis episodes and observation time following rehydration.

A hypomagnetic field (HMF) also represents extreme conditions (for review, see, e.g., [34]). The HMF results from significant decrease in strength of the Earth's magnetic field, also called the geomagnetic field (GMF, ~50 μT) or standard magnetic field (SMF). Studies concerning the effect of HMF on tardigrades are scarce and concern survival of parthenogenetic females at different stages of anhydrobiosis [18, 35]. Thus, the effect of exposure to HMF on survival was studied for active animals of different age and sex (**the scheme of the experiment is shown in Figure 1 in publication 3).** The survival was estimated at four different time windows i.e., 2, 6, 24 and 48 h. For the experiments, the *Pam. experimentalis* model was applied, described in more details in **publication 2**. Namely, females and males representing three age classes (30-60 days, 150-180 days and over 300 days, described as growing, mature and old adults, respectively) were selected and exposed to SMF and HMF for three different durations. The exposure to SMF was performed using climate chamber PolLab Q-Cell 140, whereas, exposure to HMF was achieved using a special anti-magnetic chamber known as a CIMF (**for details see publication 3**).

Obtained results indicated that the effect of HMF on the *Pam. experimentalis* survival rate depended on the exposure duration and the animal age and sex. The estimated survival was high, but males, particularly the oldest ones, appeared to be more sensitive to HMF. Namely, for females, only the duration of HMF treatment was a statistically-significant factor influencing the survival, while for males both the duration of exposure to HMF and their age influenced the survival rate in a statistically-significant way. Moreover, for both females and males, shorter exposure to HMF resulted in comparable effects on survival that differed significantly from the longest exposure effect, while for males significant differences in the survival rate were observed between younger and oldest animals. Although no statistically significant differences in the survival rate were obtained between females and males after exposure to HMF, statistically significant difference was obtained for the oldest age class males

for the longest HMF duration when compared with the relevant control SMF variant of the exposure. The survival of exposure to HMF of different duration for animals of different age and/or sex should be also performed for other tardigrade species. Moreover, the long-term effect of the treatment on *Pam. experimentalis* should be performed because for the longest duration of exposure to HMF initial reduced mobility of females and males was observed transiently, but after its quick restoration the mobility did not change until the last time of observation following the end of the exposure (48h). Nevertheless, at the basis of obtained results, it could be assumed that the survival could serve as the aging marker for *Pam. experimentalis* males.

On the basis of the presented results, age of individuals appears as the most important factors affecting their survival under the studied extreme conditions whereas sex contribution is more complex and unequivocal.

4.3 The mitochondrial inner membrane potential could serve as a marker of tardigrade age

3. Nagwani NK., Budka A., Łacka A., Kaczmarek Ł., Kmita H. (2023) The effect of hypomagnetic field on survival and mitochondrial functionality of active *Paramacrobiotus experimentalis* females and males of different age. *Frontiers in Physiology*, 14: 1253483. https://doi.org/10.3389/fphys.2023.1253483

As mentioned above, HMF is considered as extreme conditions. Importantly, it is known that mitochondria play an important role in an organism's response to extreme conditions [36]. Simultaneously, a decrease of the mitochondrial inner membrane potential ($\Delta\psi$), a well-known marker of mitochondria functionality, has been shown to occur after exposure to HMF [37]. This denotes that mitochondria are sensitive to HMF. However, the effect of exposure to HMF have never been studied for tardigrade mitochondria. Moreover, the contribution of sex and age of animals to the effect of exposure to HMF was neglected. Therefore, qualitative and quantitative analysis of the effect of HMF exposure on $\Delta\psi$ was performed for *Pam. experimentalis* females and males of different age. For the experiments, the *Pam. experimentalis* model was applied, **described also in more details in publication 2**. Namely, females and males of different sex and representing three age classes (30-60 days, 150-180 days and over 300 days, described as growing, mature and old adults, respectively) were selected and exposed to GMF and HMF for three different durations as described in p. 2 (**the scheme of the experiment is shown in Figure 1 in publication 3**). For the qualitative analysis of $\Delta\psi$, individuals were treated with 2 μM TMRM (a fluorescence probe that accumulates in

mitochondria, dependently on the level of Δψ) and 1 μg/ml of DAPI (a cell-permeable fluorescence probe that binds to DNA to assure the specificity of the TMRM staining) and observed under a Nikon A1Rsi confocal microscope connected to a digital camera. In order to achieve data for the quantitative analysis, the exposed individuals were stained with 2 μM TMRM. After removing of the probe excess, individuals were transferred to multi-well plate and multiple reading per well was performed. The measurement was repeated after incubation with 50 μM FCCP (an uncoupler used as a control in the determination of Δψ level). The data were recorded using the Tecan i-control software and recalculated to the FI_{TMRM}, representing Δψ sensitivity to FCCP. Because of the differences in body size and the resulting differences in the number of animals in each sample, FI_{TMRM} was recalculated for one animal (**for details see publication 3**).

Similar to other animals, *Pam. experimentalis* responded to exposure to HMF with Δψ decrease, but the decrease did not result in stable impaired survival (**see p. 2 for the effect of exposure to HMF on *Pam. experimentalis* survival**). Moreover, following the exposure to HMF, age- and sex-related differences in Δψ level were observed. Namely, the confocal microscopic images indicated that exposure to HMF decreased the intensity of the TMRM staining when compared with SMF (control) exposure. The staining intensity also correlated with the age of active females and males, suggesting age-and sex-dependent changes in Δψ level. The intensity of the TMRM staining appeared to be higher for females and the highest for mature adults and the lowest for growing adults of both sexes, suggesting Δψ level changes related to age. Importantly, the differences in Δψ levels, dependent on age and sex, were also observed for animals after exposure to SMF. These observations were confirmed by results of quantitative analysis of TMRM staining, i.e. in values of FI_{TMRM}. Relevant values of FI_{TMRM} after exposure to SMF and HMF were statistically different supporting the relevant decrease in Δψ level after exposure to HMF. Comparison of FI_{TMRM} between females and males after exposure to SMF as well as between females and males after exposure to HMF indicated statistically significant differences for all age classes. Moreover, for females higher values of FI_{TMRM} were calculated, also after exposure to HMF. Additionally, after exposure to SMF, the age of animals affected the value of FI_{TMRM} in a statistically-significant way, whereas after HMF treatment both duration and animal age affected the value of FI_{TMRM} in a statistically-significant way. The values of FI_{TMRM} after exposure to SMF and HMF were the highest for mature adults and the lowest for growing adults. This was in agreement with the confocal microscopic images and the apparent change of Δψ level related to the animal age. For females, shorter exposures to HMF resulted in a comparable effect on FI_{TMRM} value that differed

significantly from the effect of the longest exposure, while for males significant differences in FI_{TMRM} value were observed between the shortest and the longer exposure. This suggested sex-dependent differences in the effect of exposure to HMF on Δψ level.

The results indicate that the Δψ level could be a useful marker of tardigrade age as well as could be used to differentiate between females and males that is in line with some data indicating a higher Δψ level in female mitochondria [38]. Moreover, the sensitivity of Δψ to HMF exposure may also serve as the marker of aging. Because of the observed relationship between the decrease in Δψ level and duration of exposure to HMF treatment, it is clear that HMF effect on mitochondria functionality increases over time. Therefore, the formulated conclusions not only contribute to determination of tardigrade aging markers but may also help in creating models relevant to space mission where the presence of HMF is rather dominant.

4.4 The level of ROS could serve as another marker of tardigrade age

Appendix 1: unpublished data obtained during internship at Prof. Thomas C. Boothby's Laboratory at Department of Molecular Biology, University of Wyoming, Laramie, WY, USA:

At present it is well known that the main source of reactive oxygen species (ROS) is the mitochondrial respiratory chain. Moreover, besides being important signaling molecules, ROS can be very harmful to cells under condition of uncontrolled generation and/or elimination. This results from signaling impairment and accumulation of oxidative damage to cellular molecules [e.g., 39] (**the issue is reviewed in publication 1**). It is generally assumed that throughout cell life the level of intracellular ROS increases continuously and efficiency of antioxidant mechanisms, including the levels of several antioxidant molecules, gradually decreases [e.g., 40]. Consequently, ROS are widely used as an aging marker. However, for tardigrades the role of ROS has never been validated although it is known that under stress condition the level of ROS or ROS-mediated oxidative modifications increases in tardigrades [e.g., 21, 41-42, **for review, see also publication 1**]. Therefore, the level of ROS was studied in intact females and males of different age as well as in their released storage cells. For the experiments, *Pam. experimentalis* model was applied (**described in more details in publications 2 and 3**). Namely, intact females and males representing three age classes (30-60 days, 150-180 days and over 300 days, described as growing, mature and old adults, respectively) were selected and stained with 10 μM $DCFH_2$-DA being a common probe to detect intracellular ROS [43]. Some of the stained animals were used to release storage cells

(for the total number of intact animals and released storage cells applied in the study **see Table 1 in Appendix 1**). Storage cells, known also as coelomocytes, are round free-floating cells in the body cavity of tardigrades which act as reservoirs of lipids, polysaccharides and proteins [e.g., 44]. Because the cells are easy to obtain and manipulate [e.g., 41], they were used to verify whether the marker could be applied for intact animals.

The obtained results indicated that differences in the fluorescent signal, corresponding to intracellular ROS level, which was emitted by intact animals and released storage cells were comparable (**see Figure 1 and 2 in Appendix 1**). Specifically, for intact animals and released storage cells, the intensity of the fluorescence signal gradually increased with age of both females and males. It was the highest for old adults (the age of over 300 days) and the lowest for growing adults (the age of 30-60 days). Moreover, the signal intensity was higher for males, independently of animals age.

The results clearly indicate that detection of intracellular ROS level by the applied specific fluorescence probe can be used for intact animals. Moreover, the level of ROS could be used as aging marker allowing also for sex differentiation. The results are also partially in line with data on $\Delta\psi$ level in females and males from the same age classes (**for details see publication 3**), but full understanding of the relationship between tardigrade age, the level of $\Delta\psi$ and the level of intracellular ROS requires quantitative data on ROS level and further experiments allowing for estimation of mitochondria dysfunction. In addition, the obtained results should also be validated for other dioecious tardigrade species.

5. Summary

My PhD thesis consists of one review paper and two experimental papers of which one is still under review in *Heliyon* and available as preprint (*bioRxiv*). The papers are inclined with the proposed stages of the thesis and focus on potential tardigrade multilevel aging markers. The markers are inevitable for estimation of the effect of tardigrade anhydrobiosis on aging which is explained differently by the "Sleeping Beauty" and "Picture of Dorian Gray" hypotheses, and could have important applicative consequences.

The dioecious reproductive mode and long average lifespan of *Pam. experimentalis* allowed for developing of a model that includes reproducing females and males of different age and classified to five different age classes that is of crucial meaning in terms of determining aging markers. In the studies, for the first time the effect of combination of different number and duration of anhydrobiosis episodes on survival was performed for five age classes of females and males, dehydrated and rehydrated as single individuals or in the presence of other

individuals, i.e., in groups. In addition, the effect of exposure to HMF on survival and mitochondria functionality was studied for females and males of different age. Furthermore, the level of intracellular ROS in animals of different age and sex was also evaluated. Obtained results allow for proposing of the following new markers of tardigrade aging: the vitality rate, average number of laid eggs per female, survival of the extreme conditions (anhydrobiosis and exposure to hypomagnetic field), $\Delta\psi$ level and ROS level. The markers' indication can be regarded as the main achievement of the thesis besides analysis of tardigrade diversity in terms of their life-history traits and anhydrobiosis performance as well as the *Pam. experimentalis* model that could be also used to validate aging theories. This may result in developing of anti-aging strategies and efficient approaches for preservation of biological materials. Nevertheless, the obtained results should be further verified for other dioecious tardigrade species.

6. References

1. Jørgensen A., Kristensen R.M., Møbjerg N. (2018). "Phylogeny and Integrative Taxonomy of Tardigrada," in (eds) *Water Bears: The Biology of Tardigrades*, eds. R.O. Schill, (Cham, Switzerland: Springer, 95-114). https://doi.org/10.1007/978-3-319-95702-9_3
2. Yoshida Y., Koutsovoulos G., Laetsch D.R., Stevens L., Kumar S., Horikawa D.D., et. al. (2017). Comparative genomics of the tardigrades *Hypsibius dujardini* and *Ramazzottius varieornatus*. *PLoS Biology*, 15: p.e2002266. https://doi.org/10.1371/journal.pbio.2002266
3. Degma P., Bertolani R., Guidetti R. (2023). Actual checklist of tardigrada species. 42th. Available at: https://iris.unimore.it/retrieve/bf8e14a4-625f-4cdd-8100-347e5cbc5f63/Actual%20checklist%20of%20Tardigrada%2042th%20Edition%2009-01-23.pdf.
4. Nelson D.R., Guidetti R., Rebecchi L. (2010). "Tardigrada," in (eds) *Ecology and classification of North American freshwater invertebrates*, eds. J.H. Thorp, and A.P. Covich, (Cambridge, Massachusetts, USA: Academic Press, 455-484). https://doi.org/10.1016/B978-0-12-374855-3.00014-5
5. Jönsson K.I., Holm I., Tassidis H. (2019). "Cell biology of the tardigrades: current knowledge and perspectives," in (eds) *Evo-Devo: Non-model Species in Cell and Developmental Biology. Results and Problems in Cell Differentiation*, eds. W. Tworzydlo, and S. Bilinski, (Cham, Switzerland: Springer, 231-249). https://doi.org/10.1007/978-3-030-23459-1_10
6. Koutsovoulos G., Kumar S., Laetsch D.R., Stevens L., Daub J., Conlon C., et. al. (2016). No evidence for extensive horizontal gene transfer in the genome of the tardigrade *Hypsibius dujardini*. *PNAS* 11318: 5053-5058. https://doi.org/10.1073/pnas.160033811
7. Hashimoto T., Horikawa D.D., Saito Y., Kuwahara H., Kozuka-Hata H., Shin-i T. et. al. (2016). Extremotolerant tardigrade genome and improved radiotolerance of human cultured cells by tardigrade-unique protein. *Nat. Commun*. 7: 12808. https://doi.org/10.1038/ncomms12808
8. Bemm F., Burleigh L., Förster F., Schmucki R., Ebeling M., Janzen C.J., et.al. (2017). Draft genome of the Eutardigrade *Milnesium tardigradum* sheds light on ecdysozoan evolution. *bioRxiv*, 4:122309. https://doi.org/10.1101/122309

9. Hara Y., Shibahara R., Kondo K., Abe W., Kunieda T. (2021). Parallel evolution of trehalose production machinery in anhydrobiotic animals via recurrent gene loss and horizontal transfer. *Open Biol*. 11:200413. https://doi.org/10.1098/rsob.200413
10. Kamilari M., Jørgensen A., Schiøtt M., Møbjerg N. (2019). Comparative transcriptomics suggest unique molecular adaptations within tardigrade lineages. *BMC Genom*. 20:1-9. https://doi.org/10.1186/s12864-019-5912-x
11. Mali B., Grohme M.A., Förster F., Dandekar T., Schnölzer M., Reuter D., et. al. (2010). Transcriptome survey of the anhydrobiotic tardigrade *Milnesium tardigradum* in comparison with *Hypsibius dujardini* and *Richtersius coronifer*. *BMC Genom*. 11:1-1. https://doi.org/10.1186/1471-2164-11-168
12. Kamilari M., Jørgensen A., Schiøtt M., Møbjerg N. (2019). Comparative transcriptomics suggest unique molecular adaptations within tardigrade lineages. *BMC Genom*. 20:1-9. https://doi.org/10.1186/s12864-019-5912-x
13. Neves R.C., Hvidepil L.K., Sørensen-Hygum T.L., Stuart R.M., Møbjerg N. (2020). Thermotolerance experiments on active and desiccated states of *Ramazzottius varieornatus* emphasize that tardigrades are sensitive to high temperatures. *Sci. Rep*. 10:94. https://doi.org/10.1038/s41598-019-56965-z
14. Wełnicz W., Grohme M.A., Kaczmarek Ł., Schill R.O., Frohme M. (2011). Anhydrobiosis in tardigrades—the last decade. *J. Insect Physiol*. 57:577-83. https://doi.org/10.1016/j.jinsphys.2011.03.019
15. Crowe J.H. (1975). The physiology of cryptobiosis in tardigrades. *Mem. Ist. Ital. Idrobiol*. 32: 37–59.
16. Jönsson K.I., Rebecchi L. (2002). Experimentally induced anhydrobiosis in the tardigrade *Richtersius coronifer*: phenotypic factors affecting survival. *J. Exp. Zool*. 293:578-84. https://doi.org/10.1002/jez.10186
17. Rebecchi L., Boschetti C., Nelson D.R. (2020). Extreme-tolerance mechanisms in meiofaunal organisms: a case study with tardigrades, rotifers and nematodes. *Hydrobiologia* 847:2779-99. https://doi.org/10.1007/s10750-019-04144-6
18. Erdmann W., Idzikowski B., Kowalski W., Kosicki J.Z., Kaczmarek Ł. (2021). Tolerance of two anhydrobiotic tardigrades *Echiniscus testudo* and *Milnesium inceptum* to hypomagnetic conditions. *PeerJ* 9: e10630. https://doi.org/10.7717/peerj.10630
19. Roszkowska M., Gołdyn B., Wojciechowska D., Księżkiewicz Z., Fiałkowska E., Pluskota M., et al. (2023). How long can tardigrades survive in the anhydrobiotic state?

A search for tardigrade anhydrobiosis patterns. *PLoS One*. 18: e0270386. https://doi.org/10.1371/journal.pone.0270386

20. Tsujimoto M., Komori O., Imura S. (2016). Effect of lifespan and age on reproductive performance of the tardigrade *Acutuncus antarcticus*: minimal reproductive senescence. *Hydrobiologia* 772:93-102. https://doi.org/10.1007/s10750-016-2643-8
21. Kuzmic M., Richaud M., Cuq P., Frelon S., Galas S. (2018). Carbonylation accumulation of the *Hypsibius exemplaris* anhydrobiote reveals age-associated marks. *PLoS One* 13: e0208617. https://doi.org/10.1371/journal.pone.0208617
22. Hengherr S., Brümmer F., Schill R.O. (2008). Anhydrobiosis in tardigrades and its effects on longevity traits. *J. Zool*. 275:216-20. https://doi.org/10.1111/j.1469-7998.2008.00427.x
23. Sieger J., Brümmer F., Ahn H., Lee G., Kim S., Schill R.O. (2022). Reduced ageing in the frozen state in the tardigrade *Milnesium inceptum* (Eutardigrada: Apochela). *J. Zool*. 318:253-9. https://doi.org/10.1111/jzo.13018
24. Ricci C., Covino C. (2005). Anhydrobiosis of *Adineta ricciae*: Costs and Benefits. *Hydrobiologia* 546: 307–314. https://doi.org/10.1007/s10750-005-4238-7
25. Kaczmarek Ł., Roszkowska M., Fontaneto D., Jezierska M., Pietrzak B., Wieczorek R., et al. (2019). Staying young and fit? Ontogenetic and phylogenetic consequences of animal anhydrobiosis. *J. Zool*. 309:1-1. https://doi.org/10.1111/jzo.12677
26. Kaczmarek Ł., Roszkowska M., Poprawa I., Janelt K., Kmita H., Gawlak M., et al. (2020). Integrative description of bisexual *Paramacrobiotus experimentalis* sp. nov. (Macrobiotidae) from republic of Madagascar (Africa) with microbiome analysis. *Mol. Phylogenet. Evol*. 145:106730. https://doi.org/10.1016/j.ympev.2019.106730
27. Bertolani R. (2001). Evolution of the reproductive mechanisms in tardigrades—a review. *Zool. Anz*. 240:247-52. https://doi.org/10.1078/0044-5231-00032
28. Golden T.R., Hinerfeld D.A., Melov S. (2002). Oxidative stress and aging: beyond correlation. *Aging cell* 1:117-23. https://doi.org/10.1046/j.1474-9728.2002.00015.x
29. Rahman T., Hosen I., Islam M.M., Shekhar H.U. (2012). Oxidative stress and human health. *Adv. biosci. biotechnol*. 3:997-1019. http://doi.org/10.4236/abb.2012.327123
30. Levine R.L., Stadtman E.R. (2001). Oxidative modification of proteins during aging. *Exp. Gerontol*. 36:1495-502. https://doi.org/10.1016/S0531-5565(01)00135-8
31. Wang Y., Yuan Q., Xie L. (2018). Histone modifications in aging: the underlying mechanisms and implications. *Curr. Stem Cell Res. Ther*. 13:125-35. https://doi.org/10.2174/1574888X12666170817141921

32. Srinivasan S., Guha M., Kashina A., Avadhani N.G. (2017). Mitochondrial dysfunction and mitochondrial dynamics-The cancer connection. *Biochim. Biophys. Acta - Bioenerg*. 1858:602-14. https://doi.org/10.1016/j.bbabio.2017.01.004
33. Ivarsson H., JÖnsson K.I. (2004). Aggregation effects on anhydrobiotic survival in the tardigrade *Richtersius coronifer*. *Zool. A Ecol. Integr. Physiol*. 301:195-9. https://doi.org/10.1002/jez.a.20018
34. Erdmann W., Kmita H., Kosicki J.Z., Kaczmarek Ł. (2021). How the geomagnetic field influences life on Earth–an integrated approach to geomagnetobiology. *Orig. Life Evol. Biosph*. 51:231-57. https://doi.org/10.1007/s11084-021-09612-5
35. Erdmann W., Idzikowski B., Kowalski W., Szymański B., Kosicki J.Z., Kaczmarek Ł. (2017). Can the tardigrade *Hypsibius dujardini* survive in the absence of the geomagnetic field? *PLoS One* 12: e0183380. https://doi.org/10.1371/journal.pone.0183380
36. Heine K.B., Hood W.R. (2020). Mitochondrial behaviour, morphology, and animal performance. *Biol. Rev*. 95:730-7. https://doi.org/10.1111/brv.12584
37. Wang G.M., Fu J.P., Mo W.C., Zhang H.T., Liu Y, He R.Q. (2022). Shielded geomagnetic field accelerates glucose consumption in human neuroblastoma cells by promoting anaerobic glycolysis. *Biochem. Biophys. Res. Commun*. 601:101-8. https://doi.org/10.1016/j.bbrc.2022.01.114
38. Damacena de A.C., Endoni B.T., Nuno D., Lamping K., Ledolter J., Koval O.M., et. al. (2022). Sex-Specific Differences in Endothelial Function Are Driven by Divergent Mitochondrial Ca2+ Handling. *J. Am. Heart Assoc*. 11:e023912. https://doi.org/10.1161/JAHA.121.023912
39. Zhao R.Z., Jiang S., Zhang L., Yu Z.B. (2019). Mitochondrial electron transport chain, ROS generation and uncoupling. *Int. J. Mol. Med*. 44:3-15. https://doi.org/10.3892/ijmm.2019.4188
40. Fang W., Chen S., Jin X., Liu S., Cao X., Liu B. (2023). Metabolomics in aging research: aging markers from organs. *Front. Cell Dev. Biol*. 11:1198794. https://doi.org/10.3389/fcell.2023.1198794
41. Giovannini I., Boothby T.C., Cesari M., Goldstein B., Guidetti R., Rebecchi L. (2022). Production of reactive oxygen species and involvement of bioprotectants during anhydrobiosis in the tardigrade *Paramacrobiotus spatialis*. *Sci. Rep*. 12:1938. https://doi.org/10.1038/s41598-022-05734-6

42. Schokraie E., Hotz-Wagenblatt A., Warnken U., Mali B., Frohme M., Förster F., et. al. (2010) Proteomic analysis of tardigrades: towards a better understanding of molecular mechanisms by anhydrobiotic organisms. *PLoS One* 5: e9502. https://doi.org/10.1371/journal.pone.0009502

43. Yu D., Zha Y., Zhong Z., Ruan Y., Li Z., Sun L., Hou S. (2021). Improved detection of reactive oxygen species by DCFH-DA: New insight into self-amplification of fluorescence signal by light irradiation. *Sens. Actuators B: Chem*. 339:129878. https://doi.org/10.1016/j.snb.2021.129878

44. Møbjerg N., Jørgensen A., Kristensen R.M., Neves R.C. (2018). "Phylogeny and Integrative Taxonomy of Tardigrada," in (eds) *Water Bears: The Biology of Tardigrades*, eds. R.O. Schill, (Cham, Switzerland, Springer, 57-94) https://doi.org/10.1007/978-3-319-95702-9_2

7. **Publication 1:** Nagwani AK., Kaczmarek Ł., Kmita, H. (2022) Applicable life-history and molecular traits for studying the effects of anhydrobiosis on aging in tardigrades. *Diversity*, 14(8): 664. https://doi.org/10.3390/d14080664

diversity

MDPI

Review

Applicable Life-History and Molecular Traits for Studying the Effects of Anhydrobiosis on Aging in Tardigrades

Amit Kumar Nagwani [1], Łukasz Kaczmarek [2] and Hanna Kmita [1,*,†]

[1] Department of Bioenergetics, Faculty of Biology, Adam Mickiewicz University in Poznań, Uniwersytetu Poznańskiego 6, 61-614 Poznan, Poland
[2] Department of Animal Taxonomy and Ecology, Faculty of Biology, Adam Mickiewicz University in Poznań, Uniwersytetu Poznańskiego 6, 61-614 Poznan, Poland
* Correspondence: hanna.kmita@amu.edu.pl; Tel.: +61-829-5902
† Current Address: Department of Bioenergetics, Institute of Molecular Biology and Biotechnology, Faculty of Biology, Adam Mickiewicz University in Poznań, Uniwersytetu Poznańskiego 6, 61-614 Poznan, Poland.

Abstract: Anhydrobiosis is induced by loss of water and indicates dehydration tolerance. Survival of dehydration is possible through changes at different levels of organism organization, including a remarkable reduction in metabolic activity at the cellular level. Thus, anhydrobiosis may be regarded as an anti-aging strategy. Accordingly, two hypotheses named after popular stories, "Sleeping Beauty" and "The Picture of Dorian Gray", were proposed to explain the effect of anhydrobiosis on aging. The two hypotheses predict the presence (The Picture of Dorian Gray) or absence (Sleeping Beauty) of observable aging symptoms for animals undergoing anhydrobiosis. Predictions of these hypotheses have rarely been tested, and the cellular level has not been addressed. Tardigrades appear to be a useful model for studying the effect of anhydrobiosis on aging, as they are able to enter and survive anhydrobiosis at any stage of life, although not with the same success for all species. In this review, we discuss anhydrobiosis and aging mechanisms as well as tardigrade diversity and indicate possible multilevel markers that can be used to study the impact of anhydrobiosis on tardigrade aging. This review provides data on tardigrade diversity that may also be useful for human aging studies.

Keywords: tardigrades; anhydrobiosis; aging; Sleeping Beauty; The Picture of Dorian Gray; life-history traits; cellular traits

Citation: Nagwani, A.K.; Kaczmarek, Ł.; Kmita, H. Applicable Life-History and Molecular Traits for Studying the Effects of Anhydrobiosis on Aging in Tardigrades. *Diversity* **2022**, *14*, 664. https://doi.org/10.3390/d14080664

Academic Editor: Michael Wink

Received: 31 July 2022
Accepted: 16 August 2022
Published: 17 August 2022

1. Introduction

Tardigrades (water bears) are microinvertebrates found in marine, freshwater, and limno-terrestrial habitats [1]. The number of known tardigrade species has been steadily increasing over the past decades. Currently, 1019 freshwater and 217 marine species are reported in the World Register of Marine Species (WoRMS) database, and ca. 1400 species are described in the Actual Checklist of Tardigrada Species (41st edition: 16 May 2022) [2,3]. The phylum is divided into two classes, Eutardigrada and Heterotardigrada, distinguished mainly on the basis of claws, dorsal and cephalic cuticles, body appendages, and reproductive structures [4].

Tardigrades differ in reproduction modes; they can be dioecious, parthenogenetic, or hermaphroditic [5]. The known life-history traits of tardigrades, including total lifespan, number of molts, hatching time, and hatching success, do not appear to be strictly correlated with a certain reproduction mode and vary between species [6]. A total lifespan ranging from several weeks to several years has been observed in different tardigrade species with similar reproductive modes [7]. Food preferences are also diverse in tardigrades, including plant cells, algae, bacteria, nematodes, rotifers, or other tardigrades [8–10]. Tardigrades differ in their ability to survive in extreme environmental conditions through cryptobiosis [11]. Several types of cryptobiosis are distinguished according to the triggering factor: anhydrobiosis (lack of water), cryobiosis (low temperature), anoxybiosis (lack of

oxygen), and osmobiosis (high or low osmotic pressure) [12–15]. During cryptobiosis, tardigrades reduce their metabolic activity, restoring it when conditions again become favorable [16]. Thus, cryptobiosis may extend their lifespan by many years [17,18].

One of the most important impacts of cryptobiosis on tardigrade lifespan is its impact on aging. For anhydrobiosis, the prevalent form of cryptobiosis [19], two hypotheses, denoted as "Sleeping Beauty" and "The Picture of Dorian Gray", were proposed [20,21] to explain its effect on aging. The "Sleeping Beauty" hypothesis assumes complete exclusion of the time spent in anhydrobiosis; aging does not occur. The "The Picture of Dorian Gray" hypothesis predicts that the anhydrobiotic organism ages, at least in the initial stages of anhydrobiosis, such that aging proceeds or is slowed down [19]. Thus, the time spent in anhydrobiosis increases ("Sleeping Beauty") or does not increase ("The Picture of Dorian Gray") the lifespan of anhydrobiotic animals compared with non-dehydrated (active) animals, possibly due to differences in metabolic rate and protection against aging-imposed damages, although this has not been analyzed. Predictions of these hypotheses have rarely been tested. The lifespan of active specimens is currently the main parameter used to verify the hypotheses; total or age-specific fecundity, specimen vitality, and—rarely—morphology are also used [22]. The "Sleeping Beauty" hypothesis seems to apply to the bdelloid rotifers *Macrotrachela quadricornifera* Milne, 1886 [22] and *Adineta ricciae* Segers & Shiel, 2005 [20]; the free-living nematode *Panagrolaimus rigidus* Schneider, 1866 appears to follow "The Picture of Dorian Gray" [19]. For tardigrades, only one species (*Milnesium tardigradum* Doyere, 1840) [17] has been studied in this context; it was shown to follow the "Sleeping Beauty" hypothesis. The "Sleeping Beauty" hypothesis, in relation to the effect of anhydrobiosis on aging, seems to support complete suspension of metabolism [23]. However, respiration-based metabolism was detectable in anhydrobiotic animals at a low level for the tardigrade *Macrobiotus hufelandi* C.A.S. Schultze, 1834 [24] and the stem nematode *Ditylenchus dipsaci* (Kühn, 1857) [25,26]. Moreover, the activity of a mitochondrial protein known as alternative oxidase (AOX) during dehydration likely contributes to anhydrobiosis survival of *Milnesium inceptum* Morek, Suzuki, Schill, Georgiev, Yankova, Marley, & Michalczyk, 2019 [27]. Considering the available data, verification of these hypotheses remains an intriguing possibility. More research is required to determine the effect of anhydrobiosis on animal aging. However, markers that can verify these aging hypotheses are limited. The life-history traits could be used, but understanding the impact of anhydrobiosis on aging also requires study at the cellular level.

Extensive previous investigations including comparative genomics, transcriptomic analysis, and proteomic analysis have provided useful information concerning the mechanism of anhydrobiosis in several tardigrade species [16,28–30]. However, they indicated a high degree of divergence of these mechanisms among tardigrade species, suggesting unique molecular adaptations [29–31]. Previous studies focused on the identification of genes and encoded proteins involved in anhydrobiosis, and information related to tardigrade aging is minimal. Thus, we present available data on tardigrade life-history traits; highlight the features useful for studying the effect of anhydrobiosis on aging; and indicate the cellular traits that could serve as markers for analysis. Successful identification of markers will help to explain the anhydrobiosis effect in aging and contribute to a better understanding of cell death and the development of applicative solutions. Moreover, differences in anhydrobiosis ability observed for tardigrade species provide a great opportunity for research on the involved mechanisms.

2. Anhydrobiosis

Anhydrobiosis indicates "life without water" and is also known as "dehydration tolerance". Anhydrobiosis is induced by loss of water. As with other invertebrates, such as nematodes and rotifers, tardigrades exhibit a remarkable ability to enter and survive in an anhydrobiotic state at any stage of life [12,32]. The apparent decrease in metabolism with low water content is the most challenging aspect of anhydrobiosis. The relationship between hydration and metabolic rate, and whether anhydrobiotic animals

should be classified as living (metabolically active) or dead (ametabolic), has been debated. However, despite years of research on anhydrobiotic invertebrates (e.g., [23,31–33]), the metabolic status and preservation of molecular integrity with low water content are not completely understood.

During anhydrobiosis, tardigrades form a tun-shaped structure to reduce their evaporation surface [16]. This ability is present in all tardigrade lineages, including marine echiniscoideans and arthrotardigrades, indicating that it is an ancient and homologous trait and a morphological and behavioral adaption to dehydration [16,34,35]. The process of tun formation is generally accompanied by contraction of the longitudinal intersegmental cuticle and invagination of the legs [36,37]. Intracellular lipids may be responsible for reduced transpiration rates and decreased cuticle permeability [38]. Tun formation is an active process requiring energy supply; thus, only active animals with functional mitochondria can achieve it [16,38]. Species inhabiting different microenvironments often exhibit differences in tun formation. For instance, limno-terrestrial species usually form tuns within half an hour; marine-tidal species may accomplish it in seconds [39]. A study of *Echiniscoides sigismundi* (M. Schultze, 1865), a marine tardigrade species, revealed that tun formation is not a prerequisite for dehydration tolerance in all tardigrade species and may be an adaptation to elevated external pressure rather than desiccation [35]. Moreover, it was reported that marine and true freshwater tardigrades cannot survive dehydration and undergo anhydrobiosis [7].

Dehydration generally causes severe damage to cellular structures, resulting in cell death; tardigrades have the ability to withstand such extremes. Available data indicate that tardigrade resistance to dehydration is based on mechanisms highly conserved within eukaryotes and mechanisms specific to the animals [28,30]. These mechanisms are mediated by oxidative stress response proteins (superoxide dismutase glutathione peroxidase, glutathione reductase, glutathione transferase, and catalase), chaperones (heat shock proteins), DNA repair enzymes (recombinases involved in DNA homologous recombination), water transporters (aquaporins), and intrinsically disordered proteins, such as late embryogenesis abundant proteins (LEA) and tardigrade-specific proteins, including tardigrade-specific intrinsically disordered proteins (TDP) and damage suppressor proteins (Dsup) [13,30,40,41]. Available data indicate that the mechanisms overlap, ensuring different molecule shielding and metabolic reprogramming and supporting glass formation by different molecules and water replacement. The latter is also assisted by non-protein molecules such as trehalose, although not all tardigrades rely on this disaccharide [16,31,42,43]. Further study of other non-protein protectants may provide additional useful information concerning dehydration tolerance in anhydrobiotic tardigrades. The same applies to TDP and Dsup; the results of multiomic studies indicate different numbers of paralogs for these proteins [28] and a lack of conservation of these proteins between Eutardigrada and Heterotardigrada but also a possibility of convergent evolution of anhydrobiosis machinery [29,30,44,45]. However, the role of highly conserved and ubiquitous heat shock proteins (HSPs) in managing different kinds of cellular stress and providing proteostasis is not consistent in the case of tardigrade anhydrobiosis [46–48]. With contradictory data, the role of these proteins in different anhydrobiotic tardigrades remains to be verified.

Damage caused by oxidative stress appears to be the most deleterious effect of water depletion, mediated by the formation of reactive oxygen species (ROS) [49,50]. ROS are involved in many pathological processes, including aging [51,52]. Genomic-, transcriptomic-, and proteomics-based studies have indicated the expression of a wide variety of known antioxidant enzymes in dehydrated tardigrades compared to active ones [30,53–55]. These enzymes can limit the availability of ROS and include superoxide dismutase (SOD), which transforms superoxide anions into hydrogen peroxide (H_2O_2); catalase (CAT) and glutathione peroxidase (GPx), which decompose H_2O_2 and glutathione transferase (GST), catalyzing the detoxification of endogenously derived ROS (and environmental pollutants) by glutathione conjunction; and glutathione reductase (GR), which recycles glutathione from glutathione disulfide [41,54,56]. Duplication of SOD-encoding genes was

observed as a common characteristic of anhydrobiotic tardigrades [28,30]. Additionally, in *Paramacrobiotus richtersi* (Murray, 1911), increased SOD activity was reported in response to dehydration, suggesting its importance in the process [54]. Moreover, upregulation of catalase-encoding genes during anhydrobiosis was detected in the tardigrade *Hypsibius exemplaris* Gąsiorek, Stec, Morek, & Michalczyk, 2018 [29]. Glutathione peroxidase was reported to be crucial for successful anhydrobiosis in *Pam. spatialis* Guidetti, Cesari, Bertolani, Altiero, & Rebecchi, 2019 [41]. However, despite the available data concerning antioxidant systems in anhydrobiotic tardigrades, the molecular mechanism underlying anhydrobiosis is not completely understood. Further studies are necessary to understand the role of antioxidant systems in anhydrobiotic species.

