Adam Mickiewicz University in Poznan

Faculty of Biology

Department of Animal Taxonomy and Ecology

ZTiEZ, Poznań

The genus *Paramacrobiotus* (Tardigrada): integrative taxonomy, biogeography and effects of stress factors on the selected species.

ADAM MICKIEWICZ
UNIVERSITY
POZNAŃ

Pushpalata Kayastha

Doctoral thesis

Promotor: Dr hab. Prof. UAM Łukasz Kaczmarek

Promotor pomocniczy: Dr Monika Mioduchowska

Poznan, Poland 2023

ACKNOWLEDGEMENTS

My time spent as a PhD student at the Department of Animal Taxonomy and Ecology was like a roller coaster ride. Firstly, I would like to thank my parents (Birendra Prasad Lal Karna and Lalita Karna) and my siblings (Sanandan Kayastha and Yogita Kayastha) for always cheering, supporting and being there for me. Next, I thank myself for being able to go through this journey, survive and come out victorious.

I would also like to thank my supervisor, prof. UAM dr. hab. Łukasz Kaczmarek, for giving me the opportunity to be his student and to experience a truly exceptional atmosphere of both hard work and great joy in making scientific discoveries; for his outstanding support, understanding and inspiration to excel. My co-supervisor Dr. Monika Mioduchowska for helping and guiding me at every step alongside my supervisor. The head of the Department of Animal Taxonomy and Ecology prof. UAM dr. hab. Wojciech Ł. Magowski for outstanding support during my PhD. Professor Hanna Kmita for providing the laboratory workspace to carry out the molecular biology part of the integrative taxonomy approach.

Moreover, I would like to thank all my friends, colleagues, co-workers, professors, and administration support staff (especially Dr. Karolina Cerbin & Ms. Arleta Kucz) from the Adam Mickiewicz University in Poznań, and a huge thanks to the friends from my laboratory for their (Milena Roszkowska, Tomasz Bartylak, Amit Nagwani, Magdalena Górna and Michalina Krakowiak) invaluable help, scientific, as well as social support, encouragement, and inspiration. Nonetheless, I would like to thank all my friends for supporting me and tolerating me during the highs and lows of this PhD.

All members of our collaborator's group of Dr. hab. Izabela Poprawa at the University of Katowice and a group of Prof. Marek Michalak at the University of Alberta, Canada.

Rafał Gryka and his family (Paweł Gryka, Dorota Gryka and Emilia Gryka) in Poznań played a pivotal role in my integration into the Polish culture and made me feel at home in a foreign land.

Dedicated to both my late grandmothers, whom I lost during the period of my PhD.

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STRESZCZENIE

Paramacrobiotus stanowi jeden z rodzajów należących do typu Tardigrada (grupy zwierząt potocznie nazywanej niesporczakami lub misiami wodnymi). Rodzaj ten został utworzony ponad dekadę temu. Poprzednio, przedstawiciele tego rodzaju byli zaliczani do kompleksu *richtersi-areolatus* wewnątrz rodzaju *Macrobiotus*, by w końcu, przy pomocy technik analizy molekularnej i filogenetycznej zostać wyodrębnionymi jako nowy rodzaj.

Jak sugeruje tytuł, celem niniejszej rozprawy były badania rodzaju *Paramacrobiotus*, z uwzględnieniem zastosowania taksonomii integratywnej w opisie nowych dla wiedzy gatunków, ustalenie potencjalnych zasięgów występowania gatunków partenogenetycznych oraz sprawdzenie zasadności hipotezy „wszystko jest wszędzie" względem badanych gatunków, zbadanie wpływu czynników stresowych na gatunek *Pam. experimentalis*, badania nad rozmieszczeniem poszczególnych gatunków, badania mikrobiomu, a także przyjrzenie się historiom życiowym poszczególnych gatunków z uwzględnieniem zdolności do anhydrobiozy. Ponadto sporządzony został nowy klucz diagnostyczny dla rodzaju *Paramacrobiotus* wykorzystujący cechy morfologiczne i morfometryczne osobników dorosłych i jaj.