3. Aging

Aging is a universal process that can be defined as the progressive decline in biological functions leading to increased vulnerability to disease and death [57–60]. Aging is associated with decline in behavioral (e.g., alarm reaction and sensitivity to conditioning), life-history (e.g., lifespan and fecundity), morphological (e.g., body size and body shape), and physiological traits (e.g., oxygen consumption and resistance to stress) [56]. There is great diversity in aging rates among species, geographical populations, and individuals within species [61,62]. Moreover, not all tissues and organs age at the same rate [63]. It is assumed that a variety of aging rates has evolved to meet the challenges of specific environments; understanding the underlying adaptations can provide valuable insights into aging [64], with the possibility of counteracting human physical and cognitive disability [62].

3.1. Levels of Research

It is known that aging is not dependent on a single gene and is a consequence of multiple processes that may interact and operate at different levels of functional organization [65]. Several explanations of aging have been proposed that focus on different levels of organization, programmed or adaptive aging theories (evolutionary theories) and mechanistic or damage theories (molecular, cellular, and systematic theories) [66,67].

Human aging research is difficult for many reasons, including ethical issues, a long natural lifespan, environmental influences, and demographic variability. Several animal models, including the nematode *Caenorhabditis elegans* (Maupas, 1900), fruit fly (*Drosophila melanogaster* Meigen, 1830) and rodents, and single-cell organisms such as yeast (*Saccharomyces cerevisiae* Meyen ex E.C. Hansen) have been developed to study the fundamental aspects of aging biology [63,68]. These organisms offer certain experimental advantages that make them suitable models for the study of aging. For instance, *C. elegans*, *D. melanogaster*, and *S. cerevisiae* have shorter lifespans and are easy to handle and culture [69], whereas rodents have a closer genetic proximity to humans [70] and can be genetically manipulated and phenotypically characterized [71]. Studies on such models have produced useful insights concerning molecular and cellular mechanisms underlying aging and appear to demonstrate the complexities of the process at higher levels of organization [68,70,72]. Thus, using these models to verify aging theories and in experimental examination of age-related diseases may reveal hidden aspects of aging biology.

Studies on the model organisms have identified several extracellular and intracellular aging hallmarks [73]. The models also provide the possibility of the hallmarks impacting research at higher orders of phenotype complexity, including organism morphology, physiology, and behavior [74]. Moreover, the models are useful in evolutionary studies based on life-history theory and for the explanation of variations in the timing of fertility, growth, developmental rates, and death of living organisms [75]. This requires an understanding of the life-history of an organism, defined as its pattern of survival and reproduction, along with the traits that directly affect survival and the timing and amount of reproduction. The traits include: (1) growth rate; (2) age and size at sexual maturity; (3) the temporal pattern

or schedule of reproduction; (4) the number, size, and sex ratio of offspring; (5) mortality rates; and (6) patterns of dormancy and dispersal [76].

Years of research on aging did not produce a complete understanding of the mechanism(s) of the process. An integrated multidisciplinary and multilevel approach using novel model organisms may contribute to a better outcome.

3.2. Proposed Mechanism

At the population level, aging manifests as a reduction in survival and fecundity in later stages of adulthood [77]. According to the evolutionary theory of aging, there are two approaches to explain why aging originated and is maintained in populations. The first concentrates on phenotypic life-history traits (Section 3.1) and consequences of reproductive costs; the second focuses on genetic effects that arise when natural selection pressures decrease with age [77,78]. The life-history theory claims to explain how the environment affects the survival and reproduction of organisms at different ages and how life-history traits are connected to each other [79]. Accordingly, many studies have been conducted to determine the trade-off between lifespan and fecundity and their roles in the aging process (e.g., [80–82]). Two sub-types of natural selection pressure are distinguished. The first suggests that aging is a byproduct of selection of other beneficial traits; the second suggests that natural selection is unable to prevent deterioration of older organisms because it attenuates with age. However, both sub-types embrace the common principle that natural selection affects lifespan and/or reproduction [83].

The cellular mechanism of aging was proposed by L. Hayflick in 1985, known as the telomere theory of aging [84]. It assumes that the process of cell senescence limits the number of cell divisions and can occur with the arrest of cell proliferation (replicative senescence) or from other causes (stress-induced senescence) [65]. Replicative senescence results mainly from telomere shortening [67], whereas stress-induced senescence occurs in response to stressors such as oxidative stress, mitogenic stress resulting in DNA damage, changes in heterochromatin structure, and other cellular changes, including strong mitogenic signals resulting from oncogene expression [85]. The theory assuming cellular senescence is compatible with various "accumulation theories", including free radical theory and the somatic mutation theory of aging. All living organisms produce free radicals along with ROS, which originate mainly from mitochondria and cause oxidative damage to cellular molecules, including DNA [66,86]. The somatic mutation theory proposes that mutations accumulating in cells are specific causes of senescence and that oxidative stress caused by ROS is an important cause of damage [66]. Additionally, some studies have focused on specific gene variants associated with longevity (e.g., [87]).

Studies at molecular and cellular levels have revealed several gene-mediated phenomena contributing to aging; some are categorized as key aging hallmarks, including genomic instability, telomere shortening, epigenetic alterations, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intracellular and intercellular communication [88]. The number of studies identifying "longevity genes" has increased in recent decades (e.g., [89,90]). Anhydrobiosis appears to increase lifespan [17,20], but few studies support this. Thus, an approach combining aging hallmarks and identified "longevity genes" in the context of anhydrobiosis may uncover hidden aspects of aging mechanisms, which may validate aging theories and the "Sleeping Beauty" hypothesis.

4. Markers in Research on the Effect of Anhydrobiosis on Aging

The identification of possible markers of tardigrade aging will allow the study of anhydrobiosis as an anti-aging strategy (Figure 1). Available data indicate that the markers may be identified from an analysis of life-history traits and cellular processes correlated with aging. However, the use of markers in studies on the anhydrobiosis effect on tardigrade aging requires data on the efficiency of anhydrobiosis for the species in the study. Moreover,

it should be considered that anhydrobiosis survival and recovery rate may depend on tardigrade age.

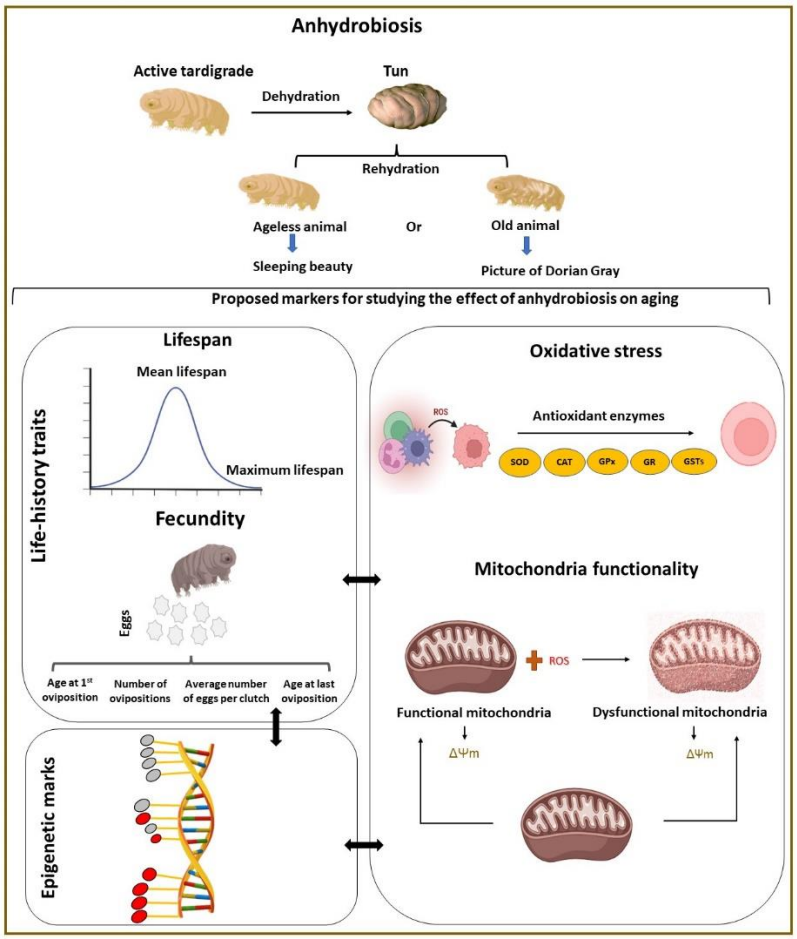


Figure 1. Life-history and cellular traits for studying the effect of anhydrobiosis on aging. ROS: reactive oxygen species; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GR: glutathione reductase; GSTs: glutathione S-transferase; ΔΨm: mitochondrial membrane potential.

4.1. Life-History Traits

Life-history traits consider the lifespan of an organism, the way it develops, reproduces, and dies [91]. For tardigrades, the following traits are considered: lifespan, number of eggs (fecundity), number of molts, clutch size, hatching success, hatching percentage, age at first oviposition, and total number of ovipositions [92,93], although their use in studying the effect of anhydrobiosis on aging is rare. The traits most often used are lifespan and fecundity (Figure 1) [17,20], both widely used in aging studies on other organisms (e.g., [80,82]). The traits for different tardigrade species and the species anhydrobiosis efficiency are summarized in Table 1. However, the conditions in these studies, including diet, ambient temperature, relative humidity, water quality, and the culture substratum, can impact the life-history traits. The same applies to anhydrobiotic success, as it can be affected by the culture conditions and other factors, such as overall body size, temperature, the mode of dehydration, and duration in the tun state, e.g., [8,16,17,37].

Table 1. Life-history traits in tardigrade species. ALS: average lifespan; ML: maximum lifespan; AFO: age at first oviposition; NO: number of ovipositions; ANEC: average number of eggs per clutch; ALO: age at last oviposition; SSLH: sample size in study of life-history traits; ND: no data; * = SD values are not reported; tardigrade genera abbreviations according to Perry et al. [94,95].

Species and Reproduction Mode	Lifespan		Fecundity				SSLH	Anhydrobiosis Capability	References
	ALS (Days) (Mean + SD)	ML (Days)	AFO (Days) (Mean + SD)	NO (Mean + SD)	ANEC (Mean + SD)	ALO (Mean ± SD)		(High/Medium/Low)	
Acu. antarcticus **generation F1; parthenogenesis**	88.8 ± 20.0	ND	17.1 ± 3.6	6.4 ± 1.0	1.8 ± 0.8	22 *	22	ND	[96]
Acu. antarcticus **generation F2; parthenogenesis**	49.5 ± 26.4	ND	16.9 ± 3.5	3.0 ± 1.9	1.7 ± 0.8	30 *	43	ND	[96]
Acu. antarcticus; **parthenogenesis**	69.2 ± 36.4	ND	9.3 ± 1.1	7.5 ± 4.3	3.4 ± 2.6	66 *	68	High	[97]
Dip. **cf.** *scoticum*; **parthenogenesis**	ND	263	ND	15	1.3 ± 4.7	ND	1	ND	[98]
Gre. myrons; **parthenogenesis**	18.8 ± 7.0	30	ND	ND	ND	ND	29	ND	[93]
Hys. exemplaris; **parthenogenesis**	61.9 ± 9.9	75	8.0 ± 3.1	10.5 ± 2.2	8 *	ND	≈100	Low	[99]
Iso. dastychi; **dioecious**	ND	29	ND	ND	ND	ND	15	ND	[100]
Mac. hefelandi; **parthenogenesis**	ND	84	31	ND	ND	ND	ND	High	[101]
Mac. joannae; **dioecious**	ND	266	ND	ND	1.7 ± 5.3	ND	ND	ND	[98]
Mac. sapiens; **dioecious**	83.0 ± 33.5	145	16.5 ± 3.8	ND	Max no 16 *	ND	66	ND	[6]
Meb. joenssoni; **dioecious**	86.5 ± 18.0	150	19.8 ± 1.7	ND	ND	ND	224	ND	[102]
Mil. tardigradum; **parthenogenesis**	82.7 ± 2.7	107	ND	ND	ND	ND	16	High	[103]
Mil. tardigradum; **parthenogenesis**	42.7 ± 11.8	58	15.3 ± 1.6	12 *	1.8 ± 11.2	ND	ND	High	[16,104]
Pam. fairbanski **clone 1 parthenogenesis**	194.9 ± 164.4	ND	76.9 ± 16.4	ND	ND	ND	16	ND	[105]
Pam. fairbanski **clone 2 parthenogenesis**	137.3 ± 136.4	ND	70.7 ± 19.4	ND	ND	ND	15	ND	[105]
Pam. kenianus, **Population (I); parthenogenesis**	125 ± 35	204	10 *	ND	ND	ND	22	ND	[92]
Pam. kenianus, **population (II); parthenogenesis**	141 ± 54	212	10 *	ND	ND	ND	22	ND	[92]
Pam. palaui; **parthenogenesis**	97 ± 31	187	10 *	ND	ND	ND	22	ND	[92]
Pam. richtersi; **parthenogenesis**	ND	ND	64.2 ± 1.7	ND	ND	ND	80	High	[106]
Pam. tonollii; **dioecious**	69.0 ± 45.1	237	24.4 ± 4.4	ND	Max no 19 *	ND	104	ND	[6]

Table 1. *Cont.*

Species and Reproduction Mode	Lifespan		Fecundity				SSLH	Anhydrobiosis Capability	References
	ALS (Days) (Mean + SD)	ML (Days)	AFO (Days) (Mean + SD)	NO (Mean + SD)	ANEC (Mean + SD)	ALO (Mean ± SD)		(High/Medium/Low)	
***Ram. oberhaeuseri*; parthenogenesis**	ND	70	ND	ND	ND	ND	ND	High	[107,108]
***Ram. varieornatus*; parthenogenesis**	13-87	87	ND	ND	ND	ND	10	High	[109]

Note: Due to lack of sufficient data on life-history traits, several tardigrade species, ***Acu. antarcticus* (generation P)**, ***Hab. crispue, Hys. convergens, Not. arcticus Ram. subanomalus, Ech. trisetosus, Ecn. sigismundi, Ric. coronifer Dip. chilenense, Ech. jenningsi, Ech. testudo, Meb.furciger, Mac. areolatus, Mil. inceptum***, and ***Pam. Spatialis***, are omitted from the table. These species differ in anhydrobiotic ability. ***Dip. chilenense, Ech. jenningsi, Ech. testudo, Meb.furciger, Mac. areolatus, Mil. inceptum***, and ***Pam. spatialis*** exhibit high anhydrobiotic ability; ***Ech. trisetosus, Ecn. sigismundi***, and ***Ric. coronifer*** are reported to exhibit moderate anhydrobiotic ability. F1 and F2 for ***Acu. antarcticus*** denote all offspring generated from two females belonging to a parental generation of adult females. The discrepancy in lifespan determined for F1 and F2 probably reflects phenotypic plasticity of life-history traits proposed as a possible survival strategy in the colonizing habitats of many invertebrates subjected to extreme and stochastic environmental conditions [95].

4.1.1. Lifespan

The maximum lifespan or total lifespan refers to the age at which the oldest member of the species or experimental group died [110]. The maximum lifespan often differs from the mean or average lifespan and longevity. The mean lifespan is a statistical measure of the average time an organism is expected to live and corresponds to life expectancy. The maximum lifespan is usually determined by the rate of aging, whereas mean lifespan varies with susceptibility to injury and disease [111]. The term "active lifespan" is generally connected to the amount of time spent in an active state, while the latent state may be represented by anhydrobiosis [112]. Longevity does not refer to the specific maximum lifespan; it refers to the relatively long lifespan of some members of a population [113]. Increased longevity directly correlates to extension of the maximum lifespan of an individual [113]. For the effect of anhydrobiosis on aging, increased longevity corresponds to the cumulative duration spent in the tun stage [17]. Accordingly, the estimation of maximum lifespan is a prerequisite to determine whether aging occurs in the tun stage. The same applies to mean lifespan, as it helps in estimating the average age at which a member of a population will die. Thus, the estimation of maximum or mean lifespan is necessary to address anhydrobiosis effects on aging.

It is known that the maximum and mean lifespan in tardigrades vary between species. The parameters are scored by estimating specimen activity, including food intake and mobility. To ensure that tardigrades are dead and not in a cryptobiotic or quiescent state, the latter caused by molting or preparing for egg laying, immobility is considered with the appearance of a straight and transparent body, although fluorescent dyes specific to dead cells are also proposed to be used [9,114]. The maximum lifespan in tardigrades is suggested as 1–24 months (excluding the period of cryptobiosis); the mean lifespan is 19–195 days [7,115]. The longest maximum lifespan was recorded for *Halobiotus crispae* Kristensen, 1982 (730 days), and the shortest maximum lifespan was recorded for *Grevenius myrops* (du Bois-Reymond Marcus, 1944) (30 days) [93,116]. The longest mean lifespan was recorded for *Pam. fairbanski* (clone 1) (194.9 ± 164.4 days); the shortest mean lifespan was recorded for *Gre. myrops* (18.8 ± 7.0 days) [93,104]. The maximum and mean lifespan of other species are presented in Table 1. Lifespan (mean or maximum) is diverse throughout the tardigrade lineage. The same applies to anhydrobiotic capability. Species with a longer lifespan do not necessarily exhibit greater anhydrobiotic capability than those with a shorter lifespan. Tardigrades with a longer lifespan require more time to grow. These data are important in determining suitable species for studying the effect of anhydrobiosis on aging.

4.1.2. Fecundity

Fecundity patterns reflect the physiological state of individuals, usually corresponding to their age [117]. The term generally refers to the total number of offspring in a particular time period [118]. Parameters associated with fecundity include number of eggs, number of reproductive days, reproductive effort, and age at first and last reproduction. These parameters are reported to be closely related to longevity [119]. Thus, fecundity and related parameters appear to be crucial for the study of anhydrobiosis in tardigrade aging.

Most marine species of tardigrades are dioecious (both female and male), whereas parthenogenesis (a self-fertilization strategy) is most common among limno-terrestrial species, although some limno-terrestrial species are also dioecious or hermaphroditic (have both types of reproductive organs) [5]. Eggs are the product of different modes of fertilization (internal and external). The total number of eggs resulting from the overall reproductivity of an individual determines the total number of offspring. The first appearance of eggs in a female ovary is considered to be an indication of sexual maturity [6]. The available food source, temperature, parasites, and number of animals in the surrounding environment directly affect egg production [98,102]. Two egg deposition patterns are observed for tardigrades; they lay eggs freely in the surrounding environment or in exuviae [120]. The number of eggs can vary in the same species, depending on age [121]. The egg-laying patterns of many tardigrade species (especially marine species) are unknown. Information on the reproduction mode and fecundity patterns is important in determining suitable species for the study of anhydrobiosis in aging. The identification of males and females in a species, time of sexual maturity (first oviposition), total number of ovipositions, and average number of eggs per clutch are some of the crucial parameters.

Unfortunately, data on changes in fecundity and other reproductive parameters over the lifespan of tardigrade species are still limited. This data could be directly linked to the understanding of species-specific or population-specific characteristics of reproduction [97]. For instance, an increase in oviposition intervals was observed to be directly correlated with lifespan in *Acutuncus antarcticus* (Richters, 1904) [97], suggesting that reproductive senescence is associated with aging. Thus, the frequency of oviposition events and age at the last oviposition could be putative indicators of aging in an organism and possible markers for studying the effect of anhydrobiosis on aging.

4.2. Possible Cellular Markers

Notwithstanding recent advancements in the understanding of mechanisms governing the aging process [74,122,123], the biological basis and factors associated with the process remain somewhat unclear. At the cellular level, aging is a process driven by accumulation of irreversible molecular and cellular damage resulting in a risk of functional decline, disease, and, ultimately, death [124]. The aging process is not dependent on a single gene and is a consequence of processes that may interact and operate at many levels of functional organization (Section 3). It is also possible that cumulative damages caused by stochastic, environmental, and genetic factors are the main drivers of lifespan variation and aging patterns [124]. The identification of molecular and cellular mechanisms underlying tardigrade aging may allow an indication of markers suitable for research on the effect of anhydrobiosis on aging.

4.2.1. Oxidative Stress

In 1928, R. Pearl proposed that lifespan is inversely related to metabolic rate [125]. In 1956, D. Harman proposed the free radical theory of aging (later known as the oxidative stress theory of aging), essentially a biochemical explanation of the theory proposed by R. Pearl [126]. According to the free radical theory, free radicals (including highly reactive derivatives of oxygen, ROS) are produced during normal metabolism. Over time, an organism becomes unable to neutralize the damage that they cause. These damages accumulate with time and threaten the homeostasis of the organism, accelerating aging and ultimately leading to death [127]. It has been assumed that a higher rate of metabolism

results in greater production of free radicals and, consequently, in faster aging and reduced lifespan. Some experiments did not confirm the simplified relationship between metabolism rate and aging [128–130]; others supported this assumption. Damage to DNA, proteins, and lipids has been shown to increase with age in many organisms, including humans, mice, flies, and *C. elegans* [130]. Reduced antioxidant defenses and increased oxidative stress have been shown to reduce lifespan [131]. Taken together, it is reasonable to consider oxidative stress and related factors in search of possible markers for studying the effect of anhydrobiosis on aging, although oxidative stress is also related to anhydrobiosis [41,103, 132]. It is proposed that the term "oxidative stress" be replaced with the term "oxidative signaling" [133].

ROS Generation and Oxidative Modifications of Different Molecules

It is commonly accepted that excessive formation of ROS and limited anti-oxidative defenses cause imbalances resulting in deleterious damage [134]. In normal metabolism, generation and elimination of ROS are balanced by antioxidant mechanisms (Figure 1) [135]. However, in stress conditions, greater ROS production occurs that cannot be removed by antioxidant defense, resulting in oxidative damage to key molecules and oxidative stress enhancement [103]. The molecular mechanisms underlying the anti-oxidative defense and the role of ROS in the biology of aging and in the development of age-related diseases remain somewhat unclear. ROS are characterized as a variety of molecules including free radicals (chemical species with one unpaired electron) derived from molecular oxygen, such as the superoxide anion ($O_2^{\bullet -}$), the hydroxyl radical ($HO^{\bullet}$), and hydrogen peroxide (H_2O_2) [136]. The main source of ROS is electron transport during ATP synthesis in mitochondria [137]. Studies on the impact of oxidative stress on anhydrobiotic tardigrades are limited. However, a study on the tardigrade *Mil. tardigradum* indicated a relationship between DNA damage and the duration of the tun stage; damage increased with the duration of the desiccated state [138]. Nevertheless, it has not been explained yet whether the DNA damage, known to be enhanced during aging, can be affected by the age of tardigrades. However, it has been shown that, in *Pam. spatialis*, ROS production significantly increases as a function of time spent in anhydrobiosis [41]. Another study on *Pam. richtersi* demonstrated that heat stress, related to oxidative stress, resulted in greater DNA damage in tuns [139]. Oxidative conditions are thought to be responsible for the death of anhydrobiotic tardigrades and for a longer required recovery time for repair of oxidative damages [139]. All data support the assumption that successful anhydrobiosis is based on effective anti-oxidative defense. Accordingly, the weakening of oxidative conditions (e.g., by application of low temperature) helps to prevent oxidative damage and suppress aging [140]. Thus, mechanisms of oxidative stress and anti-oxidative defense can be used to determine markers suitable for studies on anhydrobiosis impact on aging.

It is known that ROS oxidize all types of cellular components; the process is known as oxidative modification. This modification may be crucial for normal cell functioning [141]; however, when it prevents molecules from performing their native functions, cellular dysfunction results [133]. The modification may concern DNA, but it is also crucial for protein functioning. The oxidative modifications of proteins are characterized mainly by the addition of a carbonyl group. Increased carbonyl-bearing proteins appear to be connected with the aging process [133]. Additionally, increased protein carbonylation has been detected in a UV-stressed tardigrade *Hys. exempleris* [142]. Furthermore, oxidation of cysteine residues has been found to be linked with anhydrobiosis in *Mil. tardigradum* [143].

Another important oxidative modification is known as "lipid peroxidation". Fatty acids are a major source of energy in the cell. The composition and structure of fatty acids affect the potential for oxidative damage including peroxidation [144]. Fatty acids contain at least four carbon atoms, with a carboxylic group at the end of the molecule. They are divided into three main groups on the basis of their carbon–carbon bonds: saturated fatty acids (SFAs—single carbon–carbon bonds), monounsaturated fatty acids (MUFAs—one carbon–carbon double bond), and polyunsaturated fatty acids (PUFAs—two or more

carbon–carbon double bonds). PUFAs are abundant in cellular/organelle membranes and especially susceptible to ROS-induced peroxidation [145]. The most commonly used markers of fatty acid peroxidation are thiobarbituric acid reactive substances (TBARS), hexanoyl lysin (HEL), and total antioxidant capacity (TAC) [145]. Studies related to lipid peroxidation in tardigrades are rare. Increased TBARS were reported in anhydrobiotic *Pam. richtersi* [54]; however, the impact on lifespan and aging were not discussed.

Verification of DNA, protein, and fatty acid modifications over the lifespan appears reasonable in searching for indicators of aging in tardigrades and the role of anhydrobiosis in the process.

ROS-Scavenging Enzymes

In the course of evolution, several powerful anti-oxidative mechanisms have developed to protect against oxidative stress [146], taking advantage of endogenous antioxidant enzymes, including SOD, CAT, GPx, and GR, that have the ability to decompose ROS, providing protection against oxidative modification (Section 2 and Figure 1). Their effectiveness may vary with the physiological stages of the organism, including age [147], resulting in changes in lifespan and physiological functions [148]. There is also evidence not supporting any consistent relationship between age-related changes in antioxidant enzyme activities and lifespan [149].

In tardigrades, the activity of these enzymes during anhydrobiosis has been described (Section 2). In the tardigrade *Pam. spatialis*, GPx is reported to be critically important for desiccation tolerance. In the same study, GR and CAT are also highlighted as key components during the rehydration stage [41]. Additionally, the upregulation of two proteins, glutathione S-transferase (GST) and pirin-like protein, has been reported in response to desiccation in the tardigrade *Hys. exempleris*, indicating their role in the strategy against oxidative stress [150]. A novel manganese-dependent peroxidase (g12777) was identified as an important factor for anhydrobiosis survival in the tardigrade species *Ram. varieornatus* Bertolani & Kinchin, 1993 [149]. Recently, CAT and SOD activities were reported to be crucial for successful anhydrobiosis in *Pam. spatialis* and *Acu. antarcticus*, respectively [132]. An analysis of antioxidant enzyme performance in tardigrades, particularly in animals of different ages, may help in the verification of the enzymes as possible markers of the anhydrobiosis effect in aging.

Mitochondria Functioning

The mitochondrion is the center of cellular metabolism that controls many cell functions by mechanisms enabling the fine-tuning of gene expression levels by intracellular reduction–oxidation state and metabolite-derived nuclear epigenetic marks [151]. Thus, it is commonly accepted that aging is associated with a decline in mitochondrial functions [152]. As the mitochondrial respiratory chain is the major source of ROS (ROS Generation and Oxidative Modifications of Different Molecules), mitochondria are susceptible to excessive oxidative damage (Figure 1) [153], although they contain antioxidant enzymes, including SOD, CAT, and GPx [154]. Oxidative damage to mitochondrial DNA (mtDNA) increases its mutation rate [155], contributing to the pathophysiology of age-associated diseases, with changes in mtDNA copy number reported to coincide with aging. Impairment of mitochondrial energy transformation dysregulates nutrient sensing; alters epigenetic mechanisms; and causes a decline in cell functions [156].

Animal mitochondria contain over 1000 proteins encoded mainly by the nuclear genome. Thus, efficient communication between mitochondria and the nucleus (mitonuclear communication) is necessary to maintain correlative responses in a constantly changing intrinsic and extrinsic cellular environment [157]. Accordingly, impaired mitonuclear communication is reported to be strongly related with aging and age-related diseases [158]. Additionally, mitochondria are known to play an important role in biotic stress responses [159].

Data concerning the involvement of mitochondria in tardigrade aging are not available. However, in tardigrades, mitochondria appear to play an important role in anhydrobiosis, because uncoupling (elimination of coupling between electron transport and ATP synthesis) of mitochondria suppresses tun formation. Moreover, the organelles contribute to tun functionality and successful rehydration [24,160]. *Hys. exempleris* tun degeneration has been shown to occur with changes in mitochondrion ultrastructure [161]. Moreover, mitochondrial alternative oxidase (AOX) activity has been shown to be important for *Mil. inceptum* revival from the long-term tun stage [27]. Nevertheless, the role of mitochondria in tardigrade anhydrobiosis is still not completely understood. Studies on selected mitochondrial markers may produce a better understanding of the anhydrobiosis process and the impact of anhydrobiosis on tardigrade aging. The markers may include mtDNA copy number, metabolome changes important for epigenetic mechanisms, mitochondrial inner membrane potential, and selected mitochondrial proteins, including RvLEAM (*Ram. varieornatus* mitochondrial late embryogenesis abundant), MAHS (mitochondrial abundant heat soluble), and AOX. RvLEAM and MAHS proteins are described as potential mitochondrial protectants during anhydrobiosis [162], and AOX contribution to the state is mentioned in the Introduction.

4.2.2. Epigenetic Modifications

Epigenetics refers to reversible heritable mechanisms that affect gene expression via chromatin modifications, causing changes in DNA availability [163]. Chromatin is the polymer of nucleosomes composed of DNA and histone proteins. A single nucleosome contains a histone octamer consisting of two copies of four different histones (H2A, H2B, H3, H4) or the histone variants (e.g., macroH2A, H3.3, and H2A.Z). The octamer is wrapped by a short fragment (147 base pairs) of DNA [164] controlled by histone H1 [165], known as a linker histone, as it links adjacent nucleosomes [166]. Histones are highly conserved, basic proteins abundant in lysine and arginine residues that can be modified. Notably, both histones and DNA are modified, affecting gene expression [167].

Previous studies indicate a link between epigenetic mechanisms and aging. The loss of histones and chromatin remodeling resulting in transcriptional changes are key epigenetic hallmarks of aging [168]. Moreover, the relationship between mitochondrial functioning and epigenetic mechanisms of nuclear gene expression regulation is emerging in terms of the role of mitochondria in health and aging [169]. It should be remembered that epigenetic alterations also concern mitochondrial DNA and nucleoid proteins (as mitochondria lack histones) that may be directly linked to the stress response and the aging process, although it is not fully understood [170,171]. Studies on epigenetic mechanisms including mitochondria may provide important insight into the impact of anhydrobiosis on aging.

Epigenetics and Stress Conditions

Exposure of organisms to environmental stress can affect the fitness of their offspring for generations [20]. The effect of parental exposure to environmental stress and its transmission to offspring is known as inter-generational inheritance or epigenetic inheritance [172]. It was reported to be caused by the transmission of epigenetic markers from one generation to another, resulting in changes in traits of offspring [170]. They include histone modifications, altered expression of micro-RNAs, DNA modifications, and relevant changes in the activity of enzymes controlling epigenetic modifications [173]. Epigenetic inheritance appears to be an important issue in research on the role of the epigenetic mechanism in aging. The same applies to anhydrobiosis, which represents core survival strategies to survive harsh conditions. Therefore, studies related to epigenetic mechanisms may produce markers useful in the study of the impact of anhydrobiosis on aging.

Possible Epigenetic Markers

DNA methylation is one of the best known epigenetic modifications [174]. It is catalyzed by DNA methyltransferases (DNMTs) responsible for the formation of 5mC (5-methylcytosine) [174]. Compared with vertebrate DNA, invertebrate DNA is sparsely methylated [175]. Nevertheless, in vertebrate models, the stress response is often accompanied by DNA methylation and other chromatin modifications [176,177]. Although one DNA methyltransferase (DNMT2) has been identified in tardigrades [178], its role in anhydrobiosis or aging has not been studied. DNA methylation or DNMT2 activity may be reasonable putative markers in the study of the impact of anhydrobiosis on aging.

Histone modifications are more diverse. The most common are the methylation of arginine or lysine residues or acetylation of lysine residues. Methylation of histones can affect other proteins (transcription factors), whereas acetylation partially unwinds DNA, making it more accessible for gene expression [179]. The role of histone modifications in the aging process has been extensively investigated; however, whether these modifications are causes or effects of aging is uncertain [180]. It has been reported that alternations in histones, such as trimethylation of lysine 20 on histone H4 (H4K20me3), trimethylation of lysine 9 on histone H3 (H3K9me3), trimethylation of lysine 27 on histone H3 (H3K27me3), and acetylation of lysine 9 on histone H3 (H3K9ac), are associated with aging [179]. Moreover, combined effects of histone modifications appear to be essential for regulation of stress-responsive gene expression [181].

Epigenetic studies in tardigrades are rare; however, histone H1 has been identified in *Ram. varieornatus*, and its influence on chromatin structure has been discussed [182]. Histones H4 and H2B.2 were identified in *Mil. tardigradum* during the dormancy state [143], but their role in anhydrobiosis was not reported. Studies on histones and on histone and DNA modifications may produce possible markers to study the effect of anhydrobiosis on aging. Such studies could be supplemented by miRNAs, reported to be involved in cellular stress responses and in lifespan regulation in several organisms [183], as proper data are available for tardigrades, e.g., [29].

5. Conclusions

The impact of anhydrobiosis on aging has not been widely investigated and is not completely understood. Most previous studies have explained the extraordinary capacity of anhydrobiotic animals to survive extreme conditions; however, few data are available concerning their aging patterns and underlying processes. It is also not clear whether anhydrobiosis affects aging or suppresses possible causes of death. Most invertebrate aging research has been conducted using limited animal models; there are many opportunities for tardigrades in studies of aging biology, including molecular, cellular, and life-history traits. These studies may contribute to the verification of aging theories and hypotheses such as the "Sleeping Beauty" and "The Picture of Dorian Gray", with important applicative consequences. They may concern medicine, biotechnology, and astrobiology and result in improved anti-aging strategies and preservation of biological materials for transplantation or pharmaceutical products and dry foods.

Author Contributions: All authors contributed to writing and accepted the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: Amit Kumar Nagwani is a scholarship passport holder of the Interdisciplinary Doctoral Studies at the Faculty of Biology, Adam Mickiewicz University, POWR.03.02.00-00-I006/17. This research was supported by research grants of the National Science Centre, Poland, NCN 2016/21/B/NZ4/00131 and 2021/41/N/NZ3/01165.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The studies were performed partly in the framework of the activities of BARg (Biodiversity and Astrobiology Research group).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Kaczmarek, Ł. Tardigrada: An emerging animal model to study the Endoplasmic Reticulum stress response to environmental extremes. In *Cellular Biology of the Endoplasmic Reticulum. Progress in Molecular and Subcellular Biology*; Agellon, L.B., Michalak, M., Eds.; Springer: Cham, Switzerland, 2021; Volume 59, pp. 305–327. [CrossRef]
2. Guidetti, R.; McInnes, S.J.; Kristensen, R.M. *World List of Tardigrada*; World Register of Marine Species: Ostend, Belgium, 2022. Available online: https://www.marinespecies.org/tardigrada (accessed on 15 August 2022). [CrossRef]
3. Degma, P.; Bertolani, R.; Guidetti, R. *Actual Checklist of Tardigrada Species*, 41th ed; University of Modena and Reggio Emilia: Modena, Italy, 2022. Available online: https://iris.unimore.it/retrieve/e31e1250-6907-987f-e053-3705fe0a095a/Actual%20checklist%20of%20Tardigrada%2041th%20Edition%2016-05-22.pdf (accessed on 21 July 2022). [CrossRef]
4. Fleming, J.F.; Arakawa, K. Systematics of Tardigrada: A reanalysis of tardigrade taxonomy with specific reference to Guil et al. (2019). *Zool. Scr.* **2021**, *50*, 376–382. [CrossRef]
5. Bertolani, R. Evolution of the reproductive mechanisms in tardigrades—A review. *Zool. Anz.* **2001**, *240*, 247–252. [CrossRef]
6. Lemloh, M.; Brümmer, F.; Schill, R.O. Life-history traits of the bisexual tardigrades *Paramacrobiotus tonollii* and *Macrobiotus sapiens*. *J. Zoolog. Syst. Evol. Res.* **2011**, *49*, 58–61. [CrossRef]
7. Nelson, D.R.; Guidetti, R.; Rebecchi, L. Phylum Tardigrada. In *Thorp and Covich's Freshwater Invertebrates*; James, H., Thorp, D., Rogers, C., Eds.; Academic Press: London, UK, 2015; pp. 347–380. [CrossRef]
8. Schill, R.O.; Jönsson, K.I.; Pfannkuchen, M.; Brümmer, F. Food of tardigrades: A case study to understand food choice, intake and digestion. *J. Zoolog. Syst. Evol. Res.* **2011**, *49*, 66–70. [CrossRef]
9. Roszkowska, M.; Wojciechowska, D.; Kmita, H.; Cerbin, S.; Dziuba, M.K.; Fiałkowska, E.; Sobkowiak, R.; Szydło, W.; Kaczmarek, Ł. Tips and tricks how to culture water bears: Simple protocols for culturing eutardigrades (Tardigrada) under laboratory conditions. *Eur. Zool. J.* **2021**, *88*, 449–465. [CrossRef]
10. Roszkowska, M.; Bartels, P.J.; Gołdyn, B.; Ciobanu, D.A.; Fontoura, P.; Michalczyk, Ł.; Nelson, D.R.; Ostrowska, M.; Moreno-Talamantes, A.; Kaczmarek, Ł. Is the gut content of *Milnesium* (Eutardigrada) related to buccal tube size? *Zool. J. Linn. Soc.* **2016**, *178*, 794–803. [CrossRef]
11. Jönsson, K.I. Tardigrades—Evolutionary explorers in extreme environments. In *Extremophiles as Astrobiological Models*; Seckbach, J., Stan-Lotter, H., Eds.; Wiley: Beverly, MA, USA, 2020; pp. 255–274. [CrossRef]
12. Keilin, D. The problem of anabiosis or latent life: History and current concept. *Proc. R. Soc. Lond. B Biol. Sci.* **1959**, *150*, 149–191. [CrossRef]
13. Schill, R.O.; Hengherr, S. Environmental adaptations: Desiccation tolerance. In *Water Bears: The Biology of Tardigrades*; Schill, R.O., Ed.; Springer: Cham, Switzerland, 2018; Volume 2, pp. 273–293. [CrossRef]
14. Hengherr, S.; Schill, R.O. Environmental adaptations: Cryobiosis. In *Water Bears: The Biology of Tardigrades*; Schill, R.O., Ed.; Springer: Cham, Switzerland, 2018; Volume 2, pp. 295–310. [CrossRef]
15. Clegg, J.S. Cryptobiosis—A peculiar state of biological organization. *Comp. Biochem. Physiol. B Biochem.* **2001**, *128*, 613–624. [CrossRef]
16. Møbjerg, N.; Neves, R.C. New insights into survival strategies of tardigrades. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **2021**, *254*, 110890. [CrossRef]
17. Hengherr, S.; Brümmer, F.; Schill, R.O. Anhydrobiosis in tardigrades and its effects on longevity traits. *J. Zool.* **2008**, *275*, 216–220. [CrossRef]
18. Tsujimoto, M.; Imura, S.; Kanda, H. Recovery and reproduction of an Antarctic tardigrade retrieved from a moss sample frozen for over 30 years. *Cryobiology* **2016**, *72*, 78–81. [CrossRef]
19. Kaczmarek, Ł.; Roszkowska, M.; Fontaneto, D.; Jezierska, M.; Pietrzak, B.; Wieczorek, R.; Poprawa, I.; Kosicki, J.Z.; Karachitos, A.; Kmita, H. Staying young and fit? Ontogenetic and phylogenetic consequences of animal anhydrobiosis. *J. Zool.* **2019**, *309*, 1–11. [CrossRef]
20. Ricci, C.; Pagani, M. Desiccation of *Panagrolaimus rigidus* (Nematoda): Survival, reproduction and the influence on the internal clock. *Hydrobiologia* **1997**, *347*, 1–13. [CrossRef]
21. Ricci, C.; Covino, C. Anhydrobiosis of *Adineta ricciae*: Costs and benefits. In *Developments in Hydrobiology*; Herzig, A., Gulati, R.D., Jersabek, C.D., May, L., Eds.; Springer: Dordrecht, The Netherlands, 2005; Volume 181, pp. 307–314. [CrossRef]
22. Ricci, C.; Vaghi, L.; Manzini, M.L. Desiccation of rotifers (Macrotrachela Quadricornifera): Survival and reproduction. *Ecology* **1987**, *68*, 1488–1494. [CrossRef]
23. Clegg, J.S. Do dried cryptobiotes have a metabolism? In *Anhydrobiosis*; Crowe, J.H., Clegg, J.S., Eds.; Dowden Hutchinson and Ross: Stroudsburg, PA, USA, 1973; pp. 141–147.
24. Pigoń, A.; Weglarska, B. Rate of metabolism in tardigrades during active life and anabiosis. *Nature* **1955**, *176*, 121–122. [CrossRef]
25. Barrett, J. Metabolic responses to anabiosis in the fourth stage juveniles of *Ditylenchus dipsaci* (Nematoda). *Proc. R. Soc. Lond. B Biol. Sci.* **1982**, *216*, 159–177. [CrossRef]
26. Wharton, D.A.; Barrett, J.; Perry, R.N. Water uptake and morphological changes during recovery from anabiosis in the plant-parasitic nematode, *Ditylenchus dipsaci*. *J. Zool.* **1985**, *206*, 391–402. [CrossRef]

27. Wojciechowska, D.; Karachitos, A.; Roszkowska, M.; Rzeźniczak, W.; Sobkowiak, R.; Kaczmarek, Ł.; Kosicki, J.Z.; Kmita, H. Mitochondrial alternative oxidase contributes to successful tardigrade anhydrobiosis. *Front. Zool.* **2021**, *18*, 15. [CrossRef]
28. Murai, Y.; Yagi-Utsumi, M.; Fujiwara, M.; Tanaka, S.; Tomita, M.; Kato, K.; Arakawa, K. Multiomics study of a heterotardigrade, *Echinisicus testudo*, suggests the possibility of convergent evolution of abundant heat-soluble proteins in Tardigrada. *BMC Genom.* **2021**, *22*, 813. [CrossRef]
29. Yoshida, Y.; Koutsovoulos, G.; Laetsch, D.R.; Stevens, L.; Kumar, S.; Horikawa, D.D.; Ishino, K.; Komine, S.; Kunieda, T.; Tomita, M.; et al. Comparative genomics of the tardigrades *Hypsibius dujardini* and *Ramazzottius varieornatus*. *PLoS Biol.* **2017**, *15*, e2002266. [CrossRef]
30. Kamilari, M.; Jørgensen, A.; Schiøtt, M.; Møbjerg, N. Comparative transcriptomics suggest unique molecular adaptations within tardigrade lineages. *BMC Genom.* **2019**, *20*, 607. [CrossRef]
31. Hengherr, S.; Heyer, A.G.; Köhler, H.R.; Schill, R.O. Trehalose and anhydrobiosis in tardigrades—Evidence for divergence in responses to dehydration. *FEBS J.* **2008**, *275*, 281–288. [CrossRef]
32. Greven, H. From Johann August Ephraim Goeze to Ernst Marcus: A ramble through the history of early tardigrade research (1773 Until 1929). In *Water Bears: The Biology of Tardigrades*; Schill, R.O., Ed.; Springer: Cham, Switzerland, 2018; Volume 2, pp. 1–55. [CrossRef]
33. Pigoń, A.; Weglarska, B. The respiration of Tardigrada: A study in animal anabiosis. *Bull. Pol. Acad. Sci.* **1953**, *1*, 69–72.
34. Crowe, J. The physiology of cryptobiosis in tardigrades. *Mem. Ist. Ital. Idrobiol.* **1975**, *32*, 37–59.
35. Hygum, T.L.; Clausen, L.K.B.; Halberg, K.A.; Jørgensen, A.; Møbjerg, N. Tun formation is not a prerequisite for desiccation tolerance in the marine tidal tardigrade *Echiniscoides sigismundi*. *Zool. J. Linn. Soc.* **2016**, *178*, 907–911. [CrossRef]
36. Wright, J.C. Structural correlates of permeability and tun formation in tardigrade cuticle: An image analysis study. *J. Ultrastruct. Res.* **1988**, *101*, 23–39. [CrossRef]
37. Wełnicz, W.; Grohme, M.A.; Kaczmarek, Ł.; Schill, R.O.; Frohme, M. Anhydrobiosis in tardigrades—The last decade. *J. Insect Physiol.* **2011**, *57*, 577–583. [CrossRef]
38. Wright, J.C. The tardigrade cuticle. I. Fine structure and the distribution of lipids. *Tissue Cell* **1988**, *20*, 745–758. [CrossRef]
39. Sørensen-Hygum, T.L.; Stuart, R.M.; Jørgensen, A.; Møbjerg, N. Modelling extreme desiccation tolerance in a marine tardigrade. *Sci. Rep.* **2018**, *8*, 11495. [CrossRef]
40. Hibshman, J.D.; Clegg, J.S.; Goldstein, B. Mechanisms of desiccation tolerance: Themes and variations in brine shrimp, roundworms, and tardigrades. *Front. Physiol.* **2020**, *11*, 592016. [CrossRef]
41. Giovannini, I.; Boothby, T.C.; Cesari, M.; Goldstein, B.; Guidetti, R.; Rebecchi, L. Production of reactive oxygen species and involvement of bioprotectants during anhydrobiosis in the tardigrade *Paramacrobiotus spatialis*. *Sci. Rep.* **2022**, *12*, 1938. [CrossRef]
42. Westh, P.; Ramløv, H. Trehalose accumulation in the tardigrade *Adorybiotus coronifer* during anhydrobiosis. *J. Exp. Biol.* **1991**, *258*, 303–311. [CrossRef]
43. Crowe, L.M. Lessons from nature: The role of sugars in anhydrobiosis. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2002**, *131*, 505–513. [CrossRef]
44. Yamaguchi, A.; Tanaka, S.; Yamaguchi, S.; Kuwahara, H.; Takamura, C.; Imajoh-Ohmi, S.; Horikawa, D.D.; Toyoda, A.; Katayama, T.; Arakawa, K.; et al. Two Novel heat-soluble protein families abundantly expressed in an anhydrobiotic tardigrade. *PLoS ONE* **2012**, *7*, e44209. [CrossRef]
45. Boothby, T.C.; Tapia, H.; Brozena, A.H.; Piszkiewicz, S.; Smith, A.E.; Giovannini, I.; Rebecchi, L.; Pielak, G.J.; Koshland, D.; Goldstein, B. Tardigrades use intrinsically disordered proteins to survive desiccation. *Mol. Cell* **2017**, *65*, 975–984. [CrossRef]
46. Schill, R.O.; Steinbrück, G.H.B.; Köhler, H.-R. Stress gene (hsp70) sequences and quantitative expression in *Milnesium tardigradum* (Tardigrada) during active and cryptobiotic stages. *J. Exp. Biol.* **2004**, *207*, 1607–1613. [CrossRef]
47. Schokraie, E.; Hotz-Wagenblatt, A.; Warnken, U.; Frohme, M.; Dandekar, T.; Schill, R.O.; Schnölzer, M. Investigating heat shock proteins of tardigrades in active versus anhydrobiotic state using shotgun proteomics. *J. Zoolog. Syst. Evol. Res.* **2011**, *49*, 111–119. [CrossRef]
48. Wang, C.; Grohme, M.A.; Mali, B.; Schill, R.O.; Frohme, M. Towards decrypting cryptobiosis—Analyzing anhydrobiosis in the tardigrade *Milnesium tardigradum* using transcriptome sequencing. *PLoS ONE* **2014**, *9*, e92663. [CrossRef]
49. Wright, J.C. Cryptobiosis 300 years on from van Leuwenhoek: What have we learned about tardigrades? *Zool. Anz.* **2001**, *240*, 563–582. [CrossRef]
50. França, M.B.; Panek, A.D.; Eleutherio, E.C.A. Oxidative stress and its effects during dehydration. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2007**, *146*, 621–631. [CrossRef]
51. Hekimi, S.; Lapointe, J.; Wen, Y. Taking a "good" look at free radicals in the aging process. *Trends Cell Biol.* **2011**, *21*, 569–576. [CrossRef]
52. Zhang, J.; Wang, X.; Vikash, V.; Ye, Q.; Wu, D.; Liu, Y.; Dong, W. ROS and ROS-mediated cellular signaling. *Oxid. Med. Cell. Longev.* **2016**, *2016*, 4350965. [CrossRef]
53. Schokraie, E.; Warnken, U.; Hotz-Wagenblatt, A.; Grohme, M.A.; Hengherr, S.; Förster, F.; Schill, R.O.; Frohme, M.; Dandekar, T.; Schnölzer, M. Comparative proteome analysis of *Milnesium tardigradum* in early embryonic state versus adults in active and anhydrobiotic state. *PLoS ONE* **2012**, *7*, e45682. [CrossRef]

54. Rizzo, A.M.; Negroni, M.; Altiero, T.; Montorfano, G.; Corsetto, P.; Berselli, P.; Berra, B.; Guidetti, R.; Rebecchi, L. Antioxidant defences in hydrated and desiccated states of the tardigrade *Paramacrobiotus richtersi*. *Comp. Biochem. Physiol. B Biochem.* **2010**, *156*, 115–121. [CrossRef]
55. Förster, F.; Beisser, D.; Grohme, M.A.; Liang, C.; Mali, B.; Siegl, A.M.; Engelmann, J.C.; Shkumatov, A.V.; Schokraie, E.; Müller, T.; et al. Transcriptome analysis in tardigrade species reveals specific molecular pathways for stress adaptations. *Bioinform. Biol. Insights* **2012**, *6*, BBI-S9150. [CrossRef]
56. Rebecchi, L. Dry up and survive: The role of antioxidant defences in anhydrobiotic organisms. *J. Limnol.* **2013**, *72*, 62–72. [CrossRef]
57. Mousseau, T.A.; Roff, D.A. Natural selection and the heritability of fitness components. *Heredity* **1987**, *59*, 181–197. [CrossRef]
58. Tissenbaum, H.A. Using *C. elegans* for aging research. *Invertebr. Reprod. Dev.* **2015**, *59*, 59–63. [CrossRef]
59. Colman, R.J. Non-human primates as a model for aging. *Biochim. Biophys. Acta Mol. Basis Dis.* **2018**, *1864*, 2733–2741. [CrossRef]
60. Promislow, D.E.L.; Flatt, T.; Bonduriansky, R. The biology of aging in insects: From *Drosophila* to other insects and back. *Annu. Rev. Entomol.* **2022**, *67*, 83–103. [CrossRef]
61. Austad, S.N. Diverse aging rates in metazoans: Targets for functional genomics. *Mech. Ageing Dev.* **2005**, *126*, 43–49. [CrossRef]
62. Bloom, D.E.; Canning, D.; Lubet, A. Global population aging: Facts, challenges, solutions & perspectives. *Daedalus* **2015**, *144*, 80–92. [CrossRef]
63. Bilinski, T.; Bylak, A.; Zadrag-Tecza, R. The budding yeast *Saccharomyces cerevisiae* as a model organism: Possible implications for gerontological studies. *Biogerontology* **2017**, *18*, 631–640. [CrossRef]
64. Deweerdt, S. Comparative biology: Looking for a master switch. *Nature* **2012**, *492*, S10–S11. [CrossRef]
65. Hayflick, L. The limited in vitro lifetime of human diploid cell strains. *Exp. Cell Res.* **1965**, *37*, 614–636. [CrossRef]
66. Beckman, K.B.; Ames, B.N. The free radical theory of aging matures. *Physiol. Rev.* **1998**, *78*, 547–581. [CrossRef]
67. Blackburn, E.H. Telomere states and cell fates. *Nature* **2000**, *408*, 53–56. [CrossRef]
68. Murthy, M.; Ram, J.L. Invertebrates as model organisms for research on aging biology. *Invertebr. Reprod. Dev.* **2015**, *59*, 1–4. [CrossRef]
69. Labuschagne, C.F.; Brenkman, A.B. Current methods in quantifying ROS and oxidative damage in *Caenorhabditis elegans* and other model organism of aging. *Ageing Res. Rev.* **2013**, *12*, 918–930. [CrossRef]
70. Yuan, R.; Peters, L.L.; Paigen, B. Mice as a mammalian model for research on the genetics of aging. *ILAR J.* **2011**, *52*, 4–15. [CrossRef]
71. Brooks-Wilson, A.R. Genetics of healthy aging and longevity. *Hum. Genet.* **2013**, *132*, 1323–1338. [CrossRef]
72. He, C.; Zhou, C.; Kennedy, B.K. The yeast replicative aging model. *Biochim. Biophys. Acta Mol. Basis Dis.* **2018**, *1864*, 2690–2696. [CrossRef]
73. Kirkwood, T.B.L. Understanding the odd science of aging. *Cell J.* **2005**, *120*, 437–447. [CrossRef]
74. Bronikowski, A.M.; Meisel, R.P.; Biga, P.R.; Walters, J.R.; Mank, J.E.; Larschan, E.; Wilkinson, G.S.; Valenzuela, N.; Conard, A.M.; de Magalhães, J.P.; et al. Sex-specific aging in animals: Perspective and future directions. *Aging Cell* **2022**, *21*, e13542. [CrossRef]
75. Hill, K. Life history theory and evolutionary anthropology. *Evol. Anthropol.* **1993**, *2*, 78–88. [CrossRef]
76. Brown, J.L.; Choe, J.C. Behavioral ecology and sociobiology. In *Encyclopedia of Animal Behavior*; Breed, M.D., Moore, J., Eds.; Academic Press: Oxford, UK, 2019; Volume 1, pp. 103–108. [CrossRef]
77. Darlington, C.D. New paths in genetics. *Nature* **1942**, *149*, 317. [CrossRef]
78. Williams, G.C. Pleiotropy, natural selection and the evolution of senescence. *Evolution* **1957**, *11*, 398–411. [CrossRef]
79. Stearns, S.C. *The Evolution of Life Histories*; Oxford University Press: Oxford, UK, 1992; p. 249.
80. Snell, T.W.; King, C.E. Lifespan and fecundity patterns in rotifers: The cost of reproduction. *Evolution* **1977**, *31*, 882–890. [CrossRef]
81. Schrempf, A.; Cremer, S.; Heinze, J. Social influence on age and reproduction: Reduced lifespan and fecundity in multi-queen ant colonies. *J. Evol. Biol.* **2011**, *24*, 1455–1461. [CrossRef]
82. Monaghan, P.; Maklakov, A.A.; Metcalfe, N.B. Intergenerational transfer of ageing: Parental age and offspring lifespan. *Trends Ecol. Evol.* **2020**, *35*, 927–937. [CrossRef]
83. Kirkwood, T.B.L.; Holliday, R. Ageing as a consequence of natural selection. In *The Biology of Human Ageing*; Collins, A., Bittles, A., Eds.; Cambridge University Press: Cambridge, UK, 1986; Volume 1, pp. 1–16. [CrossRef]
84. Hayflick, L. Theories of biological aging. *Exp. Gerontol.* **1985**, *20*, 145–159. [CrossRef]
85. Campisi, J. Cellular senescence and apoptosis: How cellular responses might influence aging phenotypes. *Exp. Gerontol.* **2003**, *38*, 5–11. [CrossRef]
86. Shringarpure, R.; Davies, K.J.A. Protein turnover by the proteasome in aging and disease. *Free Radic. Biol. Med.* **2002**, *32*, 1084–1089. [CrossRef]
87. Deelen, J.; Evans, D.S.; Arking, D.E.; Tesi, N.; Nygaard, M.; Liu, X.; Wojczynski, M.K.; Biggs, M.L.; van der Spek, A.; Atzmon, G.; et al. A meta-analysis of genome-wide association studies identifies multiple longevity genes. *Nat. Commun.* **2019**, *10*, 3669. [CrossRef]
88. López-Otín, C.; Blasco, M.A.; Partridge, L.; Serrano, M.; Kroemer, G. The hallmarks of aging. *Cell J.* **2013**, *153*, 1194–1217. [CrossRef]
89. Gems, D.; Partridge, L. Genetics of longevity in model organisms: Debates and paradigm shifts. *Annu. Rev. Physiol.* **2013**, *75*, 621–644. [CrossRef]

90. Rollins, J.A.; Shaffer, D.; Snow, S.S.; Kapahi, P.; Rogers, A.N. Dietary restriction induces posttranscriptional regulation of longevity genes. *Life Sci. Alliance* **2019**, *2*, e201800281. [CrossRef]
91. Nylin, S.; Gotthard, K. Plasticity in life-history traits. *Annu. Rev. Entomol.* **1998**, *43*, 63–83. [CrossRef]
92. Schill, R.O. Life-history traits in the tardigrade species *Paramacrobiotus kenianus* and *Paramacrobiotus palaui*. *J. Limnol.* **2013**, *72*, 160–165. [CrossRef]
93. Ito, M.; Saigo, T.; Abe, W.; Kubo, T.; Kunieda, T. Establishment of an isogenic strain of the desiccation-sensitive tardigrade *Isohypsibius myrops* (Parachela, Eutardigrada) and its life history traits. *Zool. J. Linn. Soc.* **2016**, *178*, 863–870. [CrossRef]
94. Perry, E.; Miller, W.R.; Kaczmarek, Ł. Recommended abbreviations for the names of genera of the phylum Tardigrada. *Zootaxa* **2019**, *4608*, 145–154. [CrossRef] [PubMed]
95. Perry, E.; Miller, W.R.; Kaczmarek, Ł. Additional recommended abbreviations for the names of genera of the phylum Tardigrada. *Zootaxa* **2021**, *4981*, 398–400. [CrossRef] [PubMed]
96. Altiero, T.; Giovannini, I.; Guidetti, R.; Rebecchi, L. Life history traits and reproductive mode of the tardigrade *Acutuncus antarcticus* under laboratory conditions: Strategies to colonize the Antarctic environment. *Hydrobiologia* **2015**, *761*, 277–291. [CrossRef]
97. Tsujimoto, M.; Komori, O.; Imura, S. Effect of lifespan and age on reproductive performance of the tardigrade *Acutuncus antarcticus*: Minimal reproductive senescence. *Hydrobiologia* **2016**, *772*, 93–102. [CrossRef]
98. Altiero, T.; Rebecchi, L. Rearing tardigrades: Results and problems. *Zool. Anz.* **2001**, *240*, 217–221. [CrossRef]
99. Stone, J.; Vasanthan, T. Life history traits for the freshwater tardigrade species *Hypsibius exemplaris* reared under laboratory conditions. *JWB J. Wildl. Biodivers.* **2020**, *4*, 65–72. [CrossRef]
100. Bingemer, J.; Hohberg, K.; Schill, R.O. First detailed observations on tardigrade mating behaviour and some aspects of the life history of *Isohypsibius dastychi* Pilato, Bertolani & Binda 1982 (Tardigrada, Isohypsibiidae). *Zool. J. Linn. Soc.* **2016**, *178*, 856–862. [CrossRef]
101. Baumann, H. Lebenslauf und Lebensweise von *Macrobiotus hufelandi* Schultze (Tardigrada). *Veröff. Überseemus. Bremen* **1970**, *4*, 29–43.
102. Guidetti, R.; Gneuß, E.; Cesari, M.; Altiero, T.; Schill, R.O. Life-history traits and description of the new gonochoric amphimictic *Mesobiotus joenssoni* (Eutardigrada: Macrobiotidae) from the island of Elba, Italy. *Zool. J. Linn. Soc.* **2020**, *188*, 848–859. [CrossRef]
103. Suzuki, A.C. Life history of *Milnesium tardigradum* Doyère (Tardigrada) under a rearing environment. *Zool. Sci.* **2003**, *20*, 49–57. [CrossRef]
104. Neumann, S.; Reuner, A.; Brümmer, F.; Schill, R.O. DNA damage in storage cells of anhydrobiotic tardigrades. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2009**, *153*, 425–429. [CrossRef] [PubMed]
105. Altiero, T.; Rebecchi, L.; Bertolani, R. Phenotypic variations in the life history of two clones of *Macrobiotus richtersi* (Eutardigrada, Macrobiotidae). *Hydrobiologia* **2006**, *558*, 33–40. [CrossRef]
106. Hohberg, K. Tardigrade species composition in young soils and some aspects on life history of *Macrobiotus richtersi* J. Murray, 1911. *Pedobiologia* **2006**, *50*, 267–274. [CrossRef]
107. Guidetti, R.; Jönsson, K.I. Long-term anhydrobiotic survival in semi-terrestrial micrometazoans. *J. Zool.* **2002**, *257*, 181–187. [CrossRef]
108. Rebecchi, L.; Guidetti, R.; Borsari, S.; Altiero, T.; Bertolani, R. Dynamics of long-term anhydrobiotic survival of lichen-dwelling tardigrades. *Hydrobiologia* **2006**, *558*, 23–30. [CrossRef]
109. Horikawa, D.D.; Kunieda, T.; Abe, W.; Watanabe, M.; Nakahara, Y.; Yukuhiro, F.; Sakashita, T.; Hamada, N.; Wada, S.; Funayama, T.; et al. Establishment of a rearing system of the extremotolerant tardigrade *Ramazzottius varieornatus*: A new model animal for astrobiology. *Astrobiology* **2008**, *8*, 549–556. [CrossRef] [PubMed]
110. Olshansky, S.J.; Gavrilov, L.A.; Gavrilova, N.S.; Skulachev, V.P. The Biology of life span: A quantitative approach. *Popul. Dev. Rev.* **1992**, *18*, 555–558. [CrossRef]
111. Sutphin, G.L.; Kaeberlein, M. Dietary restriction by bacterial deprivation increases life span in wild-derived nematodes. *Exp. Gerontol.* **2008**, *43*, 130–135. [CrossRef]
112. Brüssow, H. What is health? *Microb. Biotechnol.* **2013**, *6*, 341–348. [CrossRef]
113. Kirkwood, T.B.L.; Holliday, R. The evolution of ageing and longevity. *Proc. R. Soc. Lond. B Biol. Sci.* **1979**, *205*, 531–546. [CrossRef]
114. Richaud, M.; Galas, S. Defining the viability of tardigrades with a molecular sensor related to death. *PLoS ONE* **2018**, *13*, e0206444. [CrossRef] [PubMed]
115. Nelson, D.R.; Guidetti, R.; Rebecchi, L. Tardigrada. In *Ecology and Classification of North American Freshwater Invertebrates*; Thorp, J.H., Covich, A.P., Eds.; Elsevier: Amsterdam, The Netherlands, 2010; Volume 1, pp. 455–484. [CrossRef]
116. Kristensen, R.M. The first record of cyclomorphosis in Tardigrada based on a new genus and species from Arctic meiobenthos. *J. Zool. Syst. Evol. Res.* **1983**, *20*, 249–270. [CrossRef]
117. Ellison, P.T. Energetics and reproductive effort. *Am. J. Hum. Biol.* **2003**, *15*, 342–351. [CrossRef] [PubMed]
118. Llodra, E.R. Fecundity and life-history strategies in marine invertebrates. *Adv. Mar. Biol.* **2002**, *43*, 87–170. [CrossRef]
119. Ricci, C.; Caprioli, M.; Fontaneto, D. Stress and fitness in parthenogens: Is dormancy a key feature for bdelloid rotifers? *BMC Evol. Biol.* **2007**, *7*, S9. [CrossRef]
120. Kinchin, I.M. *The Biology of Tardigrades*; Portland Press: London, UK, 1994; Volume 75, pp. 1–186. [CrossRef]
121. Poprawa, I.; Janelt, K. Reproduction, gonad structure, and oogenesis in tardigrades. In *Evo-Devo: Non-Model Species in Cell and Developmental Biology*; Tworzydlo, W., Bilinski, S.M., Eds.; Springer: Cham, Switzerland, 2019; Volume 68, pp. 495–513. [CrossRef]

122. Baker, G.T.; Sprott, R.L. Special issue on biomarkers of aging. *Exp. Gerontol.* **1988**, *23*, 223–438. [CrossRef]
123. Sander, M.; Avlund, K.; Lauritzen, M.; Gottlieb, T.; Halliwell, B.; Stevnsner, T.; Wewer, U.; Bohr, V.A. Aging-from molecules to populations. *Mech. Ageing Dev.* **2008**, *129*, 614–623. [CrossRef]
124. Gladyshev, V.N. On the cause of aging and control of lifespan: Heterogeneity leads to inevitable damage accumulation, causing aging; control of damage composition and rate of accumulation define lifespan. *Bioessays* **2012**, *34*, 925–929. [CrossRef] [PubMed]
125. Pearl, R. Experiments on longevity. *Q. Rev. Biol.* **1928**, *3*, 391–407. [CrossRef]
126. Harman, D. Aging: A theory based on free radical and radiation chemistry. *J. Gerontol.* **1955**, *11*, 2–5. [CrossRef]
127. Arking, R.; Buck, S.; Wells, R.A.; Pretzlaff, R. Metabolic rates in genetically based long lived strains of *Drosophila*. *Exp. Gerontol.* **1988**, *23*, 59–76. [CrossRef]
128. Le Bourg, E. The rate of living theory. Spontaneous locomotor activity, aging and longevity in *Drosophila melanogaster*. *Exp. Gerontol.* **1987**, *22*, 359–369. [CrossRef]
129. Lints, F.A.; Le Bourg, E.; Lints, C.V. Spontaneous locomotor activity and life span: A test of the rate of living theory in *Drosophila melanogaster*. *Gerontology* **1984**, *30*, 376–387. [CrossRef] [PubMed]
130. Golden, T.R.; Hinerfeld, D.A.; Melov, S. Oxidative stress and aging: Beyond correlation. *Aging Cell* **2002**, *1*, 117–123. [CrossRef] [PubMed]
131. Rahman, T.; Hosen, I.; Islam, M.M.T.; Shekhar, H.U. Oxidative stress and human health. *Adv. Biosci. Biotechnol.* **2012**, *03*, 997–1019. [CrossRef]
132. Giovannini, I.; Corsetto, P.A.; Altiero, T.; Montorfano, G.; Guidetti, R.; Rizzo, A.M.; Rebecchi, L. Antioxidant response during the kinetics of anhydrobiosis in two eutardigrade species. *Life* **2022**, *12*, 817. [CrossRef]
133. Levine, R.L.; Stadtman, E.R. Oxidative modification of proteins during aging. *Exp. Gerontol.* **2001**, *36*, 1495–1502. [CrossRef]
134. Turrens, J.F. Mitochondrial formation of reactive oxygen species. *Physiol. J.* **2003**, *552*, 335–344. [CrossRef]
135. Monaghan, P.; Metcalfe, N.B.; Torres, R. Oxidative stress as a mediator of life history trade-offs: Mechanisms, measurements and interpretation. *Ecol. Lett.* **2009**, *12*, 75–92. [CrossRef]
136. Winston, G.W.; Di Giulio, R.T. Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquat. Toxicol.* **1991**, *19*, 137–161. [CrossRef]
137. Zhao, R.-Z.; Jiang, S.; Zhang, L.; Yu, Z.-B. Mitochondrial electron transport chain, ROS generation and uncoupling (Review). *Int. J. Mol. Med.* **2019**, *44*, 3–15. [CrossRef] [PubMed]
138. Rebecchi, L.; Cesari, M.; Altiero, T.; Frigieri, A.; Guidetti, R. Survival and DNA degradation in anhydrobiotic tardigrades. *J. Exp. Biol.* **2009**, *212*, 4033–4039. [CrossRef] [PubMed]
139. Rebecchi, L.; Altiero, T.; Guidetti, R. Anhydrobiosis: The extreme limit of desiccation tolerance. *Invertebr. Surviv. J.* **2007**, *4*, 65–81.
140. Jones, D.P. Radical-free biology of oxidative stress. *Am. J. Physiol. Cell Physiol.* **2008**, *295*, C849–C868. [CrossRef] [PubMed]
141. Møller, I.M.; Jensen, P.E.; Hansson, A. Oxidative modifications to cellular components in plants. *Annu. Rev. Plant Biol.* **2007**, *58*, 459–481. [CrossRef]
142. Kuzmic, M.; Richaud, M.; Cuq, P.; Frelon, S.; Galas, S. Carbonylation accumulation of the *Hypsibius exemplaris* anhydrobiote reveals age-associated marks. *PLoS ONE* **2018**, *13*, e0208617. [CrossRef]
143. Schokraie, E.; Hotz-Wagenblatt, A.; Warnken, U.; Mali, B.; Frohme, M.; Forster, F.; Dandekar, T.; Hengherr, S.; Schill, R.O.; Schnolzer, M. Proteomic analysis of tardigrades: Towards a better understanding of molecular mechanisms by anhydrobiotic organisms. *PLoS ONE* **2010**, *5*, e9502. [CrossRef]
144. Jové, M.; Mota-Martorell, N.; Pradas, I.; Galo-Licona, J.D.; Martín-Gari, M.; Obis, È.; Sol, J.; Pamplona, R. The lipidome fingerprint of longevity. *Molecules* **2020**, *25*, 4343. [CrossRef]
145. Yavuzer, H.; Yavuzer, S.; Cengiz, M.; Erman, H.; Doventas, A.; Balci, H.; Erdincler, D.S.; Uzun, H. Biomarkers of lipid peroxidation related to hypertension in aging. *Hypertens. Res.* **2016**, *39*, 342–348. [CrossRef]
146. Ziada, A.S.; Smith, M.-S.R.; Côté, H.C.F. Updating the free radical theory of aging. *Front. Cell Dev. Biol.* **2020**, *8*, 575645. [CrossRef]
147. Davies, K.J.A.; Pomatto, L.C.D. Adaptive homeostasis and the free radical theory of ageing. *Free Radic. Biol. Med.* **2018**, *124*, 420–430. [CrossRef]
148. Fernández, C.; Ferreira, E.; Miguel, E.S.; Fernández-Briera, A. Erratum to: Superoxide dismutase and catalase: Tissue activities and relation with age in the long-lived species *Margaritifera margaritifera*. *Biol. Res.* **2016**, *49*, 35. [CrossRef]
149. Yoshida, Y.; Satoh, T.; Ota, C.; Tanaka, S.; Horikawa, D.D.; Tomita, M.; Kato, K.; Arakawa, K. Time-series transcriptomic screening of factors contributing to the cross-tolerance to UV radiation and anhydrobiosis in tardigrades. *BMC Genom.* **2022**, *23*, 405. [CrossRef] [PubMed]
150. Kondo, K.; Mori, M.; Tomita, M.; Arakawa, K. Pre-treatment with D942, a furancarboxylic acid derivative, increases desiccation tolerance in an anhydrobiotic tardigrade *Hypsibius exemplaris*. *FEBS Open Bio.* **2020**, *10*, 1774–1781. [CrossRef]
151. Amorim, J.A.; Coppotelli, G.; Rolo, A.P.; Palmeira, C.M.; Ross, J.M.; Sinclair, D.A. Mitochondrial and metabolic dysfunction in ageing and age-related diseases. *Nat. Rev. Endocrinol.* **2022**, *18*, 243–258. [CrossRef]
152. Giorgi, C.; Marchi, S.; Simoes, I.C.M.; Ren, Z.; Morciano, G.; Perrone, M.; Patalas-Krawczyk, P.; Borchard, S.; Jędrak, P.; Pierzynowska, K.; et al. Mitochondria and reactive oxygen species in aging and age-related diseases. *Int. Rev. Cell Mol. Biol.* **2018**, *340*, 209–344. [CrossRef]
153. Finaud, J.; Lac, G.; Filaire, E. Oxidative stress. *Sports Med.* **2006**, *36*, 327–358. [CrossRef]