Wykorzystując techniki taksonomii integratywnej (klasyczna morfologia i morfometria oraz badania genetyczne), nowy gatunek, *Pam. gadabouti*, został opisany na podstawie materiału pochodzącego z Ribeiro Frio na Maderze. Ponadto, eksperymentalnie zbadano sposób rozmnażania się tego gatunku, co potwierdziło wzajemne powiązania między szerokim rozprzestrzenieniem a partenogenezą.

W kolejnej pracy przeanalizowano rozmieszczenie oraz zróżnicowanie genetyczne dwóch partenogenetycznych gatunków z rodzaju *Paramacrobiotus*, tj. *Pam. gadabouti* oraz *Pam. fairbanksi* potwierdzając prawdziwość hipotezy „wszystko jest wszędzie" przynajmniej dla niektórych gatunków niesporczaków. Modelowanie niszy środowiskowych wykonane przy użyciu narzędzia MaxEnt, potwierdza szeroki zasięg obydwu partenogenetycznych gatunków.

W kolejnej publikacji zbadano przeżywalność niesporczaków z gatunku *Pam. experimentalis*, wystawianych na działanie różnych stężeń (0,25%; 0,50% oraz 1,00%) nadchloranu magnezu (uwzględniając stężenia zaobserwowane w marsjańskim regolicie) w dwóch różnych przedziałach czasowych (24h i 72h). W próbach, w których osobniki zostały poddane 24-godzinnej ekspozycji, kolejno 33,3%; 16,7% oraz 0% osobników pozostało aktywnych w stężeniach 0,25%; 0,50% oraz 1,00%. Jednakże, ponad 75% z nich powróciło do

stanu aktywnego po przeniesieniu ich do medium hodowlanego (93,3%; 76,7% oraz 86,7% osobników w stężeniach 0,25%; 0,50% oraz 1,00%). W próbach, w których osobniki zostały poddane 72-godzinnej ekspozycji, kolejno 30,0%; 26,7% oraz 0% osobników pozostało aktywnych w stężeniach 0,25%; 0,50% oraz 1,00%. Po przeniesieniu ich do medium hodowlanego kolejno 83,3%; 86,7% oraz 10,0% osobników w stężeniach 0,25%; 0,50% oraz 1,00% powróciło do stanu aktywnego.

W następnej pracy zbadano zmiany w ultrastrukturze komórek spichrzowych zarówno u aktywnych osobników, jak i u osobników w anhydrobiozie. Zbadano też poziom syntezy białek szoku cieplnego (Hsp27, Hsp60 oraz Hsp70) u aktywnych osobników *Pam. experimentalis* wystawionych na podwyższoną temperaturę (35 °C, 37 °C, 40 °C i 42 °C) przez pięć godzin, w porównaniu do optymalnej temperatury hodowlanej (20 °C). Pojedyncze komórki spichrzowe z grupy kontrolnej, znajdowały się w jamie ciała, pośród narządów wewnętrznych, przyjmując kuliste i ameboidalne kształty. Niewielkie, choć zauważalne zmiany w mitochondriach zostały zaobserwowane u osobników aktywnych wystawionych na temperaturę 35 °C. Znaczące zmiany w ultrastrukturze komórek spichrzowych zaobserwowane zostały u osobników poddanych temperaturze 37 °C. Zaobserwowano u nich liczne, uszkodzone mitochondria z zauważalnym zanikiem grzebieni oraz pojawieniem się struktur autofagicznych. Jeszcze więcej zmian zaobserwowano w temperaturze 40 °C, objawiających się nieregularnym kształtem komórek spichrzowych, uszkodzeniami organelli komórkowych oraz zwiększoną obecnością ciał autofagicznych i autolizosomów w mitochondriach. Jednakże najbardziej drastyczne zmiany zaobserwowano w 42 °C, gdzie nastąpiła pełna degradacja komórek i organelli, wskazująca na wystąpienie martwicy, do poziomu utrudniającego rozpoznanie komórek. Jednocześnie, osobniki w anhydrobiozie, poddane działaniu podwyższonej temperatury, nie wykazywały jakichkolwiek negatywnych zmian w komórkach spichrzowych na poziomie ultrastrukturalnym. Spośród pięciu zastosowanych w badaniu temperatur, trzy uznane za najważniejsze zostały wybrane (20 °C – optymalna temperatura do hodowli, 35 °C – najwyższa temperatura, po której nastąpił powrót osobników do aktywności oraz 42 °C – gdzie zaobserwowana została pełna martwica) i wykorzystane do określenia poziomów białek szoku cieplnego (Hsp27, Hsp60 oraz Hsp70) w aktywnych osobnikach. Wszystkie próbki wskazywały na wyraźny wzrost poziomu ekspresji białek wraz ze wzrostem temperatury.