154. Barja, G. The flux of free radical attack through mitochondrial DNA is related to aging rate. *Aging Clin. Exp. Res.* **2000**, *12*, 342–355. [CrossRef]
155. Kazachkova, N.; Ramos, A.; Santos, C.; Lima, M. Mitochondrial DNA damage patterns and aging: Revising the evidences for humans and mice. *Aging Dis.* **2013**, *4*, 337–350. [CrossRef]
156. Klaus, S.; Ost, M. Mitochondrial uncoupling and longevity—A role for mitokines? *Exp. Gerontol.* **2020**, *130*, 110796. [CrossRef]
157. Son, J.M.; Lee, C. Mitochondria: Multifaceted regulators of aging. *BMB Rep.* **2019**, *52*, 13. [CrossRef] [PubMed]
158. Rurek, M. Plant mitochondria under a variety of temperature stress conditions. *Mitochondrion* **2014**, *19*, 289–294. [CrossRef] [PubMed]
159. Manoli, I.; Alesci, S.; Blackman, M.R.; Su, Y.A.; Rennert, O.M.; Chrousos, G.P. Mitochondria as key components of the stress response. *Trends Endocrinol. Metab.* **2007**, *18*, 190–198. [CrossRef] [PubMed]
160. Halberg, K.A.; Jørgensen, A.; Møbjerg, N. Desiccation tolerance in the tardigrade *Richtersius coronifer* relies on muscle mediated structural reorganization. *PLoS ONE* **2013**, *8*, e85091. [CrossRef]
161. Poprawa, I.; Bartylak, T.; Kulpla, A.; Erdmann, W.; Roszkowska, M.; Chajec, Ł.; Kaczmarek, Ł.; Karachitos, A.; Kmita, H. Verification of *Hypsibius exemplaris* Gasiorek et al., 2018 (Eutardigrada; Hypsibiidae) application in anhydrobiosis research. *PLoS ONE* **2022**, *17*, e0261485. [CrossRef]
162. Tanaka, S.; Tanaka, J.; Miwa, Y.; Horikawa, D.D.; Katayama, T.; Arakawa, K.; Toyoda, A.; Kubo, T.; Kunieda, T. Novel mitochondria-targeted heat-soluble proteins identified in the anhydrobiotic tardigrade improve osmotic tolerance of human cells. *PLoS ONE* **2015**, *10*, e0118272. [CrossRef]
163. Tammen, S.A.; Friso, S.; Choi, S.W. Epigenetics: The link between nature and nurture. *Mol. Asp. Med.* **2013**, *34*, 753–764. [CrossRef]
164. Peterson, C.L.; Laniel, M.A. Histones and histone modifications. *Curr. Biol.* **2004**, *14*, R546–R551. [CrossRef]
165. Harshman, S.W.; Young, N.L.; Parthun, M.R.; Freitas, M.A. H1 histones: Current perspectives and challenges. *Nucleic Acids Res.* **2013**, *41*, 9593–9609. [CrossRef]
166. Saul, D.; Kosinsky, R.L. Epigenetics of aging and aging-associated diseases. *Int. J. Mol. Sci.* **2021**, *22*, 401. [CrossRef]
167. Sen, P.; Shah, P.P.; Nativio, R.; Berger, S.L. Epigenetic mechanisms regulating longevity and aging. *Cell J.* **2016**, *166*, 822–839. [CrossRef] [PubMed]
168. D'Aquila, P.; Bellizzi, D.; Passarino, G. Mitochondria in health, aging and diseases: The epigenetic perspective. *Biogerontology* **2015**, *16*, 569–585. [CrossRef] [PubMed]
169. Averbeck, D.; Rodriguez-Lafrasse, C. Role of mitochondria in radiation responses: Epigenetic, metabolic, and signaling impacts. *Int. J. Mol. Sci.* **2021**, *22*, 11047. [CrossRef] [PubMed]
170. Shaughnessy, D.T.; McAllister, K.; Worth, L.; Haugen, A.C.; Meyer, J.N.; Domann, F.E.; van Houten, B.; Mostoslavsky, R.; Bultman, S.J.; Baccarelli, A.A.; et al. Mitochondria, energetics, epigenetics, and cellular responses to stress. *Environ. Health Perspect.* **2014**, *122*, 1271–1278. [CrossRef]
171. Sharma, N.; Pasala, M.S.; Prakash, A. Mitochondrial DNA: Epigenetics and environment. *Environ. Mol. Mutagen.* **2019**, *60*, 668–682. [CrossRef]
172. Storey, K.B. Regulation of hypometabolism: Insights into epigenetic controls. *J. Exp. Biol.* **2015**, *218*, 150–159. [CrossRef]
173. Zhu, X.; Chen, Z.; Shen, W.; Huang, G.; Sedivy, J.M.; Wang, H.; Ju, Z. Inflammation, epigenetics, and metabolism converge to cell senescence and ageing: The regulation and intervention. *Signal Transduct. Target. Ther.* **2021**, *6*, 245. [CrossRef]
174. Cui, D.; Xu, X. Dna methyltransferases, DNA methylation, and age-associated cognitive function. *Int. J. Mol. Sci.* **2018**, *19*, 1315. [CrossRef]
175. Roberts, S.B.; Gavery, M.R. Is there a relationship between DNA methylation and phenotypic plasticity in invertebrates? *Front. Physiol.* **2012**, *2*, 116. [CrossRef]
176. Akhter, Z.; Bi, Z.; Ali, K.; Sun, C.; Fiaz, S.; Haider, F.U.; Bai, J. In response to abiotic stress, DNA methylation confers epigenetic changes in plants. *Plants* **2021**, *10*, 1096. [CrossRef]
177. Dowen, R.H.; Pelizzola, M.; Schmitz, R.J.; Lister, R.; Dowen, J.M.; Nery, J.R.; Dixon, J.E.; Ecker, J.R. Widespread dynamic DNA methylation in response to biotic stress. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, E2183–E2191. [CrossRef] [PubMed]
178. Engelhardt, J.; Scheer, O.; Stadler, P.F.; Prohaska, S.J. Evolution of DNA methylation across Ecdysozoa. *J. Mol. Evol.* **2022**, *90*, 56–72. [CrossRef] [PubMed]
179. Wang, Y.; Yuan, Q.; Xie, L. Histone modifications in aging: The underlying mechanisms and implications. *Curr. Stem Cell Res. Ther.* **2018**, *13*, 125–135. [CrossRef] [PubMed]
180. Henikoff, S.; Shilatifard, A. Histone modification: Cause or cog? *Trends Genet.* **2011**, *27*, 389–396. [CrossRef] [PubMed]
181. Yuan, L.; Liu, X.; Luo, M.; Yang, S.; Wu, K. Involvement of histone modifications in plant abiotic stress responses. *J. Integr. Plant Biol.* **2013**, *55*, 892–901. [CrossRef]
182. Chavez, C.; Cruz-Becerra, G.; Fei, J.; Kassavetis, G.A.; Kadonaga, J.T. The tardigrade damage suppressor protein binds to nucleosomes and protects DNA from hydroxyl radicals. *eLife* **2019**, *8*, e47682. [CrossRef]
183. Gonzalo, S. Epigenetic alterations in aging. *J. Appl. Physiol.* **2010**, *109*, 586–597. [CrossRef]

Author statement

Amit Kumar Nagwani
aminag@amu.edu.pl

Poznań, 17.09.2023

AUTHOR STATEMENT

for the review article 'Applicable Life-History and Molecular Traits for Studying the Effects of Anhydrobiosis on Aging in Tardigrades'. Diversity 14(8): 664 (2022).

I declare that the review article by Nagwani AK, Kaczmarek Ł, Kmita H. **Applicable Life-History and Molecular Traits for Studying the Effects of Anhydrobiosis on Aging in Tardigrades (2022) Diversity 14(8): 664** (doi: https://doi.org/10.3390/d14080664) is part of my PhD dissertation. My contribution includes writing of the draft version, preparing of figure and table and participating in the manuscript conceptualization, editing and revision.

Name: Amit Kumar Nagwani
Adress: Department of Bioenergetics,
Institute of Molecular Biology and Biotechnology,
Faculty of Biology, Adam Mickiewicz University in Poznań, Poznań
Uniwersytetu Poznańskiego 6, 61-614,
Poznań, Poland

Signature:

Kierownik
Zakładu Bioenergetyki
prof. dr hab. Hanna Kmita

Co-author statements

Prof. UAM dr hab. Łukasz Kaczmarek
kaczmar@amu.edu.pl

Poznań, 14.09.2023

CO-AUTHOR STATEMENT

I declare that I am aware that the review paper by *Nagwani AK, Kaczmarek Ł, Kmita H. Applicable Life-History and Molecular Traits for Studying the Effects of Anhydrobiosis on Aging in Tardigrades (2022) Diversity. 14(8): 664 (doi: https://doi.org/10.3390/d14080664)* is a part of Amit Kumar Nagwani PhD thesis.

At the same time, I declare that I supervised in conceptualizing the idea, finalizing the figure and table, as well as contributed in writing and editing the manuscript.

Łukasz Kaczmarek

ADAM MICKIEWICZ UNIVERSITY, POZNAŃ
Faculty of Biology
Institute of Molecular Biology and Biotechnology
Department of Bioenergetics

Prof. dr hab. Hanna Kmita
kmita@amu.edu.pl

Poznań, 5.09.2023

CO-AUTHOR STATEMENT

I declare that I am aware that the review paper by *Nagwani AK, Kaczmarek Ł, Kmita H. Applicable Life-History and Molecular Traits for Studying the Effects of Anhydrobiosis on Aging in Tardigrades (2022) Diversity. 14(8): 664 (doi: https://doi.org/10.3390/d14080664)* is a part of Amit Kumar Nagwani PhD thesis.

At the same time, I declare that I conceptualized its idea as well as supervised its writing and revision.

Kierownik
Zakładu Bioenergetyki
prof. dr hab. Hanna Kmita
Hanna Kmita

ul. Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland
tel. +48 61 829 5553, 61 829 5848
www.biologia.amu.edu.pl

8. **Publication 2:** Nagwani AK., Melosik I., Kaczmarek Ł., Kmita H. (2023) Recovery from anhydrobiosis in the tardigrade Paramacrobiotus experimentalis: better to be young than old and in a group than alone. *bioRxiv* (Preprint). https://doi.org/10.1101/2023.05.22.541721

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

Amit Kumar Nagwani[1]*, Iwona Melosik[2]*, Łukasz Kaczmarek[3], Hanna Kmita[1#]

[1]Department of Bioenergetics, Institute of Molecular Biology and Biotechnology, Faculty of Biology, Adam Mickiewicz University in Poznań, Poland

[2]Department of Genetics, Institute of Experimental Biology, Faculty of Biology, Adam Mickiewicz University in Poznań, Poland

[3]Department of Animal Taxonomy and Ecology, Institute of Environmental Biology, Faculty of Biology, Adam Mickiewicz University in Poznań, Poland

[#]Corresponding author: kmita@amu.edu.pl

*The authors have an equal contribution.

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, 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 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 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 *Pam. experimentalis* and provide background for full explanation of molecular, cellular and environmental mechanisms of anhydrobiosis.

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 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

102 individuals and in the presence of other individuals, i.e., in groups; (3) individuals were
103 divided into five age classes and (4) different number and duration of anhydrobiosis episodes
104 were applied.
105 Results obtained are important for developing a better understanding of the process of
106 anhydrobiosis, providing the background for describing underlying molecular, cellular and
107 environmental mechanisms and their response to environmental stress.
108
109 MATERIALS AND METHODS
110 *Cultures of Paramacrobiotus experimentalis*
111 Females and males of *Pam. experimentalis* [40], were cultured together in covered,
112 vented plastic Petri dishes (55 mm in diameter), with the bottom scratched with sandpaper to
113 allow the animals to move. Individuals were coated with a thin layer of the culture medium, a
114 mixture of spring water (Żywiec Zdrój S.A., Poland), and ddH_2O in a 1:3 ratio. The culture
115 medium was changed every week, and animals were fed with the rotifer *Lecane inermis*
116 (strain 1.A2.15). provided by Dr Edyta Fiałkowska (Institute of Environmental Sciences,
117 Jagiellonian University, Krakow, Poland). The Petri dishes were kept in the climate chamber
118 POL EKO KK 115 TOP+ at 20°C, in the dark (24h), and with relative humidity (RH) of
119 40%.
120 On the basis of life history traits, five age classes, defined as growing, young, mature,
121 late and old adults, were distinguished (Supplementary file, Table S1). They represent the
122 following age ranges in days: 60–90, 120–150, 150–180, 240–270 and >300, and correspond
123 to age classes 1-5, respectively. For the selected age classes an approximate ratio of 2:1
124 females to males was defined.
125
126 *Protocol for repeated episodes of anhydrobiosis*
127 The protocol includes dehydration and rehydration procedure optimized for *Par.*
128 *experimentalis* [33]. All experiments were performed in covered, vented plastic Petri dishes
129 of 35 mm diameter lined at the bottom with white filter paper (grammage 85–87, Chemland
130 Company, Poland). Females and males representing the distinguished age classes
131 (Supplementary file, Table S1) were transferred using an automatic pipette into dishes filled
132 with 450 µl of the culture medium. The dishes were placed into the climate chamber PolLab
133 Q-Cell 140, and the individuals were allowed to dry slowly at 20 °C, with 40–50% RH, and
134 in the dark for 72 h. The tun formation was checked once every 24 h by a brief 1-minute
135 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 (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 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 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 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* (see Suplementary 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) (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 combination of number and duration of anhydrobiosis episodes (Figure 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 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% more successfully than the old adults (age class 5) after repeated long and short anhydrobiosis,

204 respectively (Supplementary file, Table S4). The growing adults (age class 1) returned to
205 activity 10% and 19% slower, the mature adults (age group 3) 7% and 14% slower, and the
206 late adults (age class 4) 32% and 37% slower than individuals representing the young adults
207 (age class 2) (Supplementary file, Table S4).
208
209 *Effect of combination of the number and duration of anhydrobiosis episodes*
210 The number of active individuals decreased significantly with the increasing number
211 and duration of anhydrobiosis episodes but increased with the observation time after
212 rehydration (Figure 2). In general, significant positive correlations (p<0.05) were found
213 between the combined effect of the number and duration of anhydrobiosis episodes and
214 observation at 2h and 6h after rehydration. Although still positive, these correlations became
215 statistically insignificant as rehydration time progressed (observations at 24–48h;
216 Supplementary file, Table S5).
217 In the subsequent analysis, two episodes of long anhydrobiosis were compared with
218 the first two episodes of short anhydrobiosis to distinguish the effect of the duration of
219 anhydrobiosis from the number of anhydrobiosis episodes (2 *vs*. 5). With a 90% difference in
220 the duration of the tun state (i.e., 6 *vs*. 60 days), 25% to 36% more active individuals were
221 observed after short anhydrobiosis than after long anhydrobiosis, depending on the
222 observation time (Supplementary file, Table S6). Based on post hoc tests, significant
223 differences were found between short and long anhydrobiosis in the number of active
224 individuals after rehydration at each observation time (2–48h; one-way ANOVA, Wilk's
225 Lambda = 0.544, (F (4,75) = 15.728, p<0.001), Figure 3).
226 The comparison between consecutive short anhydrobiosis episodes showed no
227 statistically significant differences in the number of active individuals, except a significant
228 difference at 48h after rehydration between the fourth and fifth episode.
229
230 *Effect of the presence of other individuals*
231 The presence of other individuals (i.e., group influence) during anhydrobiosis
232 significantly influenced the number of active individuals after rehydration (p<0.001,
233 Supplementary file, Table S3). Furthermore, interactions between observation time after
234 rehydration, the combination of the number and duration of anhydrobiosis episodes, and the
235 group influence or interactions of two of these specified factors were significant
236 (Supplementary file, Table S7). Irrespective of observation time and combination of the
237 number and duration of anhydrobiosis episodes, more individuals were active in groups after

238 rehydration when compared to single animals (Figure 4). Based on the mean values, the
239 number of active individuals experiencing repeated long anhydrobiosis individually was 20%
240 lower than in groups, and for short anhydrobiosis, the difference between groups and
241 individuals was 6%. However, the Eta-squared indicates that the proportion of variance
242 explained by the interaction was low (Eta-squared equal to 0.03).
243
244 *Effect of sex*
245 The main effect of sex was statistically significant ($p<0.05$; Supplementary file, Table
246 S3). However, there was no general trend to conclude that one sex had a higher number of
247 active individuals after rehydration than the other one. Significant differences between
248 females and males in the number of active individuals were only observed at 2h and 6h after
249 rehydration for the repeated short and long anhydrobiosis, respectively. We also found no
250 significant differences between the sexes when analysing the different age classes, with one
251 exception concerning short anhydrobiosis for the oldest age class at 2h after rehydration
252 (Figure 5).
253 The interaction between observation time, age and sex of individuals appeared to be
254 significant ($F(12, 300) = 2.537$, $p<0.05$), meaning that both females and males differed in the
255 number of active individuals between older and younger age classes, particularly at the later
256 observation time (24–48h) after rehydration. Other interactions where sex was one of the
257 main factors were not significant (Supplementary file, Table S3). Mean numbers of active
258 individuals representing females and males in the context of age, combination of the number
259 and duration of anhydrobiosis episodes and observation time after rehydration (2–48h) are
260 presented in Supplementary file, Table S8.
261
262 DISCUSSION
263 The present study demonstrates the potential of the newly-described tardigrade
264 species *Pam. experimentalis* to return to activity after repeated short and long anhydrobiosis.
265 This potential was investigated by the determination of the activity of individuals after
266 rehydration (defined here as coordinated movements of a body and legs, i.e., the onset of
267 crawling) concerning their age, sex, and whether they occurred in the presence of other
268 individuals (i.e., in a group) or not (i.e., single) during anhydrobiosis steps. The study also
269 focuses on the combined effect of the number and duration of anhydrobiosis episodes. Some
270 of these factors have not been studied before (e.g., the effect of sex) or only limited
271 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, 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, 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, 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]. Similarly, studies on the nematode *Panagrolaimus rigidus* showed that increasing age had a negative effect on recovery from anhydrobiosis [42]. 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

306 *Macrotrachela quadricornifera*, for which adult individuals showed better recovery from
307 anhydrobiosis than juveniles and eggs [48, 49].

308 Among the tardigrades tested, ability of anhydrobiosis varies over a wide range [9, 14,
309 17, 33]. This may be related to abiotic factors, such as the moisture content in the natural
310 environment, the innate ability of the animal to recover and specific conditions for entry into
311 anhydrobiosis and rehydration [7]. Species living in constantly moist habitats tend to have a
312 lower ability to tolerate drought by anhydrobiosis than those living in dry environments [11,
313 50]. It has been suggested that the upper limit of recovery from anhydrobiosis by tardigrades
314 can be counted in years but does not exceed ten years [51]. However, other available data
315 indicate that some tardigrade species can return to activity when the tun (desiccated) state
316 lasts for up to 15–22 years [e.g., 52]. For semi-terrestrial tardigrades, also represented by
317 *Pam. experimentalis*, the ability of anhydrobiosis has been studied, among others, for the
318 eutardigrade *Ramazzottius oberhaeuseri* and the heterotardigrade *Echiniscus* spp. It was
319 shown that under natural conditions *Ram. oberhaeuseri* recovered from the tun state lasting
320 1192 days with an average revival of 21.7% but could survive in this state for up to 1604
321 days. In *Echiniscus* spp., after 706 days of anhydrobiosis, the average revival was 9.9%, but
322 the species could tolerate the tun state lasting for up to 1085 days [8].

323 Because of the different species tested and the methods used, our study cannot be
324 directly compared with previous studies. Differences include, among others, the number and
325 duration of anhydrobiosis episodes. In the case of *Pam. experimentalis*, significant
326 differences in the number of active individuals after repeated anhydrobiosis persisted during
327 the initial observation times after rehydration (2–6h), but became insignificant over time,
328 indicating important differences in the rate of recovery. The observed return to activity at 48h
329 after repeated short and long anhydrobioses was consistent with our previous experiments on
330 *Pam. experimentalis* showing that this species has a high capacity for anhydrobiosis, as the
331 average recovery rate of individuals after 240 days of tun state was 43% [33]. However,
332 representatives of other populations of the species would need to be studied to verify
333 correlations between the conditions of the natural environment and anhydrobiosis ability for
334 this species. Previous studies are contradictory regarding population differences in
335 tardigrades` recovery from anhydrobiosis, which is explained by the intraspecific variation of
336 physiology and/or habitat properties [6, 33, 53].

337 We found a significant positive correlation, at least for the initial observation time
338 after rehydration, between return to activity and combination of the number and duration of
339 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 for *Pam. 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 and 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 *Pam. 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 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 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 [56]. 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 [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. However, we did not observe typical aggregations of individuals (Supplementary file, Figure S1). Therefore, it appears that under

certain conditions, formation of 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 [50]. 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 *Pam. 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 [57-58]. On the other hand, the absence of the aggregates may explain the lower return to activity after the first episode of long anhydrobiosis (30 days) when compared with the relevant data available for *Pam. experimentalis* [33], although the deterioration was not observed for the first episode of short anhydrobiosis (3 days). However, the age and sex were not considered in that study, and well as the shortest duration of the tun state was 7 days, that does not allow for deeper comparison.

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 [57]. In both species the ratio was determined for animals not undergoing anhydrobiosis and in the case of *Pam. experimentalis* in all age classes. Additionally, we observed only a marginally lower maximum lifespan for *Pam. 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 [59]. 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. [60], 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 [61].

CONCLUSIONS

The most significant predictor of recovery from repeated anhydrobiosis in *Pam. 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 analyzed in different populations of this species and other bisexual tardigrade species.

The analyzed 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.

Acknowledgment

The study was supported by the research grants of the National Science Centre, Poland, NCN 2016/21/B/NZ4/00131 and 2021/41/N/NZ3/01165, and partially conducted in the framework of activities of BARg (Biodiversity and Astrobiology Research Group). Amit Kumar Nagwani is a scholarship passport holder of the Interdisciplinary Doctoral Studies at the Faculty of Biology, Adam Mickiewicz University, POWR.03.02.00-00-I006/17. The authors would also like to thank Cambridge Proofreading LLC (http://proofreading.org) for their linguistic assistance. Technical support of Tomasz Bartylak and Pushpalata Kayastha is highly appreciated.

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.

442

References

1. Watanabe, M. Anhydrobiosis in invertebrates. *Appl. Entomol. Zool.* **41,** 15–31. https://doi.org/10.1303/aez.2006.15 (2006).
2. Rebecchi, L., Altiero, T. & Guidetti, R. Anhydrobiosis: The extreme limit of desiccation tolerance. *Invertebr. Surviv. J.* **4,** 65–81 (2007).
3. Rebecchi, L. Dry up and survive: The role of antioxidant defences in anhydrobiotic organisms. *J. Limnol.* **72,** 62–72. https://doi.org/10.4081/jlimnol.2013.s1.e8 (2013).
4. Kaczmarek, Ł. *et al.* Staying young and fit? Ontogenetic and phylogenetic consequences of animal anhydrobiosis. *J. Zool.* **309,** 1–11. https://doi.org/10.1111/jzo.12677 (2019).
5. Arakawa, K. Examples of extreme survival: Tardigrade genomics and molecular anhydrobiology. *Annu. Rev. Anim. Biosci.* **10,** 17–37. https://doi.org/10.1146/annurev–animal–021419–083711 (2022).
6. Jönsson, K. I. The nature of selection on anhydrobiotic capacity in tardigrade. *Zool. Anz.* **240,** 409–417. https://doi.org/10.1078/0044–5231–00049 (2001).
7. Wright, J. C. Desiccation tolerance and water–retentive mechanisms in tardigrades. *J. Exp. Biol.* **142,** 267–292. https://doi.org/10.1242/jeb.142.1.267 (1989).
8. Rebecchi, L., Guidetti, R., Borsari, S., Altiero, T. & Bertolani, R. Dynamics of long-term anhydrobiotic survival of lichen-dwelling tardigrades. *Hydrobiologia* **558,** 23–30. https://doi.org/10.1007/s10750-005-1415-7 (2006).
9. Roszkowska, M. *et al.* Tolerance to anhydrobiotic conditions among two coexisting tardigrade species differing in life strategies. *Zool. Stud.* **60,** 74. https://doi.org/10.6620/ZS.2021.60–74 (2021).
10. Schill, R. O. *et al.* Molecular mechanisms of tolerance in tardigrades: New perspectives for preservation and stabilization of biological material. *Biotechnol. Adv.* **27,** 348–352. https://doi.org/10.1016/j.biotechadv.2009.01.011 (2009).
11. Guidetti, R., Rizzo, A. M., Altiero, T. & Rebecchi, T. 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,** 97–102. https://doi.org/10.1016/j.pss.2012.05.021 (2012).
12. May, M. L'évolution des tardigrades de la vie aquatique à la vie Terrestre. *Bull. fr. pêche piscic.* **168,** 93–100 (1953).
13. Nelson, D. R., Guidetti, R. & Rebecchi, L. Phylum Tardigrada in *Thorp and Covich's Freshwater Invertebrates* (eds. James, H. Thorp, D. & Rogers, C.) 347-380

(Academic Press: Cambridge) https://doi.org/10.1016/B978–0–12–385026–3.00017–6 (2015).

14. Schill, R. O. & Hengherr, S. Environmental adaptations: Desiccation tolerance in *Water Bears: The Biology of Tardigrades* (ed. Schill, R.) 273-293 (Zoological Monographs, Springer: Switzerland). https://doi.org/10.1007/978–3–319–95702–9_10 (2018).
15. Hibshman, J. D., Clegg, J. S. & Goldstein, B. Mechanisms of desiccation tolerance: themes and variations in brine shrimp, roundworms, and tardigrades, *Front. Physiol*. **11,** 592016. https://doi.org/10.3389/fphys.2020.592016 (2020).
16. Soemme, L. Anhydrobiosis and cold tolerance in tardigrades. *Eur. J. Entomol*. **93,** 349–358 (1996).
17. Nagwani, A. K., Kaczmarek, Ł. & Kmita, H. Applicable life-history and molecular traits for studying the effects of anhydrobiosis on aging in tardigrades. *Diversity* **14,** 664. https://doi.org/10.3390/d14080664 (2022).
18. Bertolani, R. Evolution of the reproductive mechanisms in tardigrades–a review. *Zool. Anz.* **240,** 247–252. https://doi.org/10.1078/0044-5231-00032 (2001).
19. Sugiura, K., & Matsumoto, M. Sexual reproductive behaviours of tardigrades: a review. *Invertebr. Reprod. Dev.* **65,** 279–287. https://doi.org/10.1080/07924259.2021.1990142 (2021).
20. Crowe, J. H. The physiology of cryptobiosis in tardigrades. *Mem. Ist. Ital. Idrobiol*. **32,** 37–59 (1975).
21. Wright, J. C., Westh. P. & Ramløv, H. Cryptobiosis in Tardigrada. *Biol. Rev.* **67,** 1–29. https://doi.org/10.1111/j.1469–185X.2013.tb01657.x (1992).
22. Guidetti, R., Altiero, T., Bertolani. R., Grazioso, P. & Rebecchi, L**.** Survival of freezing by hydrated tardigrades inhabiting terrestrial and freshwater habitats. *Zool*. **114,** 123–128. https://doi.org/10.1016/j.zool.2010.11.005 (2011).
23. Horikawa, D. D. Survival of Tardigrades in Extreme Environments: A Model Animal for Astrobiology in *Anoxia: Evidence for Eukaryote Survival and Paleontological Strategies* (eds. Altenbach, A., Bernhard, J. & Seckbach, J.) 205–217 (Dordrecht: Springer Netherlands) https://doi.org/10.1007/978-94-007-1896-8_12 (2012).
24. Giovannini, I., Altiero, T., Guidetti, R. & Rebecchi, L. Will the Antarctic tardigrade *Acutuncus antarcticus* be able to withstand environmental stresses related to global climate change? *J. Exp. Biol.* **221,** jeb160622. https://doi.org/10.1242/jeb.160622 (2018).

25. Guidetti, R., Schill, R. O., Bertolani, R., Dandekar, T. & Wolf, M. New molecular data for tardigrade phylogeny, with the erection of *Paramacrobiotus* gen. nov. *J. Zoolog. Syst. Evol.* **47,** 315–321. https://doi.org/10.1111/j.1439-0469.2009.00526.x (2009).
26. Yoshida, Y. *et al*. Comparative genomics of the tardigrades *Hypsibius dujardini* and *Ramazzottius varieornatus*. *PLoS Biol.* **15,** e2002266. https://doi.org/10.1371/journal.pbio.2002266 (2017).
27. Jørgensen, A., Kristensen, R. M. & Møbjerg, N. Phylogeny and Integrative Taxonomy of Tardigrada in *Water Bears: The Biology of Tardigrades* (ed. Schill, R.) 95–114 (Zoological Monographs, Springer: Switzerland) https://doi.org/10.1007/978–3–319–95702–9_3 (2018).
28. Hesgrove, C. & Boothby, T. C. The biology of tardigrade disordered proteins in extreme stress tolerance. *Cell Commun. Signal.* **18,** 1–15. https://doi.org/10.1186/s12964-020-00670-2 (2020).
29. Jönsson, K. I. Tardigrades as a potential model organism in space research. *Astrobiology* **7,** 757–766. https://doi.org/10.1089/ast.2006.0088 (2007).
30. Jönsson, K. I. & Rebecchi, L. Experimentally induced anhydrobiosis in the tardigrade *Richtersius coronifer*: phenotypic factors affecting survival. *J. Exp. Zool.* **293,** 578–584. https://doi.org/10.1002/jez.10186 (2002).
31. Hengherr, S., Brümmer, F. & Schill, R. O. Anhydrobiosis in tardigrades and its effects on longevity traits, *J. Zool.* **275,** 216–220. https://doi.org/10.1111/j.1469-7998.2008.00427.x (2008)
32. Møbjerg, N. & Neves, R. C. New insights into survival strategies of tardigrades. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **254,** 110890. https://doi.org/10.1016/j.cbpa.2020.110890 (2021).
33. Roszkowska, M. *et al*. How long can tardigrades survive in anhydrobiotic state? Searching on tardigrade anhydrobiosis patterns. *PLoS One* **18,** e0270386. https://doi.org/10.1371/journal.pone.0270386 (2023).
34. Degma, P. & Guidetti, R. Actual checklist of Tardigrada species. (42th Edition, 09-01-2023) (2009-2023). (accessed on 15 October 2022) https://iris.unimore.it/retrieve/bf8e14a4-625f-4cdd-8100347e5cbc5f63/Actual%20checklist%20of%20Tardigrada%2042th%20Edition%2009-01-23.pdf

35. Czernekova, M. & Jönsson K. I. Experimentally induced repeated anhydrobiosis in the eutardigrade *Richtersius coronifer*. *PLoS One* **11,** e0164062. https://doi.org/10.1371/journal.pone.0164062 (2016).
36. Giovannini, I., Boothby. T. C., Cesari, M., Goldstein, B., Guidetti, G. & Rebecchi, L. Production of reactive oxygen species and involvement of bioprotectants during anhydrobiosis in the tardigrade *Paramacrobiotus spatialis*. *Sci. Rep.* **12,** 1938. https://doi.org/10.1038/s41598–022–05734–6 (2022).
37. Suzuki, A. C. Appearance of males in a thelytokous strain of *Milnesium* cf. *tardigradum* (Tardigrada). *Zool. Sci.* **25,** 849–853. https://doi.org/10.2108/zsj.25.849 (2008).
38. Neumann, S., Reuner, A., Brümmer, F. & Schill, R. O. DNA damage in storage cells of anhydrobiotic tardigrades. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **153,** 425–429. https://doi.org/10.1016/j.cbpa.2009.04.611 (2009).
39. Kinchin, I. M. The Biology of Tardigrades. *London: Portland Press.* 1–186. https://doi.org/10.1017/s002531540001540x (1994).
40. Kaczmarek, Ł. *et al.* Integrative description of bisexual *Paramacrobiotus experimentalis* sp. nov. (Macrobiotidae) from republic of Madagascar (Africa) with microbiome analysis. *Mol. Phylogenet. Evol.* **145,** 106730. https://doi.org/10.1016/j.ympev.2019.106730 (2020).
41. Bender, R., Lange, S. Adjusting for multiple testing - when and how? *J. Clin. Epidemiol.* **54,** 343–349. https://doi.org/10.1016/S0895–4356(00)00314–0 (2001).
42. Ricci, C. & Pagani, M. Desiccation of *Panagrolaimus rigidus* (Nematoda): Survival, reproduction and the influence on the internal clock. *Hydrobiologia* **347,** 1–13. https://doi.org/10.1023/a:1002979522816 (1997).
43. Suzuki, A. C. Life history of *Milnesium tardigradum* Doyère (Tardigrada) under a rearing environment. *Zool. Sci.* **20,** 49–57. https://doi.org/10.2108/zsj.20.49 (2003).
44. Schill, R. O. Life-history traits in the tardigrade species *Paramacrobiotus kenianus* and *Paramacrobiotus palaui*, *J. Limnol.* **72,** 160–165. https://doi.org/10.4081/jlimnol.2013.s1.e20 (2013).
45. Jönsson, K. I. & Bertolani, R. Facts and fiction about long-term survival in tardigrades. *J. Zool.* **255,** 121–123. https://doi.org/10.1017/s0952836901001169 (2001).

46. Schill, R. O. & Fritz, G. B. Desiccation tolerance in embryonic stages of the tardigrade. J. Zool. **276,** 103–107. https://doi.org/10.1111/j.1469-7998.2008.00474.x (2008).
47. Rebecchi, L., Boschetti, C. & Nelson, D. R. Extreme-tolerance mechanisms in meiofaunal organisms: A case study with tardigrades, rotifers and nematodes. *Hydrobiologia* **847,** 2779–2799. https://doi.org/10.1007/s10750-019-04144-6 (2020).
48. Ricci, C. Desiccation as a switch for some microinvertebrates. *ESA J.* 390:273 (1996).
49. Ricci, C. Anhydrobiotic capabilities of bdelloid rotifers. *Hydrobiologia* **387,** 321–26. https://doi.org/10.1007/978-94-011-4782-8_42 (1998).
50. Poprawa, I. *et al.* Verification of *Hypsibius exemplaris* Gąsiorek et al., 2018 (Eutardigrada; Hypsibiidae) application in anhydrobiosis research. *PLoS One* **17,** e0261485. https://doi.org/10.1371/journal.pone.0261485 (2022).
51. Guidetti, R. & Jönsson, K. I. Long-term anhydrobiotic survival in semi-terrestrial micrometazoans. *J. Zool.* **257,** 181–187. https://doi.org/10.1017/s095283690200078x (2002).
52. Roszkowska, M., Kmita, H. & Kaczmarek, Ł. Long-term anhydrobiosis in two taxa of moss dwelling Eutardigrada (Tardigrada) desiccated for 12 and 15 years, respectively. *Eur. Zool. J.* **87,** 642–647. https://doi.org/10.1080/24750263.2020.1829110 (2020).
53. Horikawa, D. D. & Higashi, S**.** Desiccation tolerance of the tardigrade *Milnesium tardigradum* collected in Sapporo, Japan, and Bogor, Indonesia. *Zool. Sci*. **21,** 813–816. https://doi.org/10.2108/zsj.21.813 (2004).
54. Madin, K. A. C. & Crowe, J. H. Anhydrobiosis in nematodes: Carbohydrate and lipid metabolism during dehydration. *J. Exp. Zool.* **193,** 335–342. https://doi.org/10.1002/jez.1401930309 (1975).
55. Sieger, J., Brümmer, F., Ahn, H., Lee, G., Kim, S. & Schill, R.O. Reduced ageing in the frozen state in the tardigrade *Milnesium inceptum* (Eutardigrada: Apochela). *J. Zool*. **318,** 253-259. https://doi.org/10.1111/jzo.13018 (2022).
56. Ivarsson, H. & Jönsson, K. I. Aggregation effects on anhydrobiotic survival in the tardigrade *Richtersius coronifer*. *J. Exp. Zool. A Ecol. Integr. Physiol.* **301,** 195–199. https://doi.org/10.1002/jez.a.20018 (2004).

609 57. Sugiura, K., Minato, H., Suzuki, A. C., Arakawa, K., Kunieda, T., & Matsumoto, M..
610 Comparison of sexual reproductive behaviors in two species of Macrobiotidae
611 (Tardigrada: Eutardigrada). *Zool. Sci.* 36(2), 120-127.
612 https://doi.org/10.2108/zs180103 (2019).
613 58. Bartel, S. & Hohberg, K. Experimental investigations on the partner-finding
614 behaviour of *Isohypsibius dastychi* (Isohypsibiidae: Tardigrada). *Zool. J. Linn. Soc.*
615 **188(3),** 878–886. https://doi.org/10.1093/zoolinnean/zlz076 (2020).
616 59. Kobayashi, K., & Hasegawa, E. A female-biased sex ratio reduces the twofold cost of
617 sex. *Sci. Rep.* **6**(1), 23982. https://doi.org/10.1038/srep23982 (2016).
618 60. Bertolani, R. Sex ratio and geographic parthenogenesis in *Macrobiotus* (Tardigrada).
619 *Experientia.* **28,** 94–95. https://doi.org/10.1007/bf01928285 (1972).
620 61. Bertolani, R., Rebecchi, L. & Beccaccioli, G. Dispersal of *Ramazzottius* and other
621 tardigrades in relation to type of reproduction. *Invertebr. Reprod. Dev.* **18,** 153–57.
622 https://doi.org/10.1080/07924259.1990.9672137 (1990).
623

Figures and captions

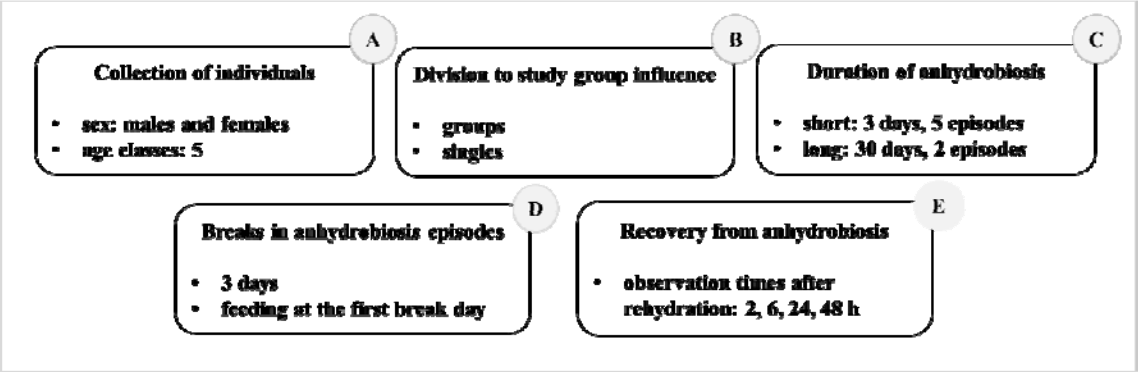


Figure 1. Graphic representation of the repeated anhydrobiosis experiment performed in *Paramacrobiotus experimenatlis*. A-E, the applied steps of the experiments. The term "anhydrobiosis duration" refers to combination of the number and duration of anhydrobiosis episodes.

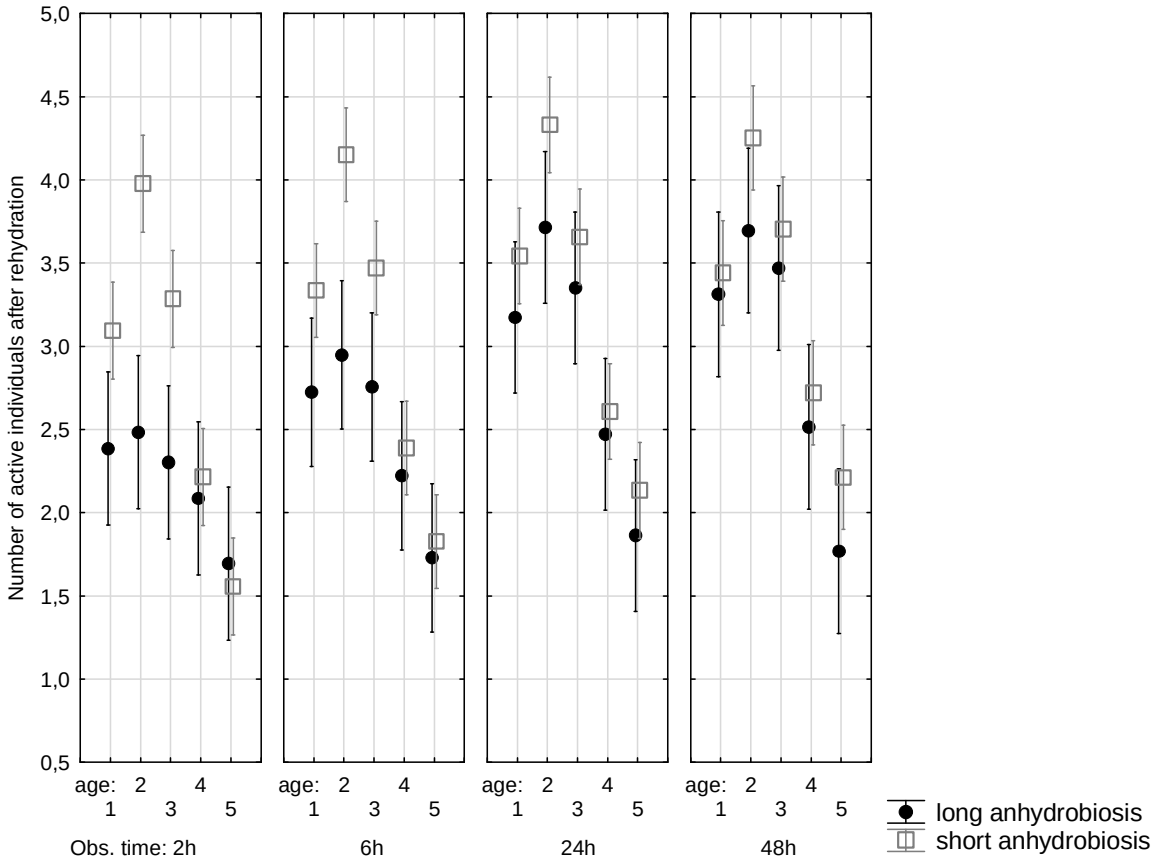


Figure 2. Number of active individuals of *Paramacrobiotus experimentalis* observed at 2h-48h after rehydration, taking into account their age and the combined effect of the number and duration of anhydrobiosis episodes (long and short anhydrobiosis). Age (1-5); the selected age classes; Obs. time -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

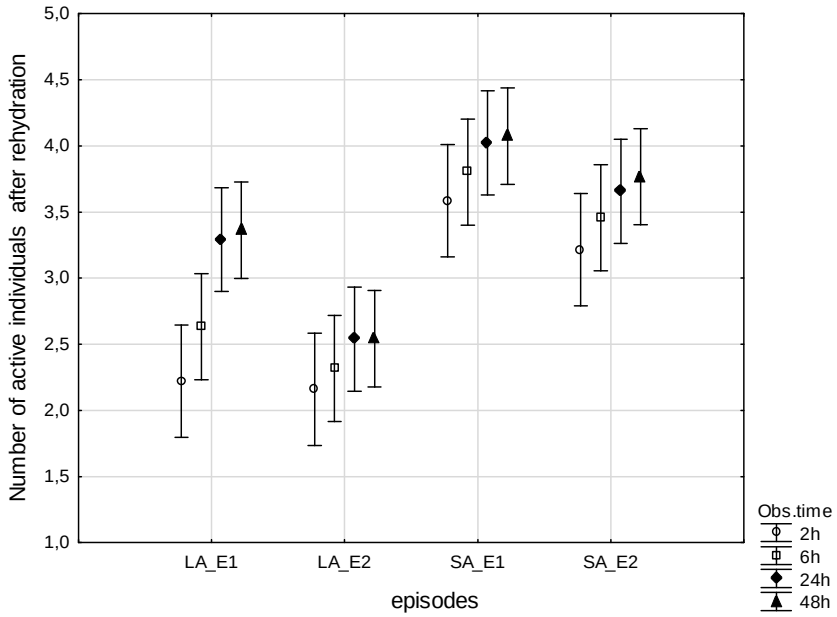


Figure 3. Number of active individuals of *Paramacrobiotus experimentalis* observed at 2h-48h after rehydration following the first two episodes of short and long anhydrobiosis. LA_E1 and LA_E2, the first and second episode of long anhydrobiosis; SA_E1 and SA_E2, the first and second episode of short anhydrobiosis; Obs. time - observation time.

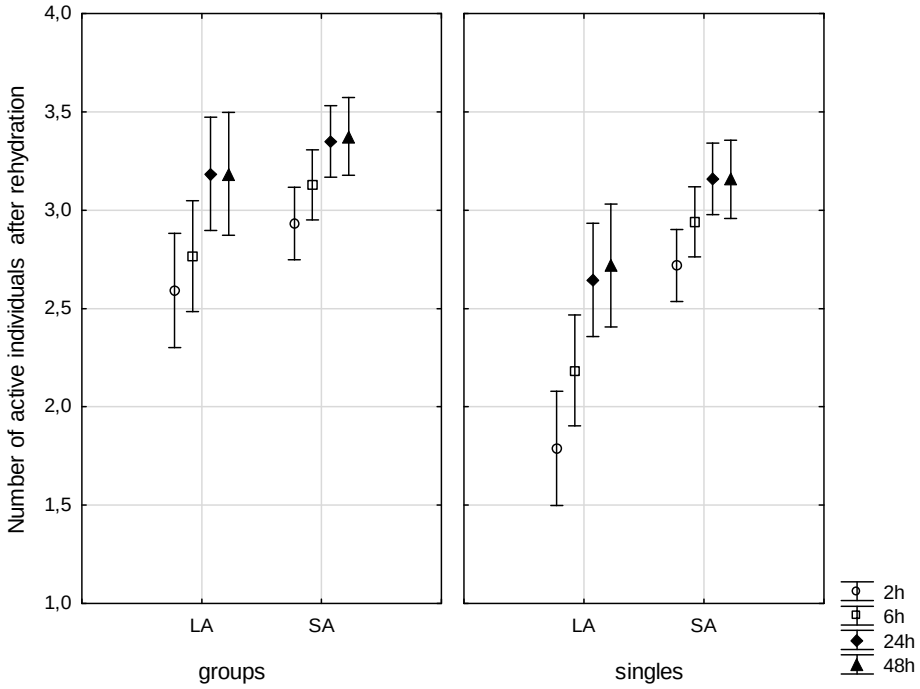


Figure 4. Number of active individuals of *Paramacrobiotus experimentalis* according to the combined effect of number and duration of anhydrobiosis episodes, and observation times after rehydration (2h-48h; F(3, 300)=2.687, *p*=0.047). LA and SA, long and short anhydrobiosis, respectively; Obs. time - observation time. Expected marginal means and 95% confidence intervals are shown.

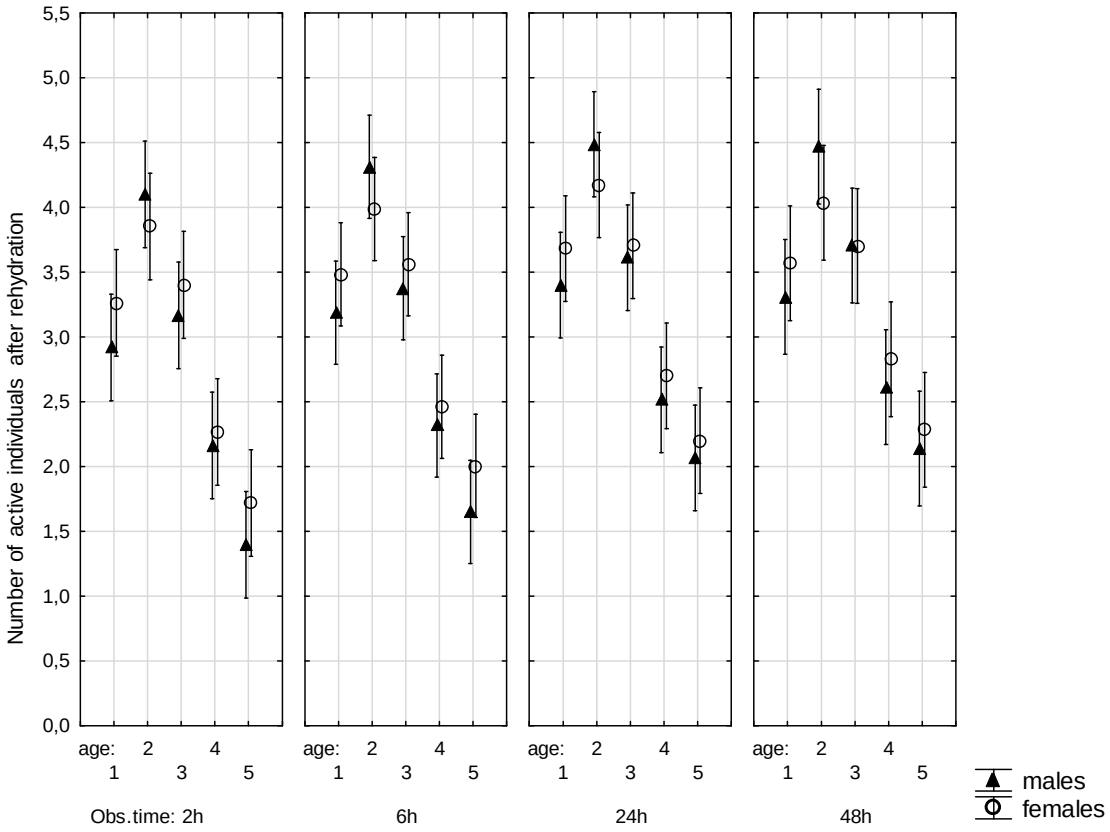


Figure 5. Number of active individuals of *Paramacrobiotus experimentalis* in the context of their sex and age, as well as observation time after rehydration. Age (1-5); the selected age classes; Obs. time; observation times. Expected marginal means and 95% confidence intervals are shown; see the Materials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

Cultures of Paramacrobiotus experimentalis

Individuals of *Pam. experimentalis* were extracted in 2019 from moss samples collected in a tropical forest located in the Toamasina Province in Eastern Madagascar (18°56′37″S, 48°30′52″E, 717 m asl). The region is known for long periods of drought that recur annually, probably due to El Niño events [1]. The Latin name "*experimentalis*" refers to the species being easy to culture and valuable in different types of research, including studies on anhydrobiosis [2]. It is a bisexual species, and sex differences between males and females concern body size and primary sexual characteristics (unpaired internal genitalia). However, the ratio of males to females in a population (the sex ratio) for this species has not been determined yet.

The set of founder individuals was extracted from the moss sample according to the standard method [3]. In short, specimens were isolated by placing moss in a beaker filled with ddH_2O up to 250 ml. After 6h, the moss was vigorously shaken with the use of metal tweezers. Then, the moss was squeezed over the beaker. The supernatant containing tardigrades as well as moss particles was stirred and poured into a 250 ml cylinder. After 30 min (when all particles fell onto the cylinder bottom), the top of ca. 150 ml of water was discarded. The remaining 50 ml was stirred and poured onto Petri dishes. Tardigrades found were extracted with a fine Pasteur pipette.

Females and males were reared together and the laid eggs were collected to prepare, and maintain a living assemblage of mixed-age animals. The assemblage was divided into groups that differed in age by a month. The collected eggs were used to form subsequent groups. The groups were kept in separate Petri dishes and characterized by selected life history traits (Table S1), including vitality rate (calculated as the ratio of active individuals and the total number of individuals, i.e., active and inactive), average total body length, and fertility (measured by the average number of eggs laid per female). Females and males were distinguished based on morphological characteristics, i.e., body shape (males are more slender than females), total body length (males are smaller than females), and the presence of eggs in the female's ovary. The accuracy of sexes identification was confirmed by observing gonads as described in [4]. Total body length and the presence of eggs were assessed using a stereomicroscope (OLYMPUS SZ61). The calibrated grid of the stereomicroscope was used to measure the total body length of animals within a given area with an accuracy of 10 µm.

1. Gautier, L., Lowry II, P. P. & Goodman, S. M. in *The New Natural History of Madagascar* (ed. Goodman, S. M.) 452–464 (Princeton University Press: New Jersey) (2022).

2. Roszkowska, M. *et al.* Tips and tricks how to culture water bears: simple protocols for culturing eutardigrades (Tardigrada) under laboratory conditions. *Eur. Zool. J.* **88,** 449–465. https://doi.org/10.1080/24750263.2021.1881631 (2021b).
3. Stec, D., Smolak, R., Kaczmarek, Ł. & Michalczyk, Ł. An integrative description of *Macrobiotus paulinae* sp. nov. (Tardigrada: Eutardigrada: Macrobiotidae: *hufelandi* group) from Kenya. *Zootaxa*, **4052,** 501–526 https://doi.org/10.11646/zootaxa.4052.5.1 (2015).
4. Kaczmarek, Ł. *et al.* Integrative description of bisexual Paramacrobiotus experimentalis sp. nov. (Macrobiotidae) from republic of Madagascar (Africa) with microbiome analysis. *Mol. Phylogenet. Evol.* **145**, 106730. https://doi.org/10.1016/j.ympev.2019.106730 (2020).

Table S1. Characterisation of age classes distinguished for *Paramacrobiotus experimentalis* based on the determined life history trait analysis. The vitality rate denotes the ratio of active individuals in the given age class and the total number of individuals in that class (i.e., active and inactive). Abbreviations: n, the sample size; ND, no data

Age classes	**Age-range in days (assigned group name)**	**Vitality rate (± SD) [%]**	**Average body length (± SD) [µm]**		**Average number of laid eggs per female (± SD)**
			Females	**Males**	
1	60-90 (growing adults)	75.4 ± 6.7	530 ± 30 n = 25	430 ± 20 n = 25	123 ± 12 n = 100
2	120-150 (young adults)	79.0 ± 2.4	750 ± 30 n = 25	570 ± 50 n = 25	142 ± 21 n = 80
3	150-180 (mature adults)	81.4 ± 3.8	750 ± 40 n = 20	590 ± 50 n = 20	162 ± 32 n = 70
4	240-270 (late-age adults)	52.1 ± 0.3	750 ± 30 n = 15	600 ± 30 n = 15	60 ± 8 n = 40
5	>300 (old adults)	40.3 ± 0.5	750 ± 30 n = 10	600 ± 30 n = 10	ND

Table S2. Number of *Paramacrobiotus experimentalis* individuals that returned to activity after each of anhydrobiosis episodes. Individuals that returned to activity after the previous episode were used in the next one. Each variant of the experiment included 3 replicates of 10 individuals at its beginning.

Females: five episodes of short anhydrobiosis

Females_groups								Females_singles							
				Observation time following rehydration								Observation time following rehydration			
Age	Anhydrobiosis duration	Number of anhydrobiosis episode	Replicates	2h	6h	24h	48h	Age	Anhydrobiosis duration	Number of anhydrobiosis episode	Replicates	2h	6h	24h	48h
60-90 days	3 days	1	1	7	7	8	8	60-90 days	3 days	1	1	5	5	6	6
60-90 days	3 days	1	2	7	7	7	7	60-90 days	3 days	1	2	5	5	7	7
60-90 days	3 days	1	3	6	7	8	8	60-90 days	3 days	1	3	5	6	6	6
60-90 days	3 days	2	1	6	6	6	6	60-90 days	3 days	2	1	4	5	6	6
60-90 days	3 days	2	2	5	6	6	6	60-90 days	3 days	2	2	5	6	6	6
60-90 days	3 days	2	3	6	6	6	7	60-90 days	3 days	2	3	3	4	4	4
60-90 days	3 days	3	1	4	4	5	5	60-90 days	3 days	3	1	3	3	5	5
60-90 days	3 days	3	2	4	4	4	4	60-90 days	3 days	3	2	2	3	3	3
60-90 days	3 days	3	3	5	6	6	6	60-90 days	3 days	3	3	3	4	4	4
60-90 days	3 days	4	1	2	2	3	3	60-90 days	3 days	4	1	2	3	4	4
60-90 days	3 days	4	2	2	3	3	3	60-90 days	3 days	4	2	2	2	2	2
60-90 days	3 days	4	3	3	3	4	4	60-90 days	3 days	4	3	2	3	3	3
60-90 days	3 days	5	1	1	1	1	1	60-90 days	3 days	5	1	1	1	2	2
60-90 days	3 days	5	2	1	1	2	2	60-90 days	3 days	5	2	1	1	1	1
60-90 days	3 days	5	3	1	2	2	3	60-90 days	3 days	5	3	1	2	2	2
120-150 days	3 days	1	1	8	8	9	8	120-150 days	3 days	1	1	7	7	8	8
120-150 days	3 days	1	2	9	9	9	8	120-150 days	3 days	1	2	7	7	7	8
120-150 days	3 days	1	3	7	8	9	9	120-150 days	3 days	1	3	8	8	8	8
120-150 days	3 days	2	1	6	6	7	6	120-150 days	3 days	2	1	6	7	7	7
120-150 days	3 days	2	2	6	6	7	7	120-150 days	3 days	2	2	5	6	6	6
120-150 days	3 days	2	3	6	7	8	7	120-150 days	3 days	2	3	6	6	7	7
120-150 days	3 days	3	1	4	4	5	5	120-150 days	3 days	3	1	5	5	6	6
120-150 days	3 days	3	2	5	5	5	5	120-150 days	3 days	3	2	5	5	5	5
120-150 days	3 days	3	3	5	6	6	5	120-150 days	3 days	3	3	5	5	6	6
120-150 days	3 days	4	1	3	3	4	4	120-150 days	3 days	4	1	4	4	5	5
120-150 days	3 days	4	2	2	3	4	4	120-150 days	3 days	4	2	3	4	4	4
120-150 days	3 days	4	3	3	4	3	3	120-150 days	3 days	4	3	4	5	5	5
120-150 days	3 days	5	1	2	2	3	3	120-150 days	3 days	5	1	3	3	4	4
120-150 days	3 days	5	2	2	3	3	4	120-150 days	3 days	5	2	3	3	3	4
120-150 days	3 days	5	3	2	2	2	2	120-150 days	3 days	5	3	3	3	4	4
150-180 days	3 days	1	1	7	8	8	8	150-180 days	3 days	1	1	6	6	7	7
150-180 days	3 days	1	2	7	7	8	8	150-180 days	3 days	1	2	6	7	7	7
150-180 days	3 days	1	3	7	7	7	7	150-180 days	3 days	1	3	6	6	6	8
150-180 days	3 days	2	1	5	5	6	6	150-180 days	3 days	2	1	4	5	5	6
150-180 days	3 days	2	2	5	5	5	6	150-180 days	3 days	2	2	4	4	5	5
150-180 days	3 days	2	3	5	6	6	6	150-180 days	3 days	2	3	4	6	6	7
150-180 days	3 days	3	1	3	3	4	4	150-180 days	3 days	3	1	4	4	4	5
150-180 days	3 days	3	2	4	5	5	5	150-180 days	3 days	3	2	4	4	5	5
150-180 days	3 days	3	3	4	4	4	4	150-180 days	3 days	3	3	4	4	5	5
150-180 days	3 days	4	1	2	2	2	3	150-180 days	3 days	4	1	3	3	4	4
150-180 days	3 days	4	2	2	3	3	3	150-180 days	3 days	4	2	3	4	4	4
150-180 days	3 days	4	3	2	3	3	4	150-180 days	3 days	4	3	3	3	4	4
150-180 days	3 days	5	1	2	2	2	3	150-180 days	3 days	5	1	3	3	3	3
150-180 days	3 days	5	2	2	2	3	3	150-180 days	3 days	5	2	2	2	3	4
150-180 days	3 days	5	3	1	2	2	2	150-180 days	3 days	5	3	2	3	3	3
240-270 days	3 days	1	1	3	4	4	4	240-270 days	3 days	1	1	2	3	3	4
240-270 days	3 days	1	2	3	3	4	4	240-270 days	3 days	1	2	2	2	4	5
240-270 days	3 days	1	3	4	5	5	6	240-270 days	3 days	1	3	2	3	3	3
240-270 days	3 days	2	1	2	3	3	4	240-270 days	3 days	2	1	1	2	2	3
240-270 days	3 days	2	2	2	2	2	3	240-270 days	3 days	2	2	2	2	3	4
240-270 days	3 days	2	3	3	3	4	5	240-270 days	3 days	2	3	1	2	2	3
240-270 days	3 days	3	1	2	2	2	3	240-270 days	3 days	3	1	1	1	2	3
240-270 days	3 days	3	2	1	1	2	3	240-270 days	3 days	3	2	1	2	2	3
240-270 days	3 days	3	3	2	3	3	4	240-270 days	3 days	3	3	1	1	2	2
240-270 days	3 days	4	1	1	1	2	2	240-270 days	3 days	4	1	1	1	1	2
240-270 days	3 days	4	2	1	1	1	2	240-270 days	3 days	4	2	1	1	2	3
240-270 days	3 days	4	3	1	2	2	3	240-270 days	3 days	4	3	1	1	1	1
240-270 days	3 days	5	1	1	1	2	2	240-270 days	3 days	5	1	1	1	1	1
240-270 days	3 days	5	2	1	1	1	1	240-270 days	3 days	5	2	1	1	2	2
240-270 days	3 days	5	3	1	1	2	2	240-270 days	3 days	5	3	0	0	0	1
>300 days	3 days	1	1	1	2	2	4	>300 days	3 days	1	1	0	1	1	2
>300 days	3 days	1	2	2	2	3	4	>300 days	3 days	1	2	2	4	5	4
>300 days	3 days	1	3	1	1	2	2	>300 days	3 days	1	3	1	2	3	4
>300 days	3 days	2	1	1	1	2	3	>300 days	3 days	2	1	0	2	2	2
>300 days	3 days	2	2	2	2	2	3	>300 days	3 days	2	2	1	1	1	4
>300 days	3 days	2	3	1	1	1	2	>300 days	3 days	2	3	1	1	1	1
>300 days	3 days	3	1	1	1	2	2	>300 days	3 days	3	1	1	1	1	1
>300 days	3 days	3	2	1	1	2	2	>300 days	3 days	3	2	0	1	2	4
>300 days	3 days	3	3	1	1	1	2	>300 days	3 days	3	3	0	0	0	1
>300 days	3 days	4	1	1	1	1	1	>300 days	3 days	4	1	1	1	1	1
>300 days	3 days	4	2	0	1	1	1	>300 days	3 days	4	2	0	0	1	2
>300 days	3 days	4	3	1	1	1	2	>300 days	3 days	4	3	0	0	0	0
>300 days	3 days	5	1	0	1	1	1	>300 days	3 days	5	1	0	1	1	1
>300 days	3 days	5	2	0	0	0	0	>300 days	3 days	5	2	0	0	0	0
>300 days	3 days	5	3	1	1	1	1	>300 days	3 days	5	3	0	0	0	0

Males: five episodes of short anhydrobiosis

Males_groups

Age	Anhydrobiosis duration	Number of anhydrobiosis episode	Replicates	Observation time following rehydration			
				2h	6h	24h	48h
60-90 days	3 days	1	1	6	6	7	7
60-90 days	3 days	1	2	6	7	7	8
60-90 days	3 days	1	3	6	6	6	6
60-90 days	3 days	2	1	4	5	6	6
60-90 days	3 days	2	2	5	5	5	7
60-90 days	3 days	2	3	4	4	5	5
60-90 days	3 days	3	1	3	4	5	5
60-90 days	3 days	3	2	3	3	5	5
60-90 days	3 days	3	3	2	2	3	4
60-90 days	3 days	4	1	2	3	3	3
60-90 days	3 days	4	2	2	2	3	3
60-90 days	3 days	4	3	2	4	4	4
60-90 days	3 days	5	1	1	1	2	2
60-90 days	3 days	5	2	1	1	1	1
60-90 days	3 days	5	3	1	1	1	2
120-150 days	3 days	1	1	7	8	9	9
120-150 days	3 days	1	2	6	7	8	8
120-150 days	3 days	1	3	7	9	9	9
120-150 days	3 days	2	1	6	6	7	8
120-150 days	3 days	2	2	6	7	7	7
120-150 days	3 days	2	3	7	7	8	8
120-150 days	3 days	3	1	5	6	7	7
120-150 days	3 days	3	2	5	6	6	6
120-150 days	3 days	3	3	6	6	7	7
120-150 days	3 days	4	1	5	5	6	6
120-150 days	3 days	4	2	4	5	6	6
120-150 days	3 days	4	3	4	5	5	6
120-150 days	3 days	5	1	4	4	4	5
120-150 days	3 days	5	2	3	4	5	5
120-150 days	3 days	5	3	4	5	5	5
150-180 days	3 days	1	1	7	7	7	8
150-180 days	3 days	1	2	6	6	7	7
150-180 days	3 days	1	3	6	7	7	7
150-180 days	3 days	2	1	4	4	5	6
150-180 days	3 days	2	2	4	5	5	5
150-180 days	3 days	2	3	4	4	4	6
150-180 days	3 days	3	1	3	3	4	6
150-180 days	3 days	3	2	2	3	3	5
150-180 days	3 days	3	3	3	3	4	6
150-180 days	3 days	4	1	2	3	3	3
150-180 days	3 days	4	2	2	2	3	3
150-180 days	3 days	4	3	2	2	3	3
150-180 days	3 days	5	1	1	2	2	3
150-180 days	3 days	5	2	1	1	2	2
150-180 days	3 days	5	3	1	2	2	2
240-270 days	3 days	1	1	2	2	3	4
240-270 days	3 days	1	2	2	2	2	3
240-270 days	3 days	1	3	2	3	3	3
240-270 days	3 days	2	1	1	2	2	3
240-270 days	3 days	2	2	1	1	2	3
240-270 days	3 days	2	3	1	1	2	2
240-270 days	3 days	3	1	1	1	1	1
240-270 days	3 days	3	2	1	1	2	3
240-270 days	3 days	3	3	1	1	1	2
240-270 days	3 days	4	1	0	1	1	1
240-270 days	3 days	4	2	1	1	2	3
240-270 days	3 days	4	3	1	1	1	1
240-270 days	3 days	5	1	0	1	1	1
240-270 days	3 days	5	2	1	1	2	2
240-270 days	3 days	5	3	1	1	1	1
>300 days	3 days	1	1	0	1	2	3
>300 days	3 days	1	2	1	2	3	4
>300 days	3 days	1	3	1	1	1	1
>300 days	3 days	2	1	0	1	1	3
>300 days	3 days	2	2	1	1	2	3
>300 days	3 days	2	3	0	0	1	1
>300 days	3 days	3	1	0	0	1	2
>300 days	3 days	3	2	1	1	1	2
>300 days	3 days	3	3	0	0	1	1
>300 days	3 days	4	1	0	1	1	1
>300 days	3 days	4	2	0	0	1	2
>300 days	3 days	4	3	0	0	0	1
>300 days	3 days	5	1	0	0	0	0
>300 days	3 days	5	2	0	1	1	1
>300 days	3 days	5	3	0	0	0	0

Males_singles

Age	Anhydrobiosis duration	Number of anhydrobiosis episode	Replicates	Observation time following rehydration			
				2h	6h	24h	48h
60-90 days	3 days	1	1	5	6	6	6
60-90 days	3 days	1	2	4	5	5	5
60-90 days	3 days	1	3	4	4	5	5
60-90 days	3 days	2	1	3	4	5	5
60-90 days	3 days	2	2	3	4	4	4
60-90 days	3 days	2	3	3	4	4	4
60-90 days	3 days	3	1	2	3	4	4
60-90 days	3 days	3	2	2	3	3	3
60-90 days	3 days	3	3	3	3	3	3
60-90 days	3 days	4	1	1	2	3	3
60-90 days	3 days	4	2	1	2	2	2
60-90 days	3 days	4	3	1	1	2	2
60-90 days	3 days	5	1	1	1	1	1
60-90 days	3 days	5	2	0	1	1	1
60-90 days	3 days	5	3	1	1	1	1
120-150 days	3 days	1	1	6	7	7	7
120-150 days	3 days	1	2	7	7	7	7
120-150 days	3 days	1	3	8	9	9	9
120-150 days	3 days	2	1	6	6	6	7
120-150 days	3 days	2	2	5	6	6	6
120-150 days	3 days	2	3	8	8	9	9
120-150 days	3 days	3	1	4	4	4	6
120-150 days	3 days	3	2	4	5	5	5
120-150 days	3 days	3	3	7	7	8	8
120-150 days	3 days	4	1	4	5	5	5
120-150 days	3 days	4	2	5	5	6	6
120-150 days	3 days	4	3	6	7	7	8
120-150 days	3 days	5	1	3	3	5	5
120-150 days	3 days	5	2	3	3	4	4
120-150 days	3 days	5	3	5	6	6	7
150-180 days	3 days	1	1	5	6	6	7
150-180 days	3 days	1	2	5	5	6	7
150-180 days	3 days	1	3	5	5	6	7
150-180 days	3 days	2	1	3	4	4	5
150-180 days	3 days	2	2	3	5	5	6
150-180 days	3 days	2	3	4	4	6	6
150-180 days	3 days	3	1	3	3	4	4
150-180 days	3 days	3	2	2	3	4	4
150-180 days	3 days	3	3	3	3	4	5
150-180 days	3 days	4	1	3	3	3	3
150-180 days	3 days	4	2	2	3	3	3
150-180 days	3 days	4	3	3	3	4	4
150-180 days	3 days	5	1	2	3	3	3
150-180 days	3 days	5	2	2	2	2	3
150-180 days	3 days	5	3	2	2	3	3
240-270 days	3 days	1	1	2	2	2	2
240-270 days	3 days	1	2	2	2	2	4
240-270 days	3 days	1	3	1	2	2	2
240-270 days	3 days	2	1	1	1	1	2
240-270 days	3 days	2	2	1	2	3	3
240-270 days	3 days	2	3	1	1	1	2
240-270 days	3 days	3	1	1	1	2	2
240-270 days	3 days	3	2	1	1	1	2
240-270 days	3 days	3	3	1	1	1	2
240-270 days	3 days	4	1	1	1	1	1
240-270 days	3 days	4	2	0	0	2	2
240-270 days	3 days	4	3	1	1	1	2
240-270 days	3 days	5	1	1	1	1	1
240-270 days	3 days	5	2	0	1	1	2
240-270 days	3 days	5	3	1	1	1	1
>300 days	3 days	1	1	0	0	2	3
>300 days	3 days	1	2	1	1	2	3
>300 days	3 days	1	3	0	0	0	2
>300 days	3 days	2	1	1	1	3	3
>300 days	3 days	2	2	0	0	1	2
>300 days	3 days	2	3	0	1	1	1
>300 days	3 days	3	1	0	0	1	2
>300 days	3 days	3	2	0	1	1	1
>300 days	3 days	3	3	1	1	1	1
>300 days	3 days	4	1	0	0	0	0
>300 days	3 days	4	2	0	0	1	1
>300 days	3 days	4	3	1	1	1	1
>300 days	3 days	5	1	0	0	0	0
>300 days	3 days	5	2	0	0	0	0
>300 days	3 days	5	3	0	1	1	1

Females: two episodes of long anhydrobiosis

Females_groups

Age	Anhydrobiosis duration	Number of anhydrobiosis episode	Replicates	Observation time following rehydration 2h	6h	24h	48h
60-90 days	30 days	1	1	3	3	4	5
60-90 days	30 days	1	2	2	4	6	6
60-90 days	30 days	1	3	3	4	6	6
60-90 days	30 days	2	1	2	3	3	3
60-90 days	30 days	2	2	1	2	2	4
60-90 days	30 days	2	3	2	2	2	2
120-150 days	30 days	1	1	3	4	6	7
120-150 days	30 days	1	2	2	3	5	6
120-150 days	30 days	1	3	3	3	5	6
120-150 days	30 days	2	1	2	4	5	5
120-150 days	30 days	2	2	3	4	4	5
120-150 days	30 days	2	3	3	4	5	5
150-180 days	30 days	1	1	2	3	5	6
150-180 days	30 days	1	2	2	3	5	6
150-180 days	30 days	1	3	3	3	5	6
150-180 days	30 days	2	1	2	3	4	4
150-180 days	30 days	2	2	2	3	4	4
150-180 days	30 days	2	3	2	3	4	5
240-270 days	30 days	1	1	2	2	2	4
240-270 days	30 days	1	2	2	2	3	3
240-270 days	30 days	1	3	2	2	3	3
240-270 days	30 days	2	1	2	2	2	3
240-270 days	30 days	2	2	1	1	2	2
240-270 days	30 days	2	3	2	2	2	2
>300 days	30 days	1	1	1	2	2	2
>300 days	30 days	1	2	1	1	1	1
>300 days	30 days	1	3	1	2	3	3
>300 days	30 days	2	1	0	1	1	1
>300 days	30 days	2	2	0	0	0	0
>300 days	30 days	2	3	0	1	1	1

Females_singles

Age	Anhydrobiosis duration	Number of anhydrobiosis episode	Replicates	Observation time following rehydration 2h	6h	24h	48h
60-90 days	30 days	1	1	2	3	5	5
60-90 days	30 days	1	2	2	3	4	4
60-90 days	30 days	1	3	2	3	4	5
60-90 days	30 days	2	1	1	2	3	3
60-90 days	30 days	2	2	1	1	2	3
60-90 days	30 days	2	3	1	2	2	4
120-150 days	30 days	1	1	2	2	5	6
120-150 days	30 days	1	2	2	5	4	3
120-150 days	30 days	1	3	2	2	5	6
120-150 days	30 days	2	1	3	4	5	5
120-150 days	30 days	2	2	1	1	2	3
120-150 days	30 days	2	3	3	4	4	5
150-180 days	30 days	1	1	2	3	4	5
150-180 days	30 days	1	2	2	2	4	3
150-180 days	30 days	1	3	2	2	3	5
150-180 days	30 days	2	1	2	3	4	4
150-180 days	30 days	2	2	2	2	3	3
150-180 days	30 days	2	3	2	3	3	4
240-270 days	30 days	1	1	1	2	2	3
240-270 days	30 days	1	2	1	1	2	2
240-270 days	30 days	1	3	1	2	2	3
240-270 days	30 days	2	1	1	1	1	2
240-270 days	30 days	2	2	0	1	1	1
240-270 days	30 days	2	3	1	1	2	2
>300 days	30 days	1	1	1	1	1	1
>300 days	30 days	1	2	0	1	1	1
>300 days	30 days	1	3	0	2	4	5
>300 days	30 days	2	1	0	0	0	0
>300 days	30 days	2	2	0	0	0	0
>300 days	30 days	2	3	0	1	1	2

Males: two episodes of long anhydrobiosis

Males_groups

Age	Anhydrobiosis duration	Number of anhydrobiosis episode	Replicates	Observation time following rehydration 2h	6h	24h	48h
60-90 days	30 days	1	1	1	2	4	5
60-90 days	30 days	1	2	1	3	5	5
60-90 days	30 days	1	3	1	3	5	6
60-90 days	30 days	2	1	1	2	2	3
60-90 days	30 days	2	2	1	1	3	3
60-90 days	30 days	2	3	2	3	3	4
120-150 days	30 days	1	1	0	3	6	6
120-150 days	30 days	1	2	0	4	8	6
120-150 days	30 days	1	3	4	4	9	8
120-150 days	30 days	2	1	2	3	3	3
120-150 days	30 days	2	2	5	5	5	4
120-150 days	30 days	2	3	6	6	6	5
150-180 days	30 days	1	1	1	2	4	5
150-180 days	30 days	1	2	1	3	5	6
150-180 days	30 days	1	3	1	3	5	6
150-180 days	30 days	2	1	2	3	3	4
150-180 days	30 days	2	2	3	3	3	3
150-180 days	30 days	2	3	2	3	4	4
240-270 days	30 days	1	1	1	2	3	3
240-270 days	30 days	1	2	2	2	2	3
240-270 days	30 days	1	3	2	2	2	2
240-270 days	30 days	2	1	1	1	2	2
240-270 days	30 days	2	2	1	0	1	2
240-270 days	30 days	2	3	1	1	1	1
>300 days	30 days	1	1	1	1	2	2
>300 days	30 days	1	2	0	1	1	1
>300 days	30 days	1	3	1	1	1	2
>300 days	30 days	2	1	0	0	0	0
>300 days	30 days	2	2	0	0	0	0
>300 days	30 days	2	3	0	0	1	1

Males_singles

Age	Anhydrobiosis duration	Number of anhydrobiosis episode	Replicates	Observation time following rehydration 2h	6h	24h	48h
60-90 days	30 days	1	1	1	1	2	4
60-90 days	30 days	1	2	1	1	3	4
60-90 days	30 days	1	3	0	2	3	3
60-90 days	30 days	2	1	1	1	1	1
60-90 days	30 days	2	2	0	1	1	2
60-90 days	30 days	2	3	0	1	1	1
120-150 days	30 days	1	1	0	0	2	3
120-150 days	30 days	1	2	0	0	4	5
120-150 days	30 days	1	3	0	0	3	4
120-150 days	30 days	2	1	1	1	2	1
120-150 days	30 days	2	2	0	1	2	3
120-150 days	30 days	2	3	0	1	1	2
150-180 days	30 days	1	1	0	1	2	3
150-180 days	30 days	1	2	0	1	3	4
150-180 days	30 days	1	3	1	1	3	4
150-180 days	30 days	2	1	0	1	2	2
150-180 days	30 days	2	2	1	0	1	1
150-180 days	30 days	2	3	0	1	1	2
240-270 days	30 days	1	1	1	2	2	2
240-270 days	30 days	1	2	1	1	1	1
240-270 days	30 days	1	3	1	2	2	3
240-270 days	30 days	2	1	1	1	1	1
240-270 days	30 days	2	2	0	0	1	1
240-270 days	30 days	2	3	0	0	0	1
>300 days	30 days	1	1	0	2	2	2
>300 days	30 days	1	2	1	1	2	2
>300 days	30 days	1	3	0	0	0	0
>300 days	30 days	2	1	0	0	0	0
>300 days	30 days	2	2	0	0	0	1
>300 days	30 days	2	3	0	0	0	0

Statistical analysis – assumptions for ANOVA

Data were tested for normal distribution using Kolmogorov-Smirnov (KS) tests [5] and Levene's tests for homogeneity of group variances [6]. 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 central limit theorem, especially for large sample sizes [e.g., 7], 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, $d = 0.134$, $p<0.05$).