W ostatniej pracy składającej się na rozprawę doktorską zebrano całkowitą dostępną wiedzę na temat rodzaju *Paramacrobiotus*. Podsumowując więc, do rodzaju *Paramacrobiotus* należy obecnie 45 gatunków (wliczając w to *Pam. gadabouti* dodany w ramach niniejszej pracy), które można znaleźć na całym świecie, popierając tezę, że rodzaj ten jest kosmopolityczny. W rodzaju występują zarówno partenogenetyczne, jak i obupłciowe gatunki, wykazujące zarówno krótką jak i długą długość życia. Gatunki w tym rodzaju mogą być wszystkożerne (jednak z przewagą drapieżników), a odżywiają się między innymi, cyjanobakteriami, algami, grzybami, wrotkami, nicieniami oraz niesporczakami. Gatunki z tego rodzaju charakteryzują się dość dobrą zdolnością do kryptobiozy, dzięki czemu często stanowią obiekt badań w pracach nad anhydrobiozą.

ABSTRACT

Paramacrobiotus is one of the genera of the phylum Tardigrada (commonly referred as water bears). This genus was erected more than a decade ago. Previously all representatives of this genus were included in the *richtersi-areolatus* complex within the genus *Macrobiotus,* but with the help of molecular and phylogenetic analysis, this new genus was identified and established.

As the title of thesis suggests, the goal of this dissertation was the overall study of the genus *Paramacrobiotus*, including: incorporating integrative taxonomy in new species descriptions, working out the distribution patterns of parthenogenetic *Pramacrobiotus* species, testing if the 'everything is everywhere' hypothesis is true for them, testing the influence of different types of stressors on the species *Pam. experimentalis*, assessing biogeography, distribution, microbiome, reproduction, feeding behaviour, life history, *Wolbachia* endosymbiont identification and cryptobiotic abilities of the species from the genus *Pramacrobiotus* and providing a new diagnostic key for the genus using morphological and morphometric characters of adults and eggs.

Using an integrative taxonomy approach (classical morphology and morphometry, as well as, genotypic using DNA barcodes and phylogenetic tree), a new species: *Pam. gadabouti* was described from a moss sample collected in Ribeiro Frio, Madeira. Furthermore, the mode of reproduction of this species was studied experimentally, which corroborates the interrelatedness between wide distribution and parthenogenesis.

In the subsequent paper constituting the doctoral dissertation, two parthenogenetic species of the *Paramacrobiotus*, i.e., *Pam. gadabouti* and *Pam. fairbanksi* were analysed to study the distribution, as well as genetic variability which showed that the 'everything is everywhere' hypothesis is true for, at least, some tardigrade species. Environmental niche modelling performed using MaxEnt supports the wide distribution of these two parthenogenetic species.

In the next publication, survivability of the *Pam. experimentalis* was tested at various concentrations (0.25%, 0.50% and 1.00%) of magnesium perchlorates (in range with the concentration present in Martian regolith) for two different time points (24h and 72h). In experiments where specimens were exposed to 24h time period, 33.3%, 16.7% and 0% were active in 0.25%, 0.50% and 1.00% solutions, respectively. However, more than 75% returned to activity after transferring them to the culture medium (93.3%, 76.7% and 86.7% of

specimens exposed to 0.25%, 0.50% and 1.00% solutions, respectively). In experiments where specimens were exposed to 72h time period, 30.0%, 26.7% and 0% were active in 0.25%, 0.50% and 1.00% solutions, respectively and after transferring them to the culture medium, 83.3%, 86.7% and 10.0% of specimens exposed to 0.25%, 0.50% and 1.00% solutions, respectively, returned to activity.