5. Lilliefors, H. W. On the Kolmogorov-Smirnov Test for normality with mean and variance unknown. *J. Am. Stat. Assoc.* **62,** 399–402. https://doi.org/10.1080/01621459.1967.10482916 (1967).
6. Levene, H. Robust tests for equality of variances in *Contributions to probability and statistics* (eds. Olkin, I., Ghurye, S. G., Hoeffding, W., Madow, W. G. & Mann, H. B.) 278–292 (Stanford University Press: Stanford) (1960).
7. Keselman, J. C., Lix, L. M., & Keselman, H. J. The analysis of repeated measurements: A quantitative research synthesis. *Br. J. Math. Stat. Psychol.* **49,** 275–298. https://doi.org/10.1111/j.2044-8317.1996.tb01089 (1996).

Table S3. Multivariate repeated measures 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 *P. experimentalis* observed at 2h-48h 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.

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	7,159	1	7,159	4,507	0,0362302
{4} age	196,546	4	49,136	30,931	0,0000000
anhydrobiosis duration*group influence	4,488	1	4,488	2,825	0,0959270
anhydrobiosis duration*sex	2,631	1	2,631	1,656	0,2010482
group influence*sex	2,736	1	2,736	1,722	0,1924293
anhydrobiosis duration*age	10,134	4	2,533	1,595	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	2,514	1	2,514	1,582	0,2113643
anhydrobiosis duration*group influence*age	4,241	4	1,060	0,667	0,6161241
anhydrobiosis duration*sex*age	3,140	4	0,785	0,494	0,7400715
group influence*sex*age	2,060	4	0,515	0,324	0,8611674
anhydrobiosis duration*group influence*sex*age	1,080	4	0,270	0,170	0,9532145
Error	158,859	100	1,589		
{5}OBS.TIME	28,290	3	9,430	172,376	0,0000000
OBS.TIME*anhydrobiosis duration	2,182	3	0,727	13,298	0,0000000
OBS.TIME*group influence	0,473	3	0,158	2,883	0,0360823
OBS.TIME*sex	0,209	3	0,070	1,271	0,2844629
OBS.TIME*age	2,305	12	0,192	3,511	0,0000698
OBS.TIME*anhydrobiosis duration*group influence	0,441	3	0,147	2,687	0,0466582
OBS.TIME*anhydrobiosis duration*sex	0,329	3	0,110	2,004	0,1134477
OBS.TIME*group influence*sex	0,107	3	0,036	0,650	0,5833932
OBS.TIME*anhydrobiosis duration*age	5,761	12	0,480	8,776	0,0000000
OBS.TIME*group influence*age	1,067	12	0,089	1,625	0,0837031
OBS.TIME*sex*age	1,665	12	0,139	2,537	0,0033537
5*1*2*3	0,466	3	0,155	2,837	0,0383341
5*1*2*4	0,765	12	0,064	1,166	0,3072984
5*1*3*4	1,712	12	0,143	2,608	0,0025540
5*2*3*4	0,682	12	0,057	1,039	0,4131341
5*1*2*3*4	0,964	12	0,080	1,468	0,1351782
Error	16,412	300	0,055		

Table S4. Number of active individuals of *P. experimentalis* observed at 2h-48h after rehydration, with an emphasis on their age and duration of anhydrobiosis ((long anhydrobiosis - two episodes lasting 30 days each; short anhydrobiosis – five episodes lasting 3 days each). Based on the square root transformed reflected variable, x(transf.) = sqrt{1+max(x)-x}. Mean, SE - Standard Error, and 95% confidence interval for the population mean are given.

Subclass	anhydrobiosis	age class	OBS.TIME	Mean	SE	-95%	95%	N
1	long	1	2h	2,385	0,232	1,925	2,846	8
2	long	1	6h	2,723	0,224	2,278	3,169	8
3	long	1	24h	3,174	0,229	2,719	3,629	8
4	long	1	48h	3,313	0,250	2,818	3,808	8
5	long	2	2h	2,483	0,232	2,023	2,944	8
6	long	2	6h	2,948	0,224	2,503	3,393	8
7	long	2	24h	3,715	0,229	3,260	4,170	8
8	long	2	48h	3,695	0,250	3,200	4,190	8
9	long	3	2h	2,303	0,232	1,842	2,763	8
10	long	3	6h	2,756	0,224	2,311	3,201	8
11	long	3	24h	3,351	0,229	2,896	3,807	8
12	long	3	48h	3,470	0,250	2,975	3,965	8
13	long	4	2h	2,086	0,232	1,626	2,547	8
14	long	4	6h	2,223	0,224	1,778	2,669	8
15	long	4	24h	2,472	0,229	2,017	2,927	8
16	long	4	48h	2,515	0,250	2,020	3,010	8
17	long	5	2h	1,695	0,232	1,235	2,155	8
18	long	5	6h	1,729	0,224	1,284	2,174	8
19	long	5	24h	1,864	0,229	1,409	2,319	8
20	long	5	48h	1,770	0,250	1,275	2,265	8
21	short	1	2h	3,093	0,147	2,802	3,384	20
22	short	1	6h	3,336	0,142	3,054	3,618	20
23	short	1	24h	3,542	0,145	3,255	3,830	20
24	short	1	48h	3,440	0,158	3,127	3,754	20
25	short	2	2h	3,978	0,147	3,686	4,269	20
26	short	2	6h	4,151	0,142	3,870	4,433	20
27	short	2	24h	4,331	0,145	4,043	4,619	20
28	short	2	48h	4,252	0,158	3,939	4,566	20
29	short	3	2h	3,285	0,147	2,994	3,577	20
30	short	3	6h	3,471	0,142	3,189	3,752	20
31	short	3	24h	3,658	0,145	3,370	3,946	20
32	short	3	48h	3,704	0,158	3,391	4,017	20
33	short	4	2h	2,215	0,147	1,924	2,506	20
34	short	4	6h	2,389	0,142	2,107	2,671	20
35	short	4	24h	2,608	0,145	2,320	2,896	20
36	short	4	48h	2,721	0,158	2,408	3,034	20
37	short	5	2h	1,556	0,147	1,265	1,848	20
38	short	5	6h	1,827	0,142	1,546	2,109	20
39	short	5	24h	2,134	0,145	1,846	2,422	20
40	short	5	48h	2,212	0,158	1,899	2,525	20

class								
1	long	all	2h-48h	2,634	0,100	2,436	2,831	40
2	short	all	2h-48h	3,095	0,063	2,970	3,220	100

Table S5. Correlations between observation time after rehydration and duration of anhydrobiosis (long vs. short). Anhydrobiosis duration denotes combination of the number and duration of anhydrobiosis episodes. Correlation coefficients in red are significant at $p < 0.05$.

Obs. time after rehydration	2h	6h	24h	48h	anhydrobiosis duration
2h	1,000	0,939	0,887	0,857	0,280
6h	0,939	1,000	0,960	0,926	0,254
24h	0,887	0,960	1,000	0,979	0,157
48h	0,857	0,926	0,979	1,000	0,145
anhydrobiosis duration	0,280	0,254	0,157	0,145	1,000

Table S6. Number of active individuals after rehydration (2h - 48h) considering the first two episodes of short (SA) and long anhydrobiosis (LA); Mean, SE - standard error, and 95% confidence interval are given.

	episodes	Mean_2h	SE_2h	-95%_6h	95%_2h	Mean_6h	SE_6h	-95%_6h	95%_6h	Mean_24h	SE_24h	-95%_24h	95%_24h	Mean_48h	SE_48h	-95%_48h	95%_48h	N
1	LA_E1	2,220	0,213	1,795	2,645	2,634	0,201	2,232	3,035	3,291	0,197	2,898	3,684	3,362	0,183	2,997	3,727	20
2	LA_E2	2,161	0,213	1,736	2,586	2,318	0,201	1,917	2,719	2,539	0,197	2,146	2,932	2,543	0,183	2,179	2,908	20
3	SA_E1	3,586	0,213	3,161	4,011	3,803	0,201	3,402	4,204	4,023	0,197	3,630	4,417	4,073	0,183	3,709	4,438	20
4	SA_E2	3,215	0,213	2,790	3,640	3,456	0,201	3,054	3,857	3,657	0,197	3,264	4,050	3,767	0,183	3,402	4,131	20

Table S7. Number of active individuals for groups and single animals by the duration of anhydrobiosis (combination of the number and duration of anhydrobiosis episodes) and observation time (Obs. time) after rehydration (2h-48h); Mean, SE - Standard Error, and 95% confidence interval for the population mean are given; LA - long anhydrobiosis; SA - short anhydrobiosis.

#	anhydrobiosis	in groups or single animals	Obs. time	Mean	SE	-95%	95%	N
1	LA	group	2h	2,592	0,147	2,301	2,884	20
2	LA	group	6h	2,767	0,142	2,485	3,049	20
3	LA	group	24h	3,186	0,145	2,898	3,473	20
4	LA	group	48h	3,186	0,158	2,873	3,499	20
5	LA	single	2h	1,789	0,147	1,498	2,080	20
6	LA	single	6h	2,185	0,142	1,903	2,466	20
7	LA	single	24h	2,645	0,145	2,357	2,933	20
8	LA	single	48h	2,720	0,158	2,407	3,033	20
9	SA	group	2h	2,932	0,093	2,748	3,116	50
10	SA	group	6h	3,129	0,090	2,951	3,307	50
11	SA	group	24h	3,349	0,092	3,167	3,531	50
12	SA	group	48h	3,375	0,100	3,177	3,573	50
13	SA	single	2h	2,719	0,093	2,535	2,903	50
14	SA	single	6h	2,941	0,090	2,763	3,119	50
15	SA	single	24h	3,160	0,092	2,978	3,342	50
16	SA	single	48h	3,157	0,100	2,959	3,355	50

Table S8. Number of active females (F) and males (M) by the duration of anhydrobiosis (combination of the number and duration of anhydrobiosis episodes) and observation time (Obs. Time) after rehydration (2h-48h). Mean, SE - Standard Error, and 95% for the population mean are given .

#	age	anhydrobiosis duration	sex	Obs. Time	Mean	SE	-95%	95%	N
1	1	LA	M	2h	2,247	0,328	1,596	2,898	4
2	1	LA	M	6h	2,470	0,317	1,841	3,100	4
3	1	LA	M	24h	2,968	0,324	2,325	3,612	4
4	1	LA	M	48h	3,120	0,353	2,420	3,820	4
5	1	LA	F	2h	2,524	0,328	1,873	3,175	4
6	1	LA	F	6h	2,976	0,317	2,346	3,606	4
7	1	LA	F	24h	3,380	0,324	2,737	4,024	4
8	1	LA	F	48h	3,507	0,353	2,807	4,207	4
9	1	SA	M	2h	2,921	0,208	2,509	3,333	10
10	1	SA	M	6h	3,189	0,201	2,790	3,587	10
11	1	SA	M	24h	3,401	0,205	2,994	3,808	10
12	1	SA	M	48h	3,311	0,223	2,868	3,754	10
13	1	SA	F	2h	3,265	0,208	2,853	3,676	10
14	1	SA	F	6h	3,483	0,201	3,085	3,882	10
15	1	SA	F	24h	3,684	0,205	3,277	4,091	10
16	1	SA	F	48h	3,570	0,223	3,127	4,013	10
17	2	LA	M	2h	2,098	0,328	1,447	2,749	4
18	2	LA	M	6h	2,584	0,317	1,954	3,214	4
19	2	LA	M	24h	3,596	0,324	2,952	4,239	4
20	2	LA	M	48h	3,462	0,353	2,762	4,163	4
21	2	LA	F	2h	2,869	0,328	2,218	3,520	4
22	2	LA	F	6h	3,312	0,317	2,682	3,942	4
23	2	LA	F	24h	3,833	0,324	3,190	4,477	4
24	2	LA	F	48h	3,928	0,353	3,228	4,628	4
25	2	SA	M	2h	4,101	0,208	3,689	4,513	10
26	2	SA	M	6h	4,314	0,201	3,915	4,712	10
27	2	SA	M	24h	4,488	0,205	4,081	4,895	10
28	2	SA	M	48h	4,469	0,223	4,026	4,911	10
29	2	SA	F	2h	3,854	0,208	3,443	4,266	10
30	2	SA	F	6h	3,989	0,201	3,590	4,387	10
31	2	SA	F	24h	4,174	0,205	3,767	4,581	10
32	2	SA	F	48h	4,036	0,223	3,593	4,479	10
33	3	LA	M	2h	1,914	0,328	1,263	2,565	4
34	3	LA	M	6h	2,474	0,317	1,844	3,103	4
35	3	LA	M	24h	3,106	0,324	2,463	3,750	4
36	3	LA	M	48h	3,248	0,353	2,548	3,948	4
37	3	LA	F	2h	2,691	0,328	2,040	3,342	4
38	3	LA	F	6h	3,038	0,317	2,408	3,668	4
39	3	LA	F	24h	3,597	0,324	2,953	4,240	4
40	3	LA	F	48h	3,693	0,353	2,993	4,393	4
41	3	SA	M	2h	3,169	0,208	2,757	3,580	10

#	age	anhydrobiosis duration	sex	Obs. Time	Mean	SE	-95%	95%	N
42	3	SA	M	6h	3,378	0,201	2,980	3,777	10
43	3	SA	M	24h	3,612	0,205	3,205	4,019	10
44	3	SA	M	48h	3,706	0,223	3,263	4,148	10
45	3	SA	F	2h	3,402	0,208	2,990	3,814	10
46	3	SA	F	6h	3,563	0,201	3,165	3,962	10
47	3	SA	F	24h	3,704	0,205	3,297	4,111	10
48	3	SA	F	48h	3,702	0,223	3,259	4,145	10
49	4	LA	M	2h	1,966	0,328	1,315	2,617	4
50	4	LA	M	6h	2,060	0,317	1,431	2,690	4
51	4	LA	M	24h	2,312	0,324	1,668	2,955	4
52	4	LA	M	48h	2,312	0,353	1,611	3,012	4
53	4	LA	F	2h	2,207	0,328	1,556	2,858	4
54	4	LA	F	6h	2,386	0,317	1,756	3,016	4
55	4	LA	F	24h	2,632	0,324	1,988	3,275	4
56	4	LA	F	48h	2,718	0,353	2,018	3,418	4
57	4	SA	M	2h	2,163	0,208	1,751	2,575	10
58	4	SA	M	6h	2,318	0,201	1,920	2,717	10
59	4	SA	M	24h	2,515	0,205	2,108	2,922	10
60	4	SA	M	48h	2,613	0,223	2,170	3,056	10
61	4	SA	F	2h	2,267	0,208	1,856	2,679	10
62	4	SA	F	6h	2,460	0,201	2,061	2,858	10
63	4	SA	F	24h	2,701	0,205	2,294	3,108	10
64	4	SA	F	48h	2,829	0,223	2,387	3,272	10
65	5	LA	M	2h	2,037	0,328	1,386	2,688	4
66	5	LA	M	6h	1,500	0,317	0,870	2,130	4
67	5	LA	M	24h	1,618	0,324	0,975	2,262	4
68	5	LA	M	48h	1,559	0,353	0,859	2,259	4
69	5	LA	F	2h	1,354	0,328	0,703	2,005	4
70	5	LA	F	6h	1,958	0,317	1,328	2,588	4
71	5	LA	F	24h	2,109	0,324	1,466	2,753	4
72	5	LA	F	48h	1,981	0,353	1,281	2,681	4
73	5	SA	M	2h	1,395	0,208	0,983	1,807	10
74	5	SA	M	6h	1,650	0,201	1,252	2,049	10
75	5	SA	M	24h	2,068	0,205	1,661	2,475	10
76	5	SA	M	48h	2,140	0,223	1,697	2,583	10
77	5	SA	F	2h	1,718	0,208	1,306	2,130	10
78	5	SA	F	6h	2,004	0,201	1,606	2,403	10
79	5	SA	F	24h	2,200	0,205	1,793	2,607	10
80	5	SA	F	48h	2,284	0,223	1,842	2,727	10

Figure S1. *Paramacrobiotus experimentalis* individuals in a group during the tun state. The young females (age group 2) are shown during the first short anhydrobiosis episode; black arrows indicate the tuns.

Author statement

Amit Kumar Nagwani
aminag@amu.edu.pl

Poznań, 17.09.2023

AUTHOR STATEMENT

for the research article 'Recovery from anhydrobiosis in the tardigrade *Paramacrobiotus experimentalis*: better to be young than old and in a group than alone'. bioRxiv (2023).

I declare that the research article by Nagwani AK, Melosik I, Kaczmarek Ł, Kmita H. **Recovery from anhydrobiosis in the tardigrade *Paramacrobiotus experimentalis*: better to be young than old and in a group than alone (2023) bioRxiv** (doi: https://doi.org/10.1101/2023.05.22.541721) is part of my PhD dissertation. My contribution includes performing of the tardigrade culture and experiments, curating of the data as well as participating in the research conceptualization, writing of the final version of the manuscript and preparing of figures and tables.

Name: Amit Kumar Nagwani
Adress: Department of Bioenergetics,
Institute of Molecular Biology and Biotechnology,
Faculty of Biology, Adam Mickiewicz University in Poznań, Poznań
Uniwersytetu Poznańskiego 6, 61-614,
Poznań, Poland

Signature:

Kierownik
Zakładu Bioenergetyki

prof. dr hab. Hanna Kmita

Co-author statements

Poznań, Sept. 8, 2023

Dr. hab. Iwona Melosik,

Department of Genetics, Faculty of Biology

Adam Mickiewicz University in Poznań

Phone (+48) 61 829 58 60; -61

Email: iwona.melosik@amu.edu.pl

CO-AUTHOR STATEMENT

I declare that I am aware that the research paper by Nagwani AK, Melosik I, Kaczmarek Ł, Kmita H. (2023), 'Recovery from anhydrobiosis in the tardigrade *Paramacrobiotus experimentalis*: better to be young than old and in a group than alone,' bioRxiv (doi: https://doi.org/10.1101/2023.05.22.541721), is a part of Amit Kumar Nagwani's PhD thesis.

I declare that I analyzed the laboratory results statistically and participated in writing. I evaluate my contribution to this paper on a par with the first author.

Prof. UAM dr hab. Łukasz Kaczmarek
kaczmar@amu.edu.pl

Poznań, 14.09.2023

CO-AUTHOR STATEMENT

I declare that I am aware that the research paper by *Nagwani AK, Melosik I, Kaczmarek Ł, Kmita H. (2023) Recovery from anhydrobiosis in the tardigrade Paramacrobiotus experimentalis: better to be young than old and in a group than alone, bioRxiv (doi: https://doi.org/10.1101/2023.05.22.541721)* is a part of Amit Kumar Nagwani PhD thesis.

At the same time, I declared that I supervised in validation of applied methodology and results as well as contributed in writing and editing the manuscript.

Łukasz Kaczmarek

ADAM MICKIEWICZ UNIVERSITY, POZNAŃ
Faculty of Biology
Institute of Molecular Biology and Biotechnology
Department of Bioenergetics

Prof. dr hab. Hanna Kmita
kmita@amu.edu.pl

Poznań, 5.09.2023

CO-AUTHOR STATEMENT

I declare that I am aware that the paper by *Nagwani AK, Melosik I, Kaczmarek Ł, Kmita H. (2023) Recovery from anhydrobiosis in the tardigrade Paramacrobiotus experimentalis: better to be young than old and in a group than alone, bioRxiv* (doi: https://doi.org/10.1101/2023.05.22.541721), is a part of Amit Kumar Nagwani PhD thesis.

At the same time, I declare that I supervised the performed experiments as well as participate in result discussion and manuscript writing.

Kierownik
Zakładu Bioenergetyki
prof. dr hab. Hanna Kmita
Hanna Kmita

ul. Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland
tel. +48 61 829 5553, 61 829 5848
www.biologia.amu.edu.pl

9. **Publication 3:** The effect of hypomagnetic field on survival and mitochondrial functionality of active Paramacrobiotus experimentalis females and males of different age. *Frontiers in Physiology*, 14: 1253483. https://doi.org/10.3389/fphys.2023.1253483

frontiers | Frontiers in Physiology

TYPE Original Research
PUBLISHED 08 September 2023
DOI 10.3389/fphys.2023.1253483

OPEN ACCESS

EDITED BY
Michele Cesari,
University of Modena and Reggio Emilia, Italy

REVIEWED BY
Frank Smith,
University of North Florida, United States
Ilaria Giovannini,
University of Modena and Reggio Emilia, Italy

*CORRESPONDENCE
Hanna Kmita,
hanna.kmita@amu.edu.pl

RECEIVED 05 July 2023
ACCEPTED 29 August 2023
PUBLISHED 08 September 2023

CITATION
Nagwani AK, Budka A, Łacka A, Kaczmarek Ł and Kmita H (2023), The effect of hypomagnetic field on survival and mitochondrial functionality of active *Paramacrobiotus experimentalis* females and males of different age.
Front. Physiol. 14:1253483.
doi: 10.3389/fphys.2023.1253483

The effect of hypomagnetic field on survival and mitochondrial functionality of active *Paramacrobiotus experimentalis* females and males of different age

Amit Kumar Nagwani[1], Anna Budka[2], Agnieszka Łacka[2], Łukasz Kaczmarek[3] and Hanna Kmita[1]*

[1]Department of Bioenergetics, Faculty of Biology, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University in Poznań, Poznań, Poland, [2]Department of Mathematical and Statistical Methods, Poznań University of Life Sciences, Poznań, Poland, [3]Department of Animal Taxonomy and Ecology, Faculty of Biology, Institute of Environmental Biology, Adam Mickiewicz University in Poznań, Poznań, Poland

Even for tardigrades, often called the toughest animals on Earth, a hypomagnetic field (HMF) is an extreme environment. However, studies on the effect of HMF on tardigrades and other invertebrates are scarce. Mitochondria play an important role in an organism's response to extreme conditions. The effect of HMF on the mitochondrial inner membrane potential ($\Delta\psi$), a well-known marker of mitochondria functionality, shows that mitochondria are very sensitive to HMF. To measure the HMF effect on *Paramacrobiotus experimentalis*, we calculated the tardigrade survival rate and $\Delta\psi$ level after HMF treatments of different durations. We also estimated the relationship between the age and sex of the tardigrade and the HMF effect. We observed age- and sex-related differences in $\Delta\psi$ and found that $\Delta\psi$ changes after HMF treatment were dependent on its duration as well as the animal's age and sex. Furthermore, active *P. experimentalis* individuals displayed a high survival rate after HMF treatment. The data may contribute to the understanding of tardigrade aging and their resistance to extreme conditions including HMF, which in turn may be useful for future space explorations.

KEYWORDS

tardigrades, *Paramacrobiotus experimentalis*, hypomagnetic field, survival, the mitochondrial inner membrane potential ($\Delta\psi$), age, sex

Introduction

The Earth's magnetic field, also called the geomagnetic field (~50 μT) or standard magnetic field (SMF), is regarded as a crucial factor for living organisms because it protects organisms living on Earth from corpuscular radiation, e.g., solar wind and cosmic radiation (for review, see, e.g., Xue et al., 2021; Erdmann et al., 2021a). However, it decreases significantly with increasing distance from the surface of the Earth and can be blocked or shielded on Earth, resulting in a hypomagnetic field (HMF; <5 μT) (e.g., Mo et al., 2014). Thus, exposure to HMF may happen not only during space travel but also on Earth, e.g., in buildings with steel walls or steel reinforcements (Sinčák and Sedlakova-Kadukova, 2023). The exposure is known to affect living organisms at different biological levels (Conley, 1970; Binhi and Prato, 2017; Erdmann et al., 2021a; Sinčák and Sedlakova-Kadukova, 2023).

Therefore, HMF can be regarded as an extreme condition but the mechanisms underlying HMF's effect on living organisms are still largely unclear (e.g., Sinčák and Sedlakova-Kadukova, 2023). At a cellular level, mitochondria have been suggested as the organelle most sensitive to HMF (e.g., Fu et al., 2016; Zhang and Tian, 2020). The mitochondrial inner membrane potential ($\Delta\psi$) is an essential component for healthy mitochondrial functioning, and mitochondrial dysfunction co-occurs with $\Delta\psi$ decrease (Cao et al., 2004; Srinivasan et al., 2017). Thus, the decrease in $\Delta\psi$ is considered to be an indicator of reduced cell health (Wang et al., 2019). Importantly, a decrease in $\Delta\psi$ has been reported under extreme conditions (e.g., Neginskaya et al., 2021), including exposure to HMF (Fu et al., 2016; Wang et al., 2022).

Studies on the effect of HMF on humans and other vertebrates as well as plants are quite common, whereas the studies on invertebrates are still limited, particularly for those displaying cryptobiosis capability (e.g., Erdmann et al., 2017; Erdmann et al., 2021b; Sinčák and Sedlakova-Kadukova, 2023). Tardigrades are microinvertebrates (mean size of ca. 500 µm) which inhabit almost all terrestrial and aquatic ecosystems throughout the world (Nelson et al., 2015). These animals are commonly known as the toughest animals on Earth, (Copley, 1999), due to their resistance to different environmental stressors. These include lack of water, low and high temperatures, high doses of radiation, low and high atmospheric pressure, low gravity, and high concentration of different toxins (e.g., Crowe, 1975; Ramlov and Westh, 2001; Altiero et al., 2011; Guidetti et al., 2012; Kaczmarek et al., 2019; Hesgrove and Boothby, 2020). Thus, tardigrades are excellent models for research on mechanisms of adaptation to the most extreme environments, including exposure to space conditions (e.g., Jönsson, 2007; 2020; Guidetti et al., 2012; Horikawa, 2012; Schill and Hengherr, 2018; Rebecchi et al., 2020; Arakawa, 2022), particularly as results obtained from studies on tardigrades can be extrapolated and applied to vertebrates, including humans (Hashimoto et al., 2016).

Up to now, ca. 1,500 tardigrade species have been described (Degma et al., 2023) but only three species have been used in studies on mortality in the presence of HMF (Erdmann et al., 2017; 2021b). They are *Hypsibius exemplaris* (misidentified in earlier works as *H. dujardini*) (Gąsiorek et al., 2018), *Echiniscus testudo* (Doyère, 1840), and *Milnesium inceptum* (Morek et al., 2019), all represented by parthenogenetic females and studied during three stages of anhydrobiosis (Erdmann et al., 2017; Erdmann et al., 2021b). The authors of these studies concluded that HMF significantly increased the mortality of the studied species and decreased their capability of anhydrobiosis, probably due to impairment of metabolic processes, although there were differences in HMF tolerance between the studied species. This aligns with the available data on tardigrade differentiation of tolerance to extreme factors as has been reviewed in relation to anhydrobiosis, (Nagwani et al., 2022), as well as the reported observation that mitochondrial functionality is a prerequisite for anhydrobiosis survival (Halberg et al., 2013; Wojciechowska et al., 2021a). Accordingly, it is well known that mitochondria play a central role in cell metabolism and control of stress responses, and mediate various cellular outcomes (e.g., Vakifahmetoglu-Norberg et al., 2017). However, the impact of HMF on tardigrade mitochondria functioning has not yet been studied.

It has been reported that the length of exposure to HMF may be an important factor for the observed effect (Sinčák and Sedlakova-Kadukova, 2023) Moreover, it appears that the tardigrade's ability to survive under extreme conditions can be influenced by many factors (e.g., McCarthy and delBarco-Trillo, 2020). Our previous results indicate that age and sex could be important to tardigrades' survival under extreme conditions represented by water deficiency resulting in dehydration (Nagwani et al., 2023). Therefore, we decided to study the impact of different durations of exposure to HMF on the survival rate and mitochondrial $\Delta\psi$ levels of active tardigrades of different ages and sex. The obtained data provide insight into age- and sex-dependent changes in tardigrade mitochondria functionality that may contribute to animals' aging, as well as their resistance to HMF.

Materials and methods

Reagents

For the detection of the mitochondrial inner membrane potential, tetramethylrhodamine methyl ester (TMRM; ThermoFisher #T668) was applied. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; Sigma-Aldrich #2920) was used to eliminate the mitochondrial inner membrane potential. To stain DNA, 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich #D9542) was applied. For confocal fluorescence microscopy, specimens were mounted on glass slides (76 mm × 26 mm; bionovo #B-1198) using the Vectashield antifade mounting medium (Vector laboratories #H-1000-10) and glass coverslips (16 mm × 16 mm; ChemLand) #04-298.202.00). For fluorescence quantitative analysis, multi-well plates (Ratiolab #6018717) were applied.

Cultures of *Paramacrobiotus experimentalis*

Females and males of *P. experimentalis* (Kaczmarek et al., 2020; Nagwani et al., 2023) were reared together in covered, vented plastic Petri dishes (55 mm in diameter), with the bottom scratched with sandpaper to allow the animals to move. The 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 exchanged every week, and animals were fed with the rotifer *Lecane inermis* (Bryce, 1892) (strain 1.A2.15), provided by Dr. Edyta Fiałkowska (Institute of Environmental Sciences, Jagiellonian University, Krakow, Poland). The Petri dishes were kept in the POL EKO KK 115 TOP + climate chamber at 20°C, in the dark, with relative humidity (RH) of 40% (Roszkowska et al., 2021).

Eggs laid by females were collected to obtain different age classes and were cultured as described above. Each of the classes contained females and males, and was kept in separate Petri dishes. The first oviposition took place 19.3 ± 3.6 days after hatching. Females and males were distinguished based on morphological characteristics, e.g., average body length (males are smaller than females), average body width (males are slimmer than females), the presence of eggs in the females' ovary (Supplementary Table S1) and the body shape. Females have a barrel shape (especially in the late stages of

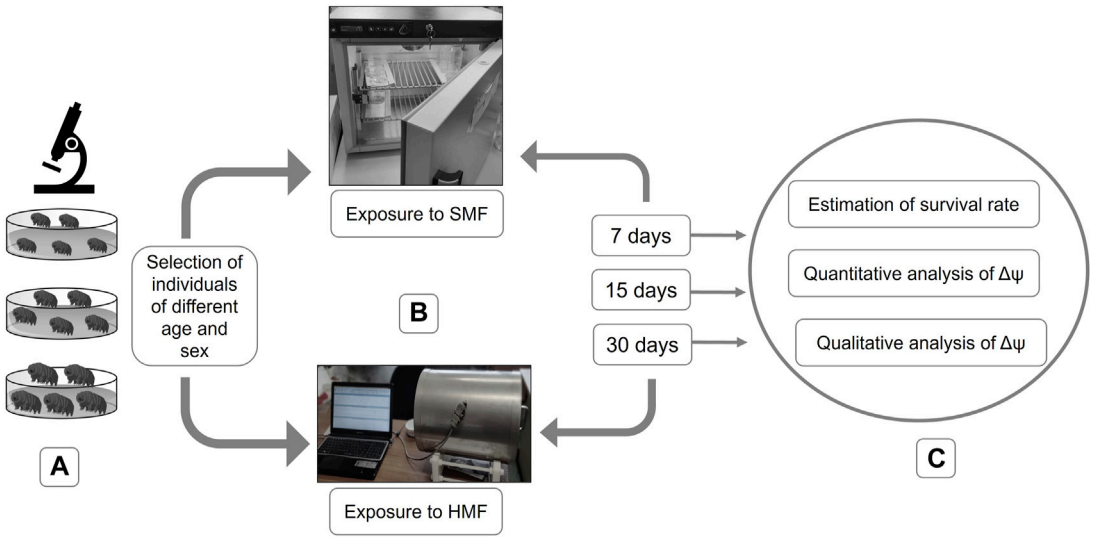


FIGURE 1
Graphical representation of performed experiments. **(A)** selection of active individuals of different age and sex; **(B)** exposure to SMF or HMF for different durations (i.e., 7, 15, and 30 days); **(C)** post-exposure analysis including estimation of the survival rate and analysis of the level of the mitochondrial inner membrane potential (Δψ).

oogenesis) and the posterior part of the body not hooked whereas males do not have the barrel shape but sometimes the posterior part of their body is slightly hooked. The accuracy of the applied approach of identification of sexes was confirmed by detecting of spermatozoa movements in the gonad using a stereomicroscope (OLYMPUS SZ61) and observing gonads using a transmission electron microscope (Hitachi H500). The number of active animals and eggs as well as their body length, body width and body shape were assessed using the stereomicroscope. The calibrated grid of the stereomicroscope was used to measure the body length and body width to an accuracy of 10 µm. Because the average lifespan of the individuals under the laboratory culture conditions is approximately 360 days (our unpublished data), three different age classes were selected, representing the following age ranges: 30–60 days, 150–180 days, and older than 300 days. These age classes were described as growing adults, mature adults, and old adults, respectively (Supplementary Table S1). To determine the average lifespan, 200 eggs were isolated from *P. experimentalis* culture from which 182 were hatched and the hatched individuals were transferred to the culture Petri dishes and cultured as described above. They were observed continuously and their activity was noted until sex distinguishing was possible (see above). Then, 30 females and 30 males were selected and further cultured as described above and observed every 3 days and then every week. The observation was performed until the last female or male died and the number of active individuals at each of the applied time windows of observation was estimated.

Exposure to a hypomagnetic field

Females and males of the selected age classes were separated and divided into experimental and control animals. They were placed in covered, vented plastic Petri dishes (55 mm in diameter) with the bottom scratched with sandpaper and coated with a thin layer of the culture medium. The feeding and exchange of culture medium were performed every week. Control females and males were exposed to standard (geomagnetic) field (SMF) using a POL EKO KK 115 TOP + climate chamber whereas experimental females and males were exposed to a hypomagnetic field (HMF) by application of a special anti-magnetic chamber, known as a Chamber Isolated from Magnetic Field (CIMF) (Erdmann et al., 2017). The chamber can deflect the force field by concentrating it inside the material's substance (Conley, 1970), leading to a 200-fold reduction of SMF (Kopcewicz et al., 1999; Miglierini et al., 2002). Importantly, for both the control and experimental animals, standard culture conditions were applied (temperature of 20°C, darkness (24 h), and RH of 40%). The air movement in the experimental chamber was verified by Erdmann et al. (2017). Importantly, the tardigrade survival rate in their control box and our control chamber during 1 month were not statistically different. The exposure to HMF was performed for 7, 15, and 30 days (Figure 1).

Estimation of hypomagnetic field effect

The impact of HMF was estimated by calculating the survival rate and evaluating the functional state of mitochondria (Figure 1). To evaluate the effect of HMF on mitochondrial functionality, the level of the mitochondrial inner membrane potential (Δψ), a well-known marker of mitochondria functionality, was estimated.

To calculate the survival rate, groups of 15 active females or males of different ages, each in three repetitions (15 × 3 = 45), were exposed to a given duration of HMF or SMF. After each of the exposure, the culture medium was exchanged and animals were observed at 0, 2, 6, 24, and 48 h following the end of the exposure. The survival rate denotes the number of still-active individuals (i.e., displaying coordinated movements of the body and legs) after a given exposure.

A Nikon A1Rsi confocal microscope connected to a digital camera was used to perform a qualitative analysis of Δψ. For each variant of HMF or SMF treatment, three active females or males from each of the selected age classes were stained for 1 h with 2 μM TMRM (a fluorescent probe that accumulates in the mitochondria, dependent on the level of Δψ) and 1 μg/mL of DAPI (a cell-permeable fluorescent probe that binds to DNA). Next, the stained animals were washed carefully by multiple replacements of the culture medium and subsequently kept for 1 h at room temperature in fresh culture medium to remove any excess probes. Since the applied probes are light-sensitive, all steps of staining and washing were carried out in the dark. After washing, the stained animals were mounted on glass slides using Vectashield antifade mounting medium and glass coverslips. The microscopic observation was performed at a wavelength of fluorescence excitation of 544 nm for TMRM and 359 nm for DAPI. Images were obtained using NIS-Elements Viewer 5.21 software and transformed using ImageJ 2.3.0 software. The NIS-Elements Viewer 5.21 software was provided by the Department of Cellular and Molecular Biology at Adam Mickiewicz University in Poznan, Poland. The ImageJ 2.3.0 software was downloaded from the official website of the National Institutes of Health (https://imagej.nih.gov/ij/download.html).

For quantitative analysis of Δψ, groups of 50-100 active females and males of different ages, each in three repetitions, were exposed to a given duration of HMF or SMF, after which they were stained with 2 μM TMRM for 1 h at room temperature in darkness. Next, the stained animals were washed as described above. After being washed, the stained animals were transferred in 100 μL of the culture medium into multi-well plates. Multiple readings per well were immediately taken using a Tecan Infinite 200 Pro microplate reader at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The measurements were repeated after incubation of the samples for 45 min with 50 μM FCCP, an uncoupler used as a control in the determination of Δψ level. The data were exported using Tecan i-control software and recalculated to the TMRM Fluorescence Index (FI_{TMRM}), representing Δψ sensitivity to FCCP (Wojciechowska et al., 2021b). Because of the differences in body size and the resulting differences in the number of animals in each sample, FI_{TMRM} was recalculated for one animal.

Statistical analysis

To analyse the effect of HMF or SMF (control) treatment, two-way ANOVA was used. The analysis was performed separately for females and males. In the analysis two dependent variables, i.e., the survival rate corresponding to the number of still-active individuals, and the level of Δψ, reflected by the value of FI_{TMRM}, as well as two independent variables, i.e., age (three age classes) and duration of the treatment (7, 15 and 30 days) were applied. Because data concerning the effect of HMF or SMF treatment on the survival rate did not meet conditions necessary for ANOVA, as detected by Fligner-Killeen test, the "aligned rank transformation" approach, available in the ARTool package of R software, was used prior to two-way ANOVA. If significant differences related to the analysed dependent variables were found, Tukey *post hoc* test was performed ($\alpha = 0.05$). Due to limited variability of the survival rate data, correlation analysis between survival and FI_{TMRM} data was not performed. Mean values of active animals and level of Δψ were additionally compared between SMF (control) and HMF treatment as well as between females and males using *t*-test (see Supplementary Tables S2, S3).

Results

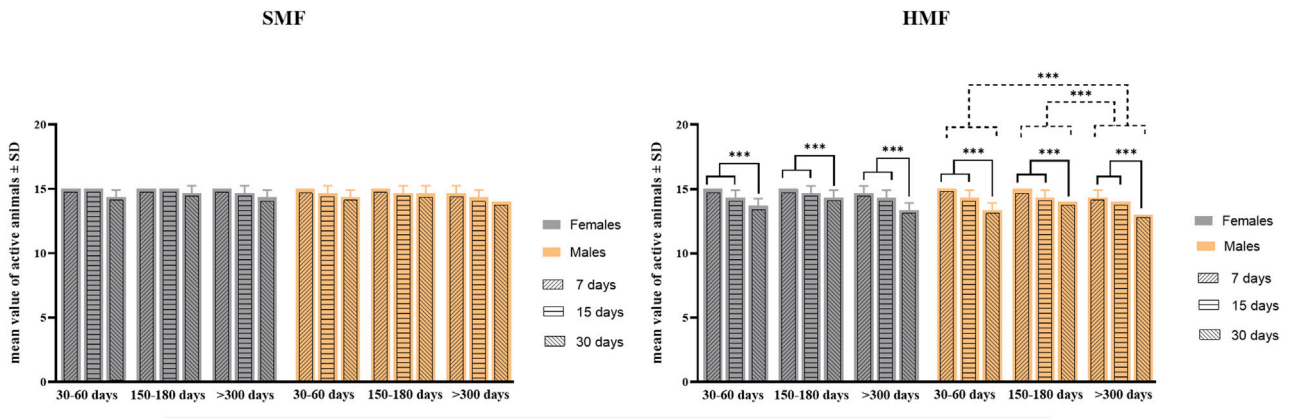
The effect of HMF on the *Paramacrobiotus experimentalis* survival rate depends on its duration and the animal age and sex

The effect of exposure to hypomagnetic field (HMF) of different durations (7, 15, and 30 days) on the survival rate was tested for active females and males classified as growing adults (the age of 30–60 days), mature adults (the age of 150–180 days) and old adults (the age of over 300 days) (see also Supplementary Table S1). Animals exposed to the standard magnetic field (SMF) were used as a control. As shown in Figure 2, the age of animals and duration of SMF treatment did not influence the survival rate in a statistically-significant way. However, after HMF treatment, both the duration of exposure and the animal's age affected the survival rate in a statistically-significant ways that differed between females and males. For females, only the duration of HMF treatment was found to be a statistically significant factor, while for males both the duration of HMF treatment and animal age were statistically significant. For both females and males, 7 and 15-day treatment with HMF resulted in comparable effects on the survival rate that differed significantly from 30-day treatment effect being the strongest one. The survival rate of male growing adults and male mature adults did not differ significantly, but the difference between their survival rate and old adults was significant as the latter were most sensitive to HMF exposure. Nevertheless, survival following HMF treatment by active females and males was high, i.e., no lower after any of the applied treatment variants than 87% (Supplementary Table S2). Moreover, survival of control active females and control active males was not statistically different and the absence of statistically significant difference was also observed between females and males after HMF treatment. When the survival was compared between control animals and animals after HMF treatment, statistically significant difference was only observed for the oldest age class males for the longest HMF duration (Figure 2; Supplementary Table S2). For the longest duration of HMF also initial reduced mobility of females and males was observed but the mobility was restored at the next time point of observation at 2 h following the end of the treatment and did not change until the last time point of observation at 48 h following the end of the treatment.

The effect of HMF on *Paramacrobiotus experimentalis* mitochondrial Δψ level depends on its duration and the animal's age and sex

It is well known that HMF, like other extreme conditions, affects mitochondrial functional state (Fu et al., 2016; Zhang and Tian, 2020). Therefore, we decided to estimate the level of the mitochondrial inner membrane potential (Δψ) for active females and males of different ages and treated with different durations of

10.3389/fphys.2023.1253483



AGE																	
30-60 days			150-180 days			>300 days			30-60 days			150-180 days			>300 days		
DURATION OF THE TREATMENT [days]																	
7	15	30	7	15	30	7	15	30	7	15	30	7	15	30	7	15	30
SMF vs HMF (p-values)																	
FEMALES									MALES								
-	ns	ns	-	ns	ns	ns	ns	ns	-	ns	ns	-	ns	ns	ns	ns	***
FEMALES vs MALES (p-values)																	
SMF									HMF								
-	ns	-	-	ns	-	ns	ns	ns	-	-	ns	-	ns	ns	ns	ns	ns

FIGURE 2
The survival rate of *P. experimentalis* females and males of different age exposed to SMF and HMF of different duration. The survival rate corresponds to mean values ±SD of still active individuals (i.e., displaying coordinated movements of the body and legs) after a given treatment. The age of animals: 30–60 days (growing adults), 150–180 days (mature adults) and >300 days (old adults); durations of the SMF and HMF treatment: 7, 15, and 30 days; statistically significant difference between age classes and treatment durations, dashed and solid lines, respectively. ***$p < 0.001$. The table presents comparison of the mean values-of active animas between SMF (control) and HMF treatment as well as between females and males using t-test. *** and ns denotes $p < 0.001$ and not statistically significant, respectively. When the values for control and experimental groups or for females and males were identical, the analysis was not performed ("-" in the table) (see also Supplementary Table S2).

HMF and SMF, using the intact animals' TMRM staining and confocal fluorescence microscopy. To assure the specificity of the TMRM staining, the animals were stained with DAPI (Supplementary Figure S1). As shown in Figure 3, the HMF treatment decreased the intensity of the TMRM staining when compared with SMF (control) treatment, suggesting a relevant decrease in Δψ level. The staining intensity also appeared to correlate with the age of active females and males, suggesting age-and sex-dependent changes in Δψ level. The intensity of the TMRM staining appeared to be higher for females and the highest for mature adults and the lowest for growing adults of both sexes, suggesting Δψ level changes related to age. The variation in buccal apparatus visibility could be explained by the amount of ingestion. It is possible that during the time of staining, the ingested food did not reach the gut completely and interfered with the staining and washing steps.

To confirm these observations, quantitative analysis of TMRM staining was performed on the animals after HMF and SMF (control) treatment using a microplate reader. The analysis consisted of calculating the TMRM Fluorescence Index (FI_{TMRM}) per animal (see also Supplementary Table S3). As shown in Figure 4, relevant values of FI_{TMRM} after SMF and HMF treatments were statistically different (see also Supplementary Table S3), supporting the relevant decrease in Δψ level after HMF treatment. Comparison of FI_{TMRM} between control females and control males as well as between females and males after HMF treatment indicated statistically significant differences for all age classes and treatment duration (see also Supplementary Table S3), that appeared to confirm the higher level of Δψ in females, also after HMF treatment. Moreover, after SMF treatment, the age of animals affected the value of FI_{TMRM} in a statistically-significant way, whereas after HMF treatment both duration and animal age affected the value of FI_{TMRM} in a statistically-significant way. The values of FI_{TMRM} after SMF and HMF treatments were the highest for mature adults and the lowest for growing adults. This accords with the confocal microscopic images and the apparent change of Δψ level related to the animal age. After HMF treatment, the value of FI_{TMRM} was lower for all age classes, indicating the treatment effect on Δψ level. However, for females, 7 and 15-day treatment with HMF resulted in a comparable effect on FI_{TMRM} that differed significantly from the effect of 30-day treatment, while for males significant differences in FI_{TMRM} were observed between 7-day and 15-day treatment as well as between 7-day and 30-day treatment, but not between 15-day and 30-day treatment. This suggested sex-dependent differences in the effect of HMF treatment on Δψ level.

Discussion

The mitochondrial inner membrane potential (Δψ), applied as a marker of mitochondria functionality, is known to change during aging (e.g., Panel et al., 2018) and under hypomagnetic conditions (e.g., Fu et al., 2016; Zhang and Tian, 2020; Wang et al., 2022; Sinčák and Sedlakova-Kadukova, 2023). However, the age and sex of

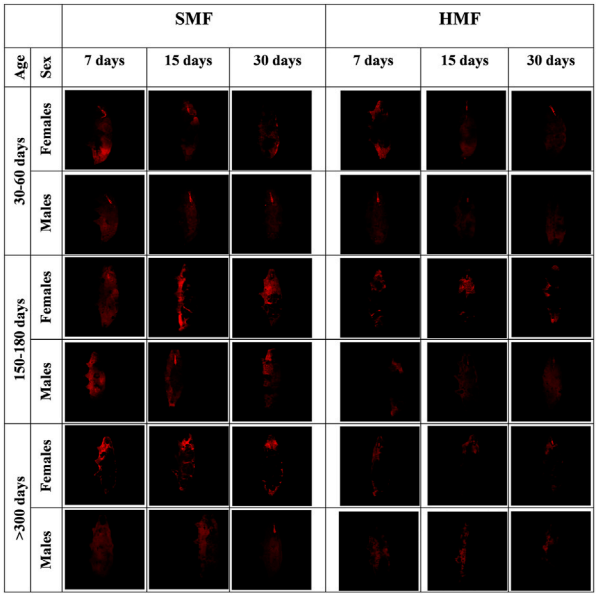


FIGURE 3
Confocal microscopy images of females and males of different age exposed to SMF and HMF of different duration and stained with TMRM. SMF, standard magnetic field; HMF, hypomagnetic field. The age of the animals: 30–60 days (growing adults), 150–180 days (mature adults), and >300 days (old adults). The duration of SMF or HMF treatment: 7, 15 and 30 days. The figure presents images representative for three animals. All confocal images are maximum projection images taken using the identical settings and later reoriented using ImageJ in order to keep the same orientation of all the captured images.

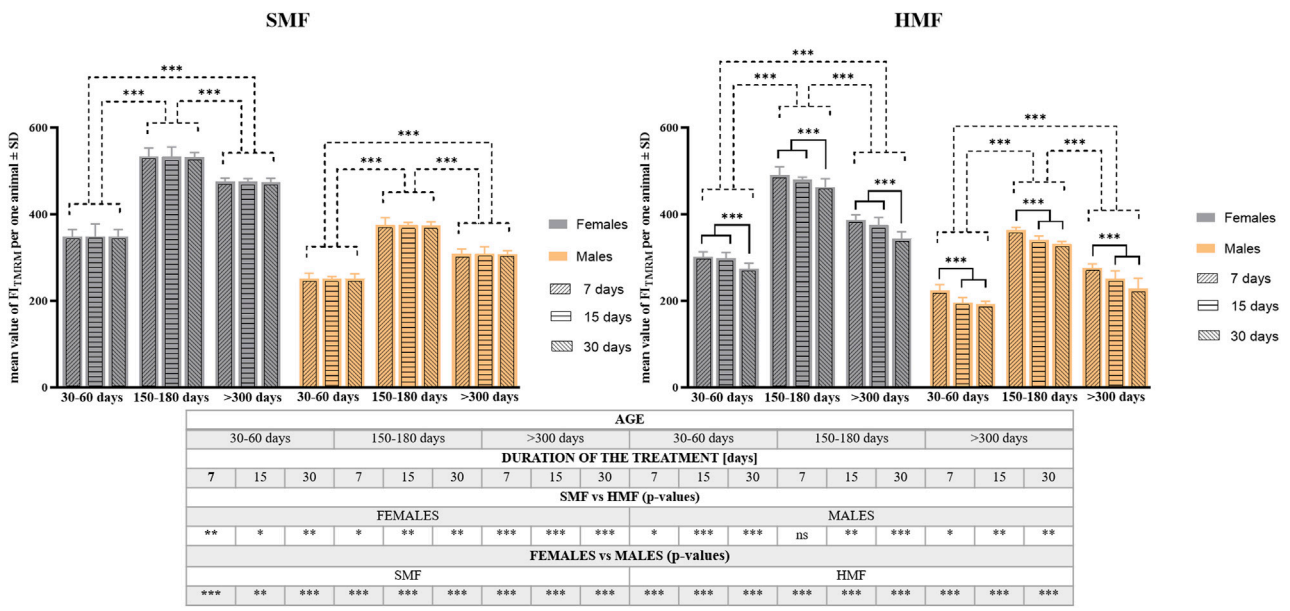
animals are omitted in HMF studies although their contributions appear to be important for better understanding the mechanisms of resistance to extreme conditions and the process of aging. The newly-described tardigrade species *P. experimentalis* was used in this study, which determined the survival rate of animals and carried out qualitative and quantitative analysis of their Δψ level after SMF (control) and HMF treatment of different durations. The obtained results indicate that tardigrade mitochondria can directly respond to HMF at a functional level by decreasing Δψ levels, and active animals have high survival (determined based on the presence of coordinated movements of the body and legs) of exposure to HMF. On the other hand, the age and sex of the animals are important factors that influence the tardigrade's response to HMF treatment.

To our knowledge, this is the first report on the survival rates of active tardigrade females and males after exposure to HMF of different durations. The determined survival rate was high, although males, particularly the oldest one, appeared to be more sensitive to HMF. This is reflected by the observation that for females, only the duration of HMF treatment was a statistically-significant factor influencing the survival rate, while for males both the duration of HMF treatment and the animal's age influenced the survival rate in a statistically-significant way. Moreover, for both females and males, shorter treatments with HMF resulted in comparable effects on the survival rate that differed significantly from the longest treatment effect, while for males significant differences in the survival rate were observed between younger and oldest animals. This accords with existing evidence that, under extreme conditions, females survive better than males (e.g., Zarulli et al., 2018). Although, no statistically significant differences in the survival rate were obtained between females and males after HMF treatment, statistically significant difference was obtained for the oldest age class males for the longest HMF duration when compared with the relevant control SMF variant of the treatment. Thus, the duration of HMF treatment may be an important factor for the observed effect (Sinčák and Sedlakova-Kadukova, 2023), although the male survivability may be affected by their age.

The known data on tardigrade treatment with HMF concern parthenogenetic females of three different species at different stages of anhydrobiosis, i.e., entering anhydrobiosis, during anhydrobiosis, and leaving anhydrobiosis (Erdmann et al., 2017; Erdmann et al., 2021b). In previous experiments (Erdmann et al., 2017; Erdmann et al., 2021b), only one duration of HMF treatment was used, and return to activity was determined. Although some differences were observed for different species, the survival rate was generally low, which was explained by the authors as being due to HMF-mediated impairment of the metabolic processes associated with anhydrobiosis. Accordingly, it has already been established that mitochondrial functionality is indispensable for anhydrobiosis survival (Halberg et al., 2013). Our qualitative and quantitative analysis of *P. experimentalis* Δψ level indicated a relationship between Δψ level and the age of active females and males that makes Δψ level an interesting marker of the tardigrade's age. The intensity of the TMRM staining was higher for females, which might be an unspecific effect resulting from the females' bigger body size and the presence of eggs or higher mitochondrial content (e.g., Cardinale et al., 2018), although some data indicate a higher Δψ level in female mitochondria (e.g., Damacena de Angelis et al., 2022).

The level of Δψ differed significantly between SMF (control) and HMF treatment as well as between females and males. However, it was the highest for mature adults and the lowest for growing adults. Besides the age of the animals, the important factor for the effect of HMF treatment was its duration. For females, shorter treatments with HMF resulted in a comparable effect on Δψ level that differed significantly from the effect of the longest treatment. This resembles the effect of HMF treatment duration on the female survival rate. For males, the effects of HMF duration on the survival rate and changes in Δψ level did not overlap; for the former, significant differences were observed between two shorter and the longest durations of HMF treatments, and for the latter, there was only a significant difference between the shortest treatment and the two longer ones. This suggested sex-related differences in sensitivity of Δψ to HMF duration. At present, we do not have an explanation for this, although the observation is in line with known sex-associated differences in mitochondrial function (e.g., Silaidos et al., 2018).

The mechanism by which the level of Δψ is modulated by SMF and HMF remains unclear. Nevertheless, it is known that HMF treatment decreases Δψ and consequently the viability of animal cells, (e.g., Fu et al., 2016; Srimai et al., 2020) which results in a range of adverse effects and severe dysfunctions in animals, including humans (for review, see, e.g., Erdman et al., 2021a). Considering that the magnetic field acts on elements with a magnetic moment, the decrease in Δψ is proposed to be caused by HMF interaction with electrons moving along the



AGE																	
30-60 days			150-180 days			>300 days			30-60 days			150-180 days			>300 days		
DURATION OF THE TREATMENT [days]																	
7	15	30	7	15	30	7	15	30	7	15	30	7	15	30	7	15	30
SMF vs HMF (p-values)																	
FEMALES									MALES								
**	*	**	*	**	**	***	***	***	*	***	***	ns	**	***	*	**	**
FEMALES vs MALES (p-values)																	
SMF									HMF								
***	**	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***

FIGURE 4
The mitochondrial inner membrane potential (Δψ) level of *P. experimentalis* females and males of different age exposed to SMF and HMF of different duration. The Δψ level corresponds to mean values of FI_{TMRM} ± SD per animal after a given treatment. The age of animals: 30–60 days (growing adults), 150–180 days (mature adults) and >300 days (old adults); durations of the SMF and HMF treatment: 7, 15, and 30 days; statistically significant difference between the age classes and treatment durations, dashed and solid lines, respectively. ***$p < 0.001$. The table presents results of comparison of Δψ mean values between SMF (control) and HMF treatment as well as between females and males using *t*-test. *, **, ***, ns denotes $p < 0.05$, $p < 0.01$, $p < 0.001$ and not statistically significant, respectively (see also Supplementary Table S3).

mitochondrial respiratory chain, ions moving along ion channels, or proton translocation by the respiratory chain (Binhi and Prato, 2017; Ogneva et al., 2020). When the external magnetic field is changed from SMF to HMF, conditions are no longer optimal for these processes and their efficiency is reduced. It has been also observed that the HMF effect on mitochondria may result in a change to the number of mitochondria and their morphology, which in the case of skeletal muscle diminishes animals' physical activity (Hu et al., 2020). Importantly, our data indicate that the physical activity of tardigrades was only initially and transiently attenuated after the longest HMF treatment. In addition, reactive oxygen species (ROS) are hypothesized to mediate the effect of HMF on mitochondria (Zhang and Tian, 2020) but available data do not allow for this hypothesis to be validated for tardigrades.

Conclusion

Our data indicate that Δψ level could be a useful marker of the tardigrade *P. experimentalis* age. Moreover, the tardigrade sensitivity to HMF treatment including Δψ changes may also serve as the marker. Using an accurate marker of tardigrade age may contribute to an explanation of tardigrade aging. The obtained results also provide important data concerning age- and sex-related changes in mitochondrial functioning under extreme conditions. Similar to other animals, *P. experimentalis* responds to HMF treatment with Δψ decrease, but the decrease does not result in impaired survival as reflected by the animal's physical activity although the activity was shortly and transiently attenuated after the longest HMF treatment. Thus, the long-term effects of the Δψ decrease on the tardigrade longevity and/or reproduction should be further studied to fully explain biological significance of HMF treatment. The observed relationship between the decrease in Δψ level and duration of HMF treatment suggests that HMF effect on mitochondria functionality increases over time. This observation is useful for creating models relevant to long-term space missions, where continuous exposure to HMF is a potential risk factor for different organisms, including humans. Nevertheless, the studied factors should also be considered in studies of HMF effect on other tardigrades species.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

Conceptualization, HK and AKN; data curation, AKN and HK; investigation, AKN, AB, and AŁ; methodology, ŁK, and HK; statistical analysis, AB and AŁ; validation, HK, and ŁK; supervision, HK and ŁK; writing, HK, and AKN. All authors accepted the final version of the manuscript.

Funding

AN is a scholarship passport holder of the Interdisciplinary Doctoral Studies at the Faculty of Biology, Adam Mickiewicz University, POWR.03.02.00-00-I006/17. This research was supported by research grants of the National Science Centre, Poland, NCN 2016/21/B/NZ4/00131 and 2021/41/N/NZ3/01165, and Initiative of Excellence-Research University Programme (ID_UB) at Adam Mickiewicz University 017/02/SNP/0006.

Acknowledgments

The studies were performed partly in the framework of the activities of BARg (Biodiversity and Astrobiology Research group). The authors would like to thank Cambridge Proofreading LLC (http://proofreading.org) for their linguistic assistance. Technical support of Wojciech Kwiatkowski, Andonis Karachitos, and Weronika Erdmann is highly appreciated. Many thanks to Prof. Izabela Poprawa (University of Silesia in Katowice) for her help in distinguishing of individuals' sex.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1253483/full#supplementary-material

References

Altiero, T., Guidetti, R., Caselli, V., Cesari, M., and Rebecchi, L. (2011). Ultraviolet radiation tolerance in hydrated and desiccated eutardigrades. *J. Zool. Syst. Evol. Res.* 49, 104–110. doi:10.1111/j.1439-0469.2010.00607.x

Arakawa, K. (2022). Examples of extreme survival: tardigrade genomics and molecular anhydrobiology. *Annu. Rev. Anim. Biosci.* 10, 17–37. doi:10.1146/annurev-animal-021419-083711

Binhi, V. N., and Prato, F. S. (2017). Biological effects of the hypomagnetic field: an analytical review of experiments and theories. *PLoS One* 12, e0179340. doi:10.1371/journal.pone.0179340

Bryce, D. (1892). On some moss-dwelling Cathypnadae; with descriptions of five new species. *Sci. Gossip Lond.* 28, 271–275.

Cao, L. C., Honeyman, T. W., Cooney, R., Kennington, L., Scheid, C. R., and Jonassen, J. A. (2004). Mitochondrial dysfunction is a primary event in renal cell oxalate toxicity. *Kidney Int.* 66 (5), 1890–1900. doi:10.1111/j.1523-1755.2004.00963.x

Cardinale, D. A., Larsen, F. J., Schiffer, T. A., Morales-Alamo, D., Ekblom, B., Calbet, J. A., et al. (2018). Superior intrinsic mitochondrial respiration in women than in men. *Front. Physiol.* 9, 1133. doi:10.3389/fphys.2018.01133

Conley, C. C. (1970). *A Review of the biological effects of very low magnetic fields*. NASA: Technical Note. TN D-5902: 1–27. Available at: http://ntrs.nasa.gov/archive/nasa/casi.ntrs.nasa.gov/19700024915.pdf.

Copley, J. (1999). *Indestructible. New Sci.* 2209, 44–46.

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

Damacena de Angelis, C., Endoni, B. T., Nuno, D., Lamping, K., Ledolter, J., Koval, O. M., et al. (2022). Sex-specific differences in endothelial function are driven by divergent mitochondrial Ca^{2+} handling. *J. Am. Heart Assoc.* 11 (13), e023912. doi:10.1161/JAHA.121.023912

Degma, P., Bertolani, R., and Guidetti, R. (2023). *Actual checklist of tardigrada species*. 42th. Available at: https://iris.unimore.it/retrieve/bf8e14a4-625f-4cdd-8100-347e5cbc5f63/Actual%20checklist%20of%20Tardigrada%2042th%20Edition%2009-01-23.pdf.

Doyère, M. (1840). Memoire sur les Tardigrades. I. Annales des Sciences Naturelles. *Zool. Ser.* 2 (14), 269–362.

Erdmann, W., Idzikowski, B., Kowalski, W., Kosicki, J. Z., and Kaczmarek, Ł. (2021b). Tolerance of two anhydrobiotic tardigrades *Echiniscus testudo* and *Milnesium inceptum* to hypomagnetic conditions. *PeerJ* 9, e10630. doi:10.7717/peerj.10630

Erdmann, W., Idzikowski, B., Kowalski, W., Szymański, B., Kosicki, J. Z., and Kaczmarek, Ł. (2017). Can the tardigrade *Hypsibius dujardini* survive in the absence of the geomagnetic field? *PLoS One* 12 (9), e0183380. doi:10.1371/journal.pone.0183380

Erdmann, W., Kmita, H., Kosicki, J. Z., and Kaczmarek, Ł. (2021a). How the geomagnetic field influences life on Earth–an integrated approach to geomagnetobiology. *Orig. Life Evol. Biosph.* 51 (3), 231–257. doi:10.1007/s11084-021-09612-5

Fu, J. P., Mo, W. C., Liu, Y., and He, R. Q. (2016). Decline of cell viability and mitochondrial activity in mouse skeletal muscle cell in a hypomagnetic field. *Bioelectromagnetics* 37 (4), 212–222. doi:10.1002/bem.21968

Gąsiorek, P., Stec, D., Morek, W., and Michalczyk, Ł. (2018). An integrative redescription of *Hypsibius dujardini* (Doyère, 1840), the nominal taxon for Hypsibioidea (Tardigrada: Eutardigrada). *Zootaxa* 4415 (1), 045–075. doi:10.11646/zootaxa.4415.1.2

Guidetti, R., Rizzo, A. M., Altiero, T., and Rebecchi, L. (2012). 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 (1), 97–102. doi:10.1016/j.pss.2012.05.021

Halberg, K. A., Jørgensen, A., and Møbjerg, N. (2013). Desiccation tolerance in the tardigrade *Richtersius coronifer* relies on muscle mediated structural reorganization. *PLoS One* 8 (12), e85091. doi:10.1371/journal.pone.0085091

Hashimoto, T., Horikawa, D. D., Saito, Y., Kuwahara, H., Kozuka-Hata, H., Shin-i, T., et al. (2016). Extremotolerant tardigrade genome and improved radiotolerance of human cultured cells by tardigrade-unique protein. *Nat. Commun.* 7 (1), 12808. doi:10.1038/ncomms12808

Hesgrove, C., and Boothby, T. C. (2020). The biology of tardigrade disordered proteins in extreme stress tolerance. *Cell. Commun. Signal.* 18, 178–215. doi:10.1186/s12964-020-00670-2

Horikawa, D. D. (2012). "Survival of tardigrades in extreme environments: A model animal for Astrobiology," in (eds) *Anoxia: Evidence for eukaryote survival and paleontological strategies*, eds. A. Altenbach, J. Bernhard, and J. Seckbach, (Dordrecht, Netherlands: Springer, 205–217. doi:10.1007/978-94-007-1896-8_12

Hu, P. D., Mo, W. C., Fu, J. P., Liu, Y., and He, R. Q. (2020). Long-term hypogeomagnetic field exposure reduces muscular mitochondrial function and exercise capacity in adult male mice. *Prog. Biochem. Biophys.* 47 (5), 426–438. doi:10.16476/j.pibb.2020.0047

Jönsson, K. I. (2007). Tardigrades as a potential model organism in space research. *Astrobiology* 7 (5), 757–766. doi:10.1089/ast.2006.0088

Jönsson, K. I. (2020). "Tardigrades – evolutionary explorers in extreme environments," in *Extremophiles as astrobiological models*. Editors J. Seckbach and Stan-Lotter (Beverly, MA, USA: Wiley), 255–274. doi:10.1002/9781119593096.ch12

Kaczmarek, Ł., Roszkowska, M., Fontaneto, D., Jezierska, M., Pietrzak, B., Wieczorek, R., et al. (2019). Two new species of the genus *Milnesium* Doyère, 1840 (Tardigrada, Apochela, Milnesiidae) from Madagascar. *J. Zool.* 309 (1), 1–22. doi:10.3897/zookeys.884.29469

Kaczmarek, Ł., Roszkowska, M., Poprawa, I., Janelt, K., Kmita, H., Gawlak, M., et al. (2020). Integrative description of bisexual *Paramacrobiotus experimentalis* sp. nov. (Macrobiotidae) from republic of Madagascar (Africa) with microbiome analysis. *Mol. Phylogenet. Evol.* 145, 106730. doi:10.1016/j.ympev.2019.106730

Kopcewicz, M., Grabias, A., Škorvánek, I., Marcin, J., Idzikowski, B., and Mössbauer, H. (1999). Mössbauer study of the magnetic properties of nanocrystalline Fe80.5Nb7B12.5 alloy. *J. Appl. Phys.* 85, 4427–4429. doi:10.1063/1.370363

McCarthy, T., and delBarco-Trillo, J. (2020). Tardigrade abundance in relation to urbanisation and highly anthropogenic substrates. *J. Urban Ecol.* 6 (1), juaa008. doi:10.1093/jue/juaa008

Miglierini, M., Tóth, I., Seberíni, M., Illeková, E., and Idzikowski, B. (2002). Structure and hyperfine interactions of melt-spun Fe80Mo7X1B12 (X = Cu or Au) before and after transformation into nanocrystalline states. *J. Phys. Condens Matter.* 14, 1249–1260. doi:10.1088/0953-8984/14/6/312

Mo, W., Liu, Y., and He, R. (2014). Hypomagnetic field, an ignorable environmental factor in space. *Sci. China Life Sci.* 57 (7), 726–728. doi:10.1007/s11427-014-4662-x

Morek, W., Suzuki, A. C., Schill, R. O., Georgiev, D., Yankova, M., Marley, N. J., et al. (2019). Redescription of *Milnesium alpigenum* Ehrenberg, 1853 (Tardigrada: Apochela) and a description of *Milnesium inceptum* sp. nov., a tardigrade laboratory model. *Zootaxa* 4586 (1), 35–64. doi:10.11646/zootaxa.4586.1.2

Nagwani, A. K., Kaczmarek, Ł., and Kmita, H. (2022). Applicable life-history and molecular traits for studying the effects of anhydrobiosis on aging in tardigrades. *Diversity* 14 (8), 664. doi:10.3390/d14080664

Nagwani, A. K., Melosik, I., Kaczmarek, L., and Kmita, H. (2023). Recovery from anhydrobiosis in the tardigrade *Paramacrobiotus experimentalis*: better to be young than old and in a group than alone. *bioRxiv*. doi:10.1101/2023.05.22.541721

Neginskaya, M. A., Pavlov, E. V., and Sheu, S. S. (2021). Electrophysiological properties of the mitochondrial permeability transition pores: channel diversity and disease implication. *Biochim. Biophys.* 1862 (3), 148357. doi:10.1016/j.bbabio.2020.148357

Nelson, D. R., Guidetti, R., and Rebecchi, L. (2015). in *Phylum Tardigrada" in Thorp and Covich's freshwater invertebrates*. Editors J. H. Thorp and D. C. Rogers (Cambridge, UK: Academic Press), 347–380.