In the following paper constituting the doctoral dissertation, changes in ultrastructure for both active animals and desiccated tuns, as well as levels of heat shock proteins (Hsp27, Hsp60 and Hsp70) in active animals of *Pam. experimentalis* were studied when exposed at higher temperatures (35 °C, 37 °C, 40 °C, and 42 °C) for 5 hours, compared to optimal temperature (20 °C). Isolated storage cells from the control group persisted in the body cavity among internal organs in an amoeboid or spherical shape. Small, but visible changes were observed in specimens exposed to 35°C in the form of alterations in the mitochondria. Significant ultrastructural changes were observed in storage cells of specimens exposed to 37 °C. There were multiple deteriorating mitochondria with the loss of its cristae and there was a presence of autophagic structures. The level of changes increased at 40°C, with the irregular and shrunken shape of storage cells, deteriorated cell organelles and mitochondria with a higher number of autophagosomes and autolysosomes. Most drastic changes were observed at 42 °C, with full degeneration of cells and organelles showing signs of necrosis, making even cell identification difficult. However, when exposed to higher temperatures, tuns exhibited absolutely no differences from the control group. Out of five temperatures tested, the three deemed most important were selected (20 °C – optimum temperature, 35 °C – the highest temperature from which the return to activity was observed and 42 °C – where full necrosis was observed) and used for quantification of heat shock proteins (Hsp27, Hsp60 and Hsp70) in active specimens. All of them showed significant upregulation with the increase of the temperature.

In the last paper, all available information on the genus *Paramacrobiotus* were summarized. Thus, in summary, the genus *Paramacrobiotus* currently includes 45 species (including *Pam. gadabouti* added as a part of the present thesis). The species of this genus can be found everywhere throughout the globe, supporting the statement that the genus is cosmopolitan. Both dioecious and unisexual species are present in the genus, with both long and short lifespan. The species in this genus are generally carnivorous with their food preference, including certain rotifers, nematodes and juvenile tardigrades. However, it was

reported that they could also feed on cyanobacteria, algae, and fungi. The species generally have a good affinity for cryptobiosis, which is why multiple studies involving anhydrobiosis have been performed to date using specimens of various species of the *Paramacrobiotus*.

LIST OF SCIENTIFIC WORKS INCLUDED IN THE DISSERTATION

The results of the experimental works are described in the following papers:

1. **Kayastha, P.**, Stec, D., Sługocki, Ł., Gawlak, M., Mioduchowska, M., & Kaczmarek, Ł. (2023). Integrative taxonomy reveals new, widely distributed tardigrade species of the genus *Paramacrobiotus* (Eutardigrada: Macrobiotidae). Scientific Reports, 13(1), 2196. https://doi.org/10.1038/s41598-023-28714-w, IF (2021): 4.997; MEiN points: 140.
2. **Kayastha, P.**, Szydło, W., Mioduchowska, M. & Kaczmarek, Ł. (after review). Morphological and genetic variability in cosmopolitan tardigrade species - *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf, 2010. Scientific Reports. https://doi.org/10.21203/rs.3.rs-2736709/v1, IF (2021): 4.997; MEiN points: 140.
3. **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, (online first). https://doi.org/10.1093/zoolinnean/zlad060. IF (2021): 3.838; MEiN points: 140.
4. **Kayastha, P.**, Wieczorkiewicz, F., Pujol, M., Robinson, A., Michalak, M., Kaczmarek, Ł. & Poprawa, I. (in review). Elevated external temperature affects cell ultrastructure and heat shock proteins (HSPs) in *Paramacrobiotus experimentalis* Kaczmarek, Mioduchowska, Poprawa, & Roszkowska, 2020, Scientific Reports. IF (2021): 4.997; MEiN points: 140.

In addition, the following review paper containing an overview of the genus *Paramacrobiotus* was published.