Ogneva, I. V., Usik, M. A., Burtseva, M. V., Biryukov, N. S., Zhdankina, Y. S., Sychev, V. N., et al. (2020). *Drosophila melanogaster* sperm under simulated microgravity and a hypomagnetic field: motility and cell respiration. *Int. J. Mol. Sci.* 21 (17), 5985. doi:10.3390/ijms21175985

Panel, M., Ghaleh, B., and Morin, D. (2018). Mitochondria and aging: A role for the mitochondrial transition pore? *Aging Cell.* 17 (4), e12793. doi:10.1111/acel.12793

Ramløv, H., and Westh, P. (2001). Cryptobiosis in the eutardigrade *Adorybiotus (Richtersius) coronifer*: tolerance to alcohols, temperature and de novo protein synthesis. *Zool. Anz.* 240 (3-4), 517–523. doi:10.1078/0044-5231-00062

Rebecchi, L., Boschetti, C., and Nelson, D. R. (2020). Extreme-tolerance mechanisms in meiofaunal organisms: A case study with tardigrades, rotifers and nematodes. *Hydrobiologia* 847 (12), 2779–2799. doi:10.1007/s10750-019-04144-6

Roszkowska, M., Wojciechowska, D., Kmita, H., Cerbin, S., Dziuba, M. K., and Fiałkowska, E. (2021). Tips and tricks how to culture water bears: simple protocols for culturing eutardigrades (Tardigrada) under laboratory conditions. *Eur. Zool.* 88 (1), 449–465. doi:10.1080/24750263.2021.1881631

Schill, R. O., and Hengherr, S. (2018). "Environmental adaptations: desiccation tolerance," in *Water bears: The Biology of tardigrades*. Editor R. O. Schill (Cham, Switzerland: Springer), 273–293. doi:10.1007/978-3-319-95702-9

Silaidos, C., Pilatus, U., Grewal, R., Matura, S., Lienerth, B., Pantel, J., et al. (2018). Sex-associated differences in mitochondrial function in human peripheral blood mononuclear cells (PBMCs) and brain. *Biol. Sex. Differ.* 9, 34–10. doi:10.1186/s13293-018-0193-7

Sinčák, M., and Sedlakova-Kadukova, J. (2023). Hypomagnetic fields and their multilevel effects on living organisms. *Processes* 11 (1), 282. doi:10.3390/pr11010282

Srimai, N., Kiatarkom, W., and Miyata, H. (2020). Evaluation of the effects of hypomagnetic fields on mouse macrophage RAW264 cells. *Int. J. Chem.* 13 (1), 12. doi:10.5539/ijc.v13n1p12

Srinivasan, S., Guha, M., Kashina, A., and Avadhani, N. G. (2017). Mitochondrial dysfunction and mitochondrial dynamics-The cancer connection. *Biochim. Biophys.* 1858 (8), 602–614. doi:10.1016/j.bbabio.2017.01.004

Vakifahmetoglu-Norberg, H., Ouchida, A. T., and Norberg, E. (2017). The role of mitochondria in metabolism and cell death. *Biochem. Biophys. Res. Commun.* 482 (3), 426–431. doi:10.1016/j.bbrc.2016.11.088

Wang, G. M., Fu, J. P., Mo, W. C., Zhang, H. T., Liu, Y., and He, R. Q. (2022). Shielded geomagnetic field accelerates glucose consumption in human neuroblastoma cells by promoting anaerobic glycolysis. *Biochem. Biophys. Res. Commun.* 601, 101–108. doi:10.1016/j.bbrc.2022.01.114

Wang, Y., Xu, E., Musich, P. R., and Lin, F. (2019). Mitochondrial dysfunction in neurodegenerative diseases and the potential countermeasure. *CNS Neurosci. Ther.* 25 (7), 816–824. doi:10.1111/cns.13116

Wojciechowska, D., Karachitos, A., Roszkowska, M., Rzeźniczak, W., Sobkowiak, R., Kaczmarek, Ł., et al. (2021a). Mitochondrial alternative oxidase contributes to successful tardigrade anhydrobiosis. *Front. Zool.* 18 (1), 15–12. doi:10.1186/s12983-021-00400-5

Wojciechowska, D., Roszkowska, M., Kaczmarek, Ł., Jarmuszkiewicz, W., Karachitos, A., and Kmita, H. (2021b). The tardigrade *Hypsibius exemplaris* has the active mitochondrial alternative oxidase that could be studied at animal organismal level. *Plos one* 16 (8), e0244260. doi:10.1371/journal.pone.0244260

Xue, X., Ali, Y. F., Luo, W., Liu, C., Zhou, G., and Liu, N. A. (2021). Biological effects of space hypomagnetic environment on circadian rhythm. *Front. Physiol.* 12, 643943. doi:10.3389/fphys.2021.643943

Zarulli, V., Barthold Jones, J. A., Oksuzyan, A., Lindahl-Jacobsen, R., Christensen, K., and Vaupel, J. W. (2018). Women live longer than men even during severe famines and epidemics. *Proc. Natl. Acad. Sci.* 115 (4), E832-E840–E840. doi:10.1073/pnas.1701535115

Zhang, B., and Tian, L. (2020). Reactive oxygen species: potential regulatory molecules in response to hypomagnetic field exposure. *Bioelectromagnetics* 41 (8), 573–580. doi:10.1002/bem.22299

Table S1: The applied age classes distinguished for *P. experimentalis* and based on the selected life history trait analysis. Body length, body width and numbers of eggs were assessed using a stereomicroscope (OLYMPUS SZ61). The calibrated grid of the stereomicroscope was used to measure the body length and body width to an accuracy of 10 µm. ND, no data recorded; n, the number of individuals.

Age classes	**Age range in days (assigned group name)**	**Average body length (± SD) [µm]**		**Average body width (± SD) [µm]**		**Average number of laid eggs per female (± SD)**
		Females	**Males**	**Females**	**Males**	
1	30-60 days (growing adults)	420 ± 20 n=25	350 ± 30 n=25	160 ± 20 n=25	110 ± 10 n=25	97 ± 18 n=100
2	150-180 days (mature adults)	750 ± 40 n=20	590 ± 50 n=20	240 ± 30 n=20	140 ± 20 n=20	162 ± 32 n=70
3	>300 days (old adults)	750 ± 30 n=10	600 ± 30 n=10	250 ± 30 n=10	150 ± 20 n=10	ND

Table S2. Raw data and statistical analysis of HMF treatment effect on the survival rate.

ANOVA: Analysis of variance using aligned rank transformation					
SMF_Females_Groups					
	Df	Df,res	F value	Pr(>F)	
Age	2	18	1.54414	0.24048	
Duration	2	18	2.72815	0.07237	
Age:Durati	4	18	0.82802	0.52456	
Results of Tukey post-hoc test (α=0,05)					
	Age				
Duration	150-180 days	30-60 days	> 300 days		
15 days	15.00	15.00	14.67	**14.89**	a
30 days	14.67	14.33	14.33	**14.44**	a
7 days	15.00	15.00	15.00	**15.00**	a
	14.89	**14.78**	**14.67**		
	a	a	a		
ANOVA: Analysis of variance using aligned rank transformation					
SMF_Males_Groups					
	Df	Df,res	F value	Pr(>F)	
Age	2	18	2.61997	0.100309	
Duration	2	18	2.88062	0.082154	
Age:Durati	4	18	0.32081	0.86032	
Results of Tukey post-hoc test (α=0,05)					
	Age				
Duration	150-180 days	30-60 days	> 300 days		
15 days	14.67	14.67	14.33	**14.56**	a
30 days	14.67	14.33	14.00	**14.33**	a
7 days	15.00	15.00	14.67	**14.89**	a
	14.78	**14.67**	**14.33**		
	a	a	a		
ANOVA: Analysis of variance using aligned rank transformation					
HMF_Females_Group					
	Df	Df,res	F value	Pr(>F)	
Age	2	18	2.03205	0.160058	
Duration	2	18	11.26757	0.000671	***
Age:Durati	4	18	0.48807	0.744431	
Results of Tukey post-hoc test (α=0,05)					
	Age				
Duration	150-180 days	30-60 days	> 300 days		
15 days	14.67	14.33	14.33	**14.44**	a
30 days	14.33	13.67	13.33	**13.78**	b
7 days	15.00	15.00	14.67	**14.89**	a
	14.67	**14.33**	**14.11**		
	a	a	a		
ANOVA: Analysis of variance using aligned rank transformation					
SMF_Males_Groups					
	Df	Df,res	F value	Pr(>F)	
Age	2	18	11.7067	0.000554	***
Duration	2	18	22.9695	1.11E-05	***
Age:Durati	4	18	1.5655	0.226266	
Results of Tukey post-hoc test (α=0,05)					
	Age				
Duration	150-180 days	30-60 days	> 300 days		
15 days	14.33	14.33	14.00	**14.22**	a
30 days	14.00	13.33	13.00	**13.44**	b
7 days	15.00	15.00	14.33	**14.78**	a
	14.44	**14.22**	**13.78**		
	a	a	b		
Codes of satistically significant differences: 0 '***'; 0,001 '**'; 0,01 '*'; 0,05 ',';					

SMF_ FEMALES_GROUPS					SMF_MALES_GROUPS			
Age	duration of treatment	R	active animals after treatment		Age	duration of treatment	R	active animals after treatment
30-60 days	7 days	R1	15		30-60 days	7 days	R1	15
30-60 days	7 days	R2	15		30-60 days	7 days	R2	15
30-60 days	7 days	R3	15		30-60 days	7 days	R3	15
30-60 days	15 days	R1	15		30-60 days	15 days	R1	15
30-60 days	15 days	R2	15		30-60 days	15 days	R2	14
30-60 days	15 days	R3	15		30-60 days	15 days	R3	15
30-60 days	30 days	R1	15		30-60 days	30 days	R1	14
30-60 days	30 days	R2	14		30-60 days	30 days	R2	14
30-60 days	30 days	R3	14		30-60 days	30 days	R3	15
150-180 days	7 days	R1	15		150-180 days	7 days	R1	15
150-180 days	7 days	R2	15		150-180 days	7 days	R2	15
150-180 days	7 days	R3	15		150-180 days	7 days	R3	15
150-180 days	15 days	R1	15		150-180 days	15 days	R1	14
150-180 days	15 days	R2	15		150-180 days	15 days	R2	15
150-180 days	15 days	R3	15		150-180 days	15 days	R3	15
150-180 days	30 days	R1	15		150-180 days	30 days	R1	15
150-180 days	30 days	R2	15		150-180 days	30 days	R2	14
150-180 days	30 days	R3	14		150-180 days	30 days	R3	15
>300 days	7 days	R1	15		>300 days	7 days	R1	14
>300 days	7 days	R2	15		>300 days	7 days	R2	15
>300 days	7 days	R3	15		>300 days	7 days	R3	15
>300 days	15 days	R1	14		>300 days	15 days	R1	14
>300 days	15 days	R2	15		>300 days	15 days	R2	15
>300 days	15 days	R3	15		>300 days	15 days	R3	14
>300 days	30 days	R1	15		>300 days	30 days	R1	14
>300 days	30 days	R2	14		>300 days	30 days	R2	14
>300 days	30 days	R3	14		>300 days	30 days	R3	14

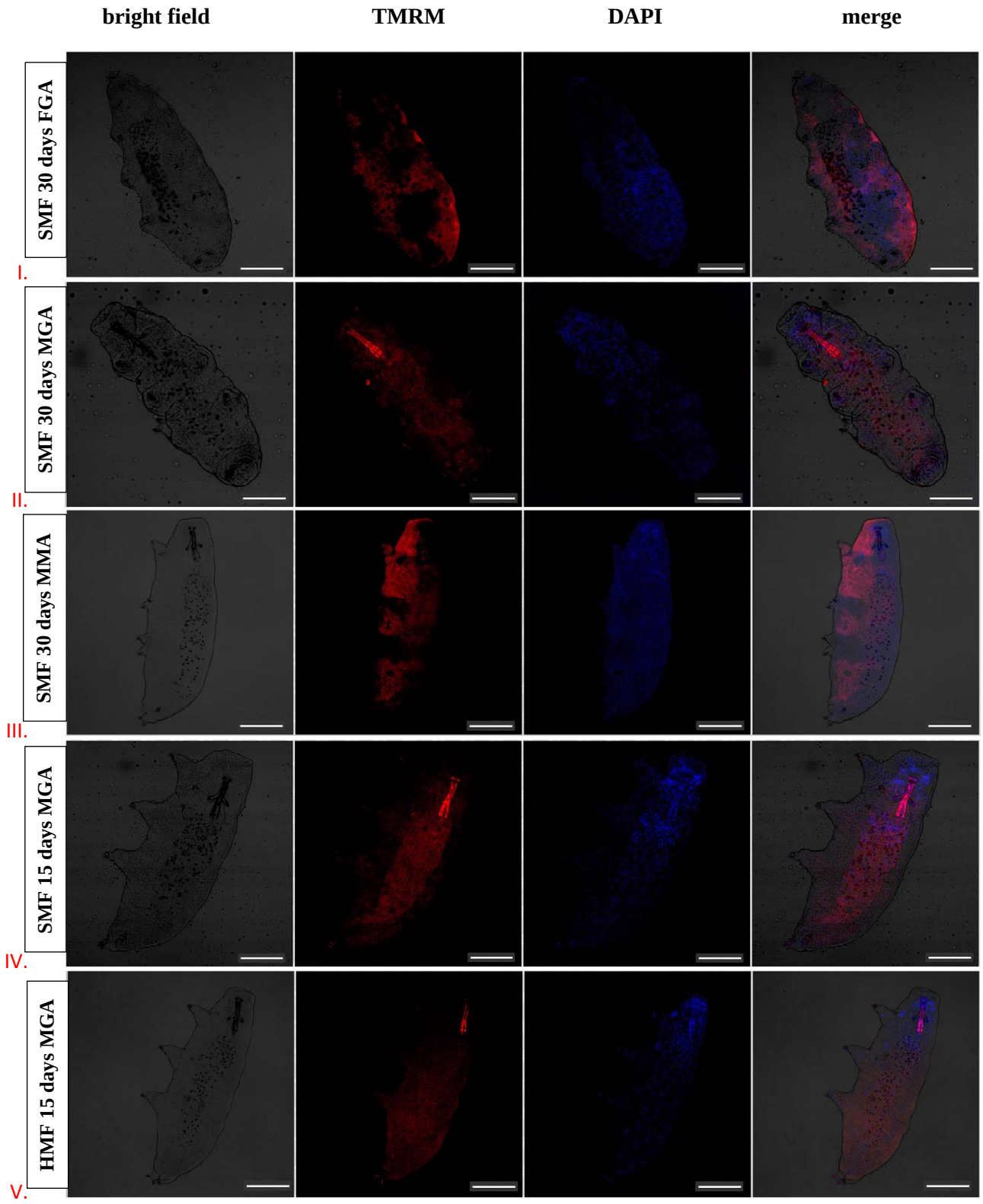
HMF_FEMALES_GROUPS					HMF_MALES-GROUPS			
Age	duration of treatment	R	active animals after treatment		Age	duration of treatment	R	active animals after treatment
30-60 days	7 days	R1	15		30-60 days	7 days	R1	15
30-60 days	7 days	R2	15		30-60 days	7 days	R2	15
30-60 days	7 days	R3	15		30-60 days	7 days	R3	15
30-60 days	15 days	R1	15		30-60 days	15 days	R1	14
30-60 days	15 days	R2	14		30-60 days	15 days	R2	15
30-60 days	15 days	R3	14		30-60 days	15 days	R3	14
30-60 days	30 days	R1	14		30-60 days	30 days	R1	13
30-60 days	30 days	R2	13		30-60 days	30 days	R2	13
30-60 days	30 days	R3	14		30-60 days	30 days	R3	14
150-180 days	7 days	R1	15		150-180 days	7 days	R1	15
150-180 days	7 days	R2	15		150-180 days	7 days	R2	15
150-180 days	7 days	R3	15		150-180 days	7 days	R3	15
150-180 days	15 days	R1	15		150-180 days	15 days	R1	14
150-180 days	15 days	R2	14.00		150-180 days	15 days	R2	15
150-180 days	15 days	R3	15.00		150-180 days	15 days	R3	14
150-180 days	30 days	R1	14.00		150-180 days	30 days	R1	14
150-180 days	30 days	R2	14		150-180 days	30 days	R2	14
150-180 days	30 days	R3	15		150-180 days	30 days	R3	14
>300 days	7 days	R1	15		>300 days	7 days	R1	14
>300 days	7 days	R2	14		>300 days	7 days	R2	14
>300 days	7 days	R3	15.00		>300 days	7 days	R3	15
>300 days	15 days	R1	14.00		>300 days	15 days	R1	14
>300 days	15 days	R2	14.00		>300 days	15 days	R2	14
>300 days	15 days	R3	15		>300 days	15 days	R3	14
>300 days	30 days	R1	14		>300 days	30 days	R1	13
>300 days	30 days	R2	13		>300 days	30 days	R2	13
>300 days	30 days	R3	13		>300 days	30 days	R3	13

Table S3. Raw data and statistical analysis of HMF treatment effect on the mitochondrial inner membrane potential.

ANOVA					
SMF_Females_Groups					
	Df	Df,res	F value	Pr(>F)	
Age	2	18	285.8045	2.303E-14	***
Duration	2	18	0.0122	0.9879	
Age:Durati	4	18	0.0016	1.0000	
Results of Tukey post-hoc test (α=0,05)					
	Age				
Duration	150-180 days	30-60 days	> 300 days		
15 days	532.64	348.70	475.12	**452.15**	a
30 days	531.96	348.36	474.25	**451.52**	a
7 days	533.75	348.74	475.77	**452.75**	a
	532.78	**348.60**	**475.05**		
	a	c	b		
ANOVA					
SMF_Males_Groups					
	Df	Df,res	F value	Pr(>F)	
Age	2	18	248.776	7.712E-14	***
Duration	2	18	0.004	0.996	
Age:Durati	4	18	0.000	1.000	
Results of Tukey post-hoc test (α=0,05)					
	Age				
Duration	150-180 days	30-60 days	> 300 days		
15 days	377.42	251.17	307.83	**312.14**	a
30 days	374.14	251.02	307.63	**310.93**	a
7 days	374.62	251.52	308.12	**311.42**	a
	375.39	**251.24**	**307.86**		
	a	c	b		
ANOVA					
HMF_Females_Group					
	Df	Df,res	F value	Pr(>F)	
Age	2	18	356.0079	3.369E-15	***
Duration	2	18	11.7166	0.0005512	***
Age:Durati	4	18	0.3132	0.8653620	
Results of Tukey post-hoc test (α=0,05)					
	Age				
Duration	150-180 days	30-60 days	> 300 days		
15 days	479.31	297.62	374.84	**383.92**	a
30 days	462.28	274.31	343.58	**360.06**	b
7 days	490.94	301.74	386.08	**392.92**	a
	477.51	**291.22**	**368.17**		
	a	c	b		
ANOVA					
HMF_Males_Group					
	Df	Df,res	F value	Pr(>F)	
Age	2	18	269.6930	3.820E-14	***
Duration	2	18	18.9799	3.686E-05	***
Age:Durati	4	18	0.5703	0.6875	
Results of Tukey post-hoc test (α=0,05)					
	Age				
Duration	150-180 days	30-60 days	> 300 days		
15 days	340.13	195.56	250.56	**262.08**	b
30 days	331.03	192.44	227.80	**250.42**	b
7 days	362.83	224.28	275.50	**287.54**	a
	344.66	**204.09**	**251.29**		
	a	c	b		
Codes of satistically significant differences: 0 '***'; 0,001 '**'; 0,01 '*'; 0,05 ','; 0,1 '1'					

SMF_ FEMALES_GROUPS					SMF_MALES_GROUPS			
Age	duration of treatment	R	FI_{TMRM}/animal		Age	duration of treatment	R	FI_{TMRM}/animal
30-60 days	7 days	R1	361.66		30-60 days	7 days	R1	239.72
30-60 days	7 days	R2	353.76		30-60 days	7 days	R2	250.92
30-60 days	7 days	R3	330.81		30-60 days	7 days	R3	263.92
30-60 days	15 days	R1	380.8		30-60 days	15 days	R1	256.53
30-60 days	15 days	R2	340.78		30-60 days	15 days	R2	247.91
30-60 days	15 days	R3	324.51		30-60 days	15 days	R3	249.07
30-60 days	30 days	R1	349.17		30-60 days	30 days	R1	251.13
30-60 days	30 days	R2	364.44		30-60 days	30 days	R2	239.4
30-60 days	30 days	R3	331.46		30-60 days	30 days	R3	262.52
150-180 days	7 days	R1	552.3		150-180 days	7 days	R1	368.48
150-180 days	7 days	R2	514.5		150-180 days	7 days	R2	394.59
150-180 days	7 days	R3	534.46		150-180 days	7 days	R3	360.79
150-180 days	15 days	R1	512.92		150-180 days	15 days	R1	367.27
150-180 days	15 days	R2	556.82		150-180 days	15 days	R2	374.84
150-180 days	15 days	R3	528.2		150-180 days	15 days	R3	381.14
150-180 days	30 days	R1	539.58		150-180 days	30 days	R1	383.92
150-180 days	30 days	R2	536.15		150-180 days	30 days	R2	368.59
150-180 days	30 days	R3	520.16		150-180 days	30 days	R3	369.92
>300 days	7 days	R1	469.78		>300 days	7 days	R1	319.38
>300 days	7 days	R2	484.24		>300 days	7 days	R2	308.65
>300 days	7 days	R3	473.28		>300 days	7 days	R3	296.33
>300 days	15 days	R1	483.04		>300 days	15 days	R1	307.97
>300 days	15 days	R2	469.12		>300 days	15 days	R2	325.13
>300 days	15 days	R3	473.2		>300 days	15 days	R3	290.4
>300 days	30 days	R1	468.24		>300 days	30 days	R1	298.22
>300 days	30 days	R2	484.32		>300 days	30 days	R2	311.98
>300 days	30 days	R3	470.2		>300 days	30 days	R3	312.68
HMF_FEMALES_GROUPS					**HMF_MALES-GROUPS**			
Age	duration of treatment	R	FI_{TMRM}/animal		Age	duration of treatment	R	FI_{TMRM}/animal
30-60 days	7 days	R1	302.03		30-60 days	7 days	R1	227.86
30-60 days	7 days	R2	313.01		30-60 days	7 days	R2	209.92
30-60 days	7 days	R3	290.17		30-60 days	7 days	R3	235.05
30-60 days	15 days	R1	296.51		30-60 days	15 days	R1	206.97
30-60 days	15 days	R2	312.34		30-60 days	15 days	R2	195.57
30-60 days	15 days	R3	284.01		30-60 days	15 days	R3	184.14
30-60 days	30 days	R1	287.23		30-60 days	30 days	R1	195.67
30-60 days	30 days	R2	272.91		30-60 days	30 days	R2	196.57
30-60 days	30 days	R3	262.8		30-60 days	30 days	R3	185.08
150-180 days	7 days	R1	507.94		150-180 days	7 days	R1	370.62
150-180 days	7 days	R2	470.56		150-180 days	7 days	R2	358.29
150-180 days	7 days	R3	494.32		150-180 days	7 days	R3	359.57
150-180 days	15 days	R1	486.86		150-180 days	15 days	R1	351.05
150-180 days	15 days	R2	475.32		150-180 days	15 days	R2	335.02
150-180 days	15 days	R3	475.76		150-180 days	15 days	R3	334.33
150-180 days	30 days	R1	442.24		150-180 days	30 days	R1	337.06
150-180 days	30 days	R2	463.08		150-180 days	30 days	R2	324.7
150-180 days	30 days	R3	481.52		150-180 days	30 days	R3	331.33
>300 days	7 days	R1	400.42		>300 days	7 days	R1	286.65
>300 days	7 days	R2	377.8		>300 days	7 days	R2	272.32
>300 days	7 days	R3	380.02		>300 days	7 days	R3	267.54
>300 days	15 days	R1	394.82		>300 days	15 days	R1	268.13
>300 days	15 days	R2	369.06		>300 days	15 days	R2	252.32
>300 days	15 days	R3	360.64		>300 days	15 days	R3	231.24
>300 days	30 days	R1	360.62		>300 days	30 days	R1	254.03
>300 days	30 days	R2	340.88		>300 days	30 days	R2	222.87
>300 days	30 days	R3	329.24		>300 days	30 days	R3	206.49

.



Supplementary Figure 1.Typical confocal microscopy images of individuals exposed to SMF and HMF for different durations (i.e. 15 and 30 days) and stained with TMRM and DAPI (scale bar, 100 μm). The images illustrate sex-specific differences (images I and II), age-specific differences (images II and III) and, differences after exposure to SMF and HMF (images IV and V). FGA, female growing adults and MGA, male growing adults (the age of 30-60 days); MMA, male mature adults (the age of 150-180 days). The figure presents images representative for three animals.

Author statement

Amit Kumar Nagwani
aminag@amu.edu.pl

Poznań, 17.09.2023

AUTHOR STATEMENT

for the research article 'The effect of hypomagnetic field on survival and mitochondrial functionality of active *Paramacrobiotus experimentalis* females and males of different age'. Front. Physiol. 14:1253483 (2023).

I declare that the research paper by Nagwani AK, Budka A, Łacka A, Kaczmarek Ł, Kmita H. **The effect of hypomagnetic field on survival and mitochondrial functionality of active *Paramacrobiotus experimentalis* females and males of different age (2023) Front. Physiol. 14:1253483** (doi: https://doi.org/10.3389/fphys.2023.1253483) is a part of my PhD thesis. My contribution includes performing of the tardigrade culture and experiments, curating of the data as well as participating in research conceptualization, writing of the final version of the manuscript and preparing all figures and tables.

Name:Amit Kumar Nagwani
Adress: Department of Bioenergetics,
Institute of Molecular Biology and Biotechnology,
Faculty of Biology, Adam Mickiewicz University in Poznań, Poznań
Uniwersytetu Poznańskiego 6, 61-614,
Poznań, Poland

Signature:

Kierownik
Zakładu Bioenergetyki

prof. dr hab. Hanna Kmita

Co-author statements

Prof. UPP dr hab. Anna Budka
anna.budka@up.poznan.pl

Poznań, 08.09.2023

CO-AUTHOR STATEMENT

I declare that I am aware that the research paper by *Nagwani AK, Budka A, Łacka A, Kaczmarek Ł, Kmita H. (2023) The effect of hypomagnetic field on survival and mitochondrial functionality of active Paramacrobiotus experimentalis females and males of different age Front. Physiol. 14:1253483 (doi: https://doi.org/10.3389/fphys.2023.1253483)* is a part of Amit Kumar Nagwani PhD thesis.

At the same time, I declare that I contributed in investigation of performed study and statistically analyzed the laboratory results.

Anna Budka

Anna Budka

Dr hab. Agnieszka Łacka
agnieszka.lacka@up.poznan.pl

Poznań, 08.09.2023

CO-AUTHOR STATEMENT

I declare that I am aware that the research paper by *Nagwani AK, Budka A, Łacka A, Kaczmarek Ł, Kmita H. (2023) The effect of hypomagnetic field on survival and mitochondrial functionality of active Paramacrobiotus experimentalis females and males of different age Front. Physiol. 14:1253483 (doi: https://doi.org/10.3389/fphys.2023.1253483)* is a part of Amit Kumar Nagwani PhD thesis.

At the same time, I declare that I contributed in investigation of performed study and statistically analyzed the laboratory results.

Agnieszka Łacka

Prof. UAM dr hab. Łukasz Kaczmarek
kaczmar@amu.edu.pl

Poznań, 14.09.2023

CO-AUTHOR STATEMENT

I declare that I am aware that the research paper by *Nagwani AK, Budka A, Łacka A, Kaczmarek Ł, Kmita H. (2023) The effect of hypomagnetic field on survival and mitochondrial functionality of active Paramacrobiotus experimentalis females and males of different age Front. Physiol. 14:1253483 (doi: https://doi.org/10.3389/fphys.2023.1253483)* is a part of Amit Kumar Nagwani PhD thesis.

At the same time, I declare that I supervised in validation of applied methodology and results.

Łukasz Kaczmarek

ADAM MICKIEWICZ UNIVERSITY, POZNAŃ
Faculty of Biology
Institute of Molecular Biology and Biotechnology
Department of Bioenergetics

Prof. dr hab. Hanna Kmita
kmita@amu.edu.pl

Poznań, 5.09.2023

CO-AUTHOR STATEMENT

I declare that I am aware that the paper by *Nagwani AK, Budka A, Łacka A, Kaczmarek Ł, Kmita H. (2023) The effect of hypomagnetic field on survival and mitochondrial functionality of active Paramacrobiotus experimentalis females and males of different age (2023) Front. Physiol. 14:1253483 (doi: https://doi.org/10.3389/fphys.2023.1253483)*, is a part of Amit Kumar Nagwani PhD thesis.

At the same time I declare that supervised the performed experiments as well as participate in data analysis, manuscript writing and made its revision.

Kierownik
Zakładu Bioenergetyki
prof. dr hab. Hanna Kmita

Hanna Kmita

ul. Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland
tel. +48 61 829 5553, 61 829 5848

www.biologia.amu.edu.pl

Appendix

10. APPENDIX 1

After 45 minutes of incubation with 10 µM $DCFH_2$-DA (prepared in methanol), animals were washed to remove the excess of the probe and then were analyzed under OLYMPUS IX81 inverted confocal microscope connected to a digital camera. For storage cells, the stained animals mounted on glass slides with Vectashield mounting medium were applied. The animals were gently broken with a pressure on a cover slip to release storage cells observed under ZEISS LSM 980 confocal microscope with Airyscan 2.

Table 1: Total number of intact animals and released storage cells applied in the study.

Age class	Sex	Total number of applied intact animals	Total number of applied released storage cells
30-60 days growing adults	**Females**	15	22
	Males	10	26
150-180 days mature adults	**Females**	15	21
	Males	10	24
>300 days old adults	**Females**	15	28
	Males	10	27

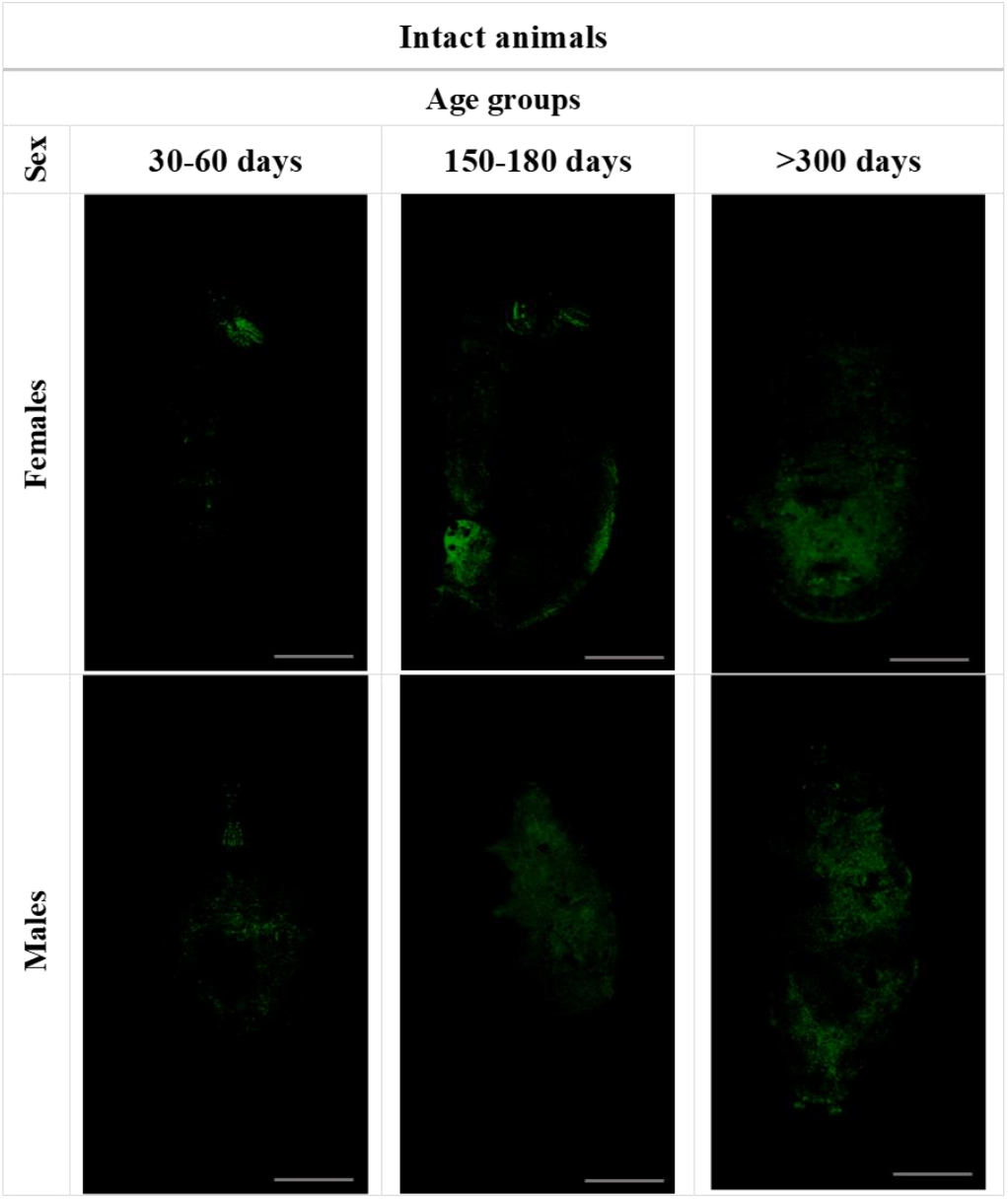


Figure 1: Confocal microscopy images of females and males of different age and stained with $DCFH_2$-DA, (scale bar, 100 μm)**.** The age of the animals: 30–60 days (growing adults), 150–180 days (mature adults), and over 300 days (old adults). The figure presents images representative for three animals. All confocal images are maximum projection images taken using the identical settings and later reoriented using ImageJ in order to keep the same orientation of all the captured images.

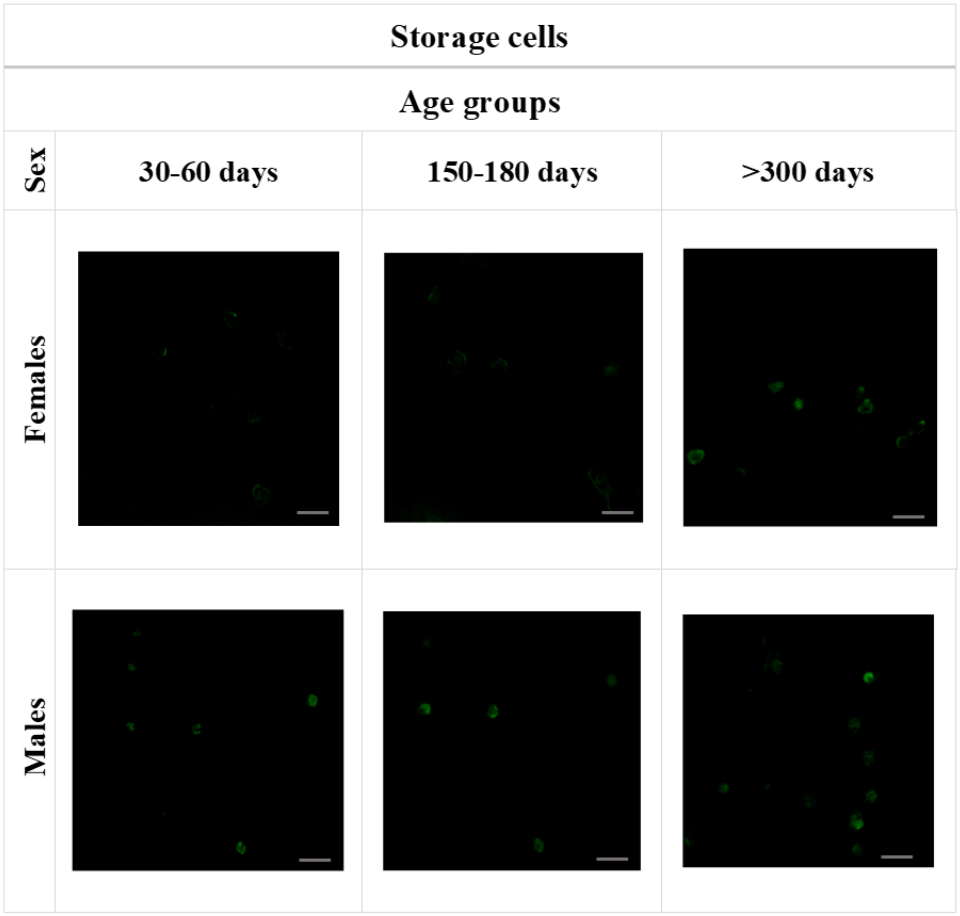


Figure 2: Confocal microscopy images of storage cells isolated from females and males of different age and stained with DCFH2-DA, (scale bar, 10 μm). The age of the animals: 30–60 days (growing adults), 150–180 days (mature adults), and over 300 days (old adults). The figure presents images representative storage cells released from for three animals. All confocal images are maximum projection images taken using the identical settings and later reoriented using ImageJ in order to keep the same orientation of all the captured images.