5. **Kayastha, P.**, Mioduchowska, M. and Kaczmarek, Ł. (after review) A review on genus *Paramacrobiotus* https://doi.org/10.20944/preprints202307.1250.v1, Diversity. IF (2021): 3.031; MEiN points: 70

OTHER PUBLICATIONS DURING MY PHD WHICH ARE NOT INCLUDED IN THESIS

1. Polishchuk, A., **Kayastha, P.**, Kovalenko, P., Parnikoza, I. & Kaczmarek, Ł. (2023). New records of tardigrades from the Danco and Graham Coasts, the maritime Antarctic. Annales Zoologici, 73: 17–28. https://doi.org/10.3161/00034541ANZ2023.73.1.002, IF (2022): 1.1; MEiN points: 100
2. Kaczmarek, Ł., Rutkowski, T., Zacharyasiewicz, M., Surmacki, A., Osiejuk, T. & **Kayastha, P.** (2023). New species of Macrobiotidae (Eutardigrada) from Cameroon (Africa), characteristics of *Macrobiotus* morpho-groups and a key to the *nelsonae* group. Annales Zoologici, 73: 1–15, https://doi.org/10.3161/00034541ANZ2023.73.1.001, IF (2022): 1.1; MEiN points: 100
3. **Kayastha, P.**, Mioduchowska, M., Gawlak, M., Sługocki, Ł., Araújo, R., Silva, J.J.G. & Kaczmarek, Ł. (2023). Integrative description of *Macrobiotus kosmali* sp. nov. (*hufelandi* group) from the Island of Madeira (Portugal). The European Zoological Journal, 90(1): 126–38. https://doi.org/10.1080/24750263.2022.2163312, IF (2022): 2.1; MEiN points: 140
4. Kaczmarek, Ł., **Kayastha, P.**, Roszkowska, M., Gawlak, M. & Mioduchowska, M. (2022). Integrative redescription of the *Minibiotus intermedius* (Plate, 1888)—The type species of the genus *Minibiotus* R.O. Schuster, 1980. Diversity, 14(5): 356. https://doi.org/10.3390/d14050356, IF (2022): 2.4; MEiN points: 70
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6. Bartylak, T., **Kayastha, P.**, Roszkowska, M., Kepel, A., Kepel, M. & Kaczmarek, Ł., (2022). Tardigrades of the Ivohibory forest (south-central Madagascar) with a description of a new *Bryodelphax* species. The European Zoological Journal, 89(1): 423–436. https://doi.org/10.1080/24750263.2022.2042404, IF(2022): 2.1; MEiN points: 140

7. Kaczmarek, Ł., **Kayastha, P.**, Gawlak, M., Mioduchowska, M. & Roszkowska, M. (2021). An integrative description of a *Diploechiniscus oihonnae* (Richters, 1903) population from near the original type locality in Merok. Zootaxa, 4964(1): 83–102. https://doi.org/10.11646/zootaxa.4964.1.4, IF (2022): 1.026; MEiN points: 70
8. **Kayastha, P.**, Wiśniewska, J., Kuzdrowska, K. & Kaczmarek, Ł. (2021). Aquatic tardigrades in Poland – a review. Limnological Review, 21(3): 147–154. https://doi.org/10.2478/limre-2021-0013, IF (2022): 0.69; MEiN points: 70
9. **Kayastha, P.**, Roszkowska, M., Mioduchowska, M., Gawlak, M. & Kaczmarek, Ł. (2021) Integrative descriptions of two new tardigrade species along with the new record of *Mesobiotus skorackii* Kaczmarek et al., 2018 from Canada. Diversit, 13(8): 394. https://doi.org/10.3390/d13080394, IF (2022): 2.4; MEiN points: 70
10. **Kayastha, P.**, Berdi, D., Mioduchowska, M., Gawlak, M., Łukasiewicz, A., Gołdyn, B., Jędrzejewski, S. & Kaczmarek, Ł. (2020). Description and molecular characterization of *Richtersius ziemowiti* sp. nov. (Richtersiidae) from Nepal (Asia) with evidence of heterozygous point mutation events in the 28S rRNA. Annales Zoologici, 70(3): 381–396. https://doi.org/10.3161/00034541ANZ2020.70.3.010, IF (2022): 1.1; MEiN points: 100
11. **Kayastha, P.**, Bartylak, T., Gawlak, M. & Kaczmarek, Ł. (2020). Integrative description of *Pseudechiniscus lalitae* sp. nov. (Tardigrada: Heterotardigrada: Echiniscidae) from the Azores Archipelago (Portugal). Annales Zoologici, 70(4): 487–505. https://doi.org/10.3161/00034541ANZ2020.70.4.002, IF (2022): 1.1; MEiN points: 100
12. **Kayastha, P.**, Berdi, D., Mioduchowska, M., Gawlak, M., Łukasiewicz, A., Gołdyn, B. & Kaczmarek, Ł. (2020). Some tardigrades from Nepal (Asia) with integrative description of *Macrobiotus wandae* sp. nov. (Macrobiotidae: hufelandi group). Annales Zoologici, 70(1): 121–142. https://doi.org/10.3161/00034541ANZ2020.70.1.007, IF (2022): 1.1; MEiN points: 100

FUNDING

This work was supported by the following sources:

- 'Support for publications in prestigious research journals'-Uczelnia Badawcza' No 085/08/POB1/0001
- 'Initiative of Excellence - Research University'- Uczelnia Badawcza' No 071/06/POB1/0022.
- 'Excellence Initiative - Research University'- Uczelnia Badawcza' No 070/13/UAM/0032
- Second edition of the "MINIGRANTY" competition as part of the Passport to the Future Project - Interdisciplinary Studies doctoral studies at the Faculty of Biology, AMU POWR.03.02.00-00-I006 / 17. No: POWER8/2021/2ed
- UNIWERSYTET JUTRA - zintegrowany program rozwoju Uniwersytetu im. Adama Mickiewicza w Poznaniu", No POWR.03.05.00-00-Z303/17
- Dean's Grant for PhD students at Faculty of Biology, Uniwersytetu im. Adama Mickiewicza w Poznaniu: No 506000/604/4102000000/BW002020 (GDWB-08/2020)

SCIENTIFIC COLLABORATION

All the experiments which are part of my PhD thesis were performed at the Department of Animal Taxonomy and Ecology, Faculty of Biology, Adam Mickiewicz University in Poznan, Poland, but also with collaboration with:

- Dr. hab. Izabela Poprawa Team (University of Silesia in Katowice, Poland)
- Prof. Marek Michalak (Michalak Lab, Department of Biochemistry, University of Alberta, Canada)

SUMMARY

Background

Tardigrades are invertebrates from the phylum Tardigrada, also known as water bears or moss piglets. There are ca. 1,500 species and subspecies in this phylum till date (Degma & Guidetti, 2009-2023). Within the phylum are currently 33 families, 159 genera, 1464 species, and 21 more subspecies (Nelson et al., 2020) (Figure 1). Tardigrades are widespread throughout the world's biomes and can be found in freshwater, marine, and terrestrial environments, despite their understudied status (Nelson et al., 2018) (Figure 2). Marine tardigrades have been reported from the intertidal and subtidal zones to the deepest depths of the sea (Kristensen & Sterrer, 1985). Freshwater tardigrades inhabit various running and standing water sources and underground habitats. Terrestrial species live in a wide variety of habitats, such as mosses, lichens, and liverworts on rocks, soil, tree trunks, leaf litter, and soil, but to be active they need to be surrounded by a film of water (Ramazzotti & Maucci, 1983). Limno-terrestrial species inhabit both freshwater and terrestrial environments. Tardigrades have adapted to their environment in many ways. The most common ones that they undergo are cryptobiosis which are anhydrobiosis (lack of water), anoxybiosis (lack of oxygen), chemobiosis (harsh chemical condition), cryobiosis (very low temperature) and osmobiosis (higher salt concentration) (Keilin, 1959). Furthermore, our understanding of tardigrade biodiversity and biogeography is fast changing due to constant discoveries of new species and the development of integrative taxonomy employing combined morphological and genetic data, which is currently deemed vital for future tardigrade studies (Nelson et al., 2010, 2015).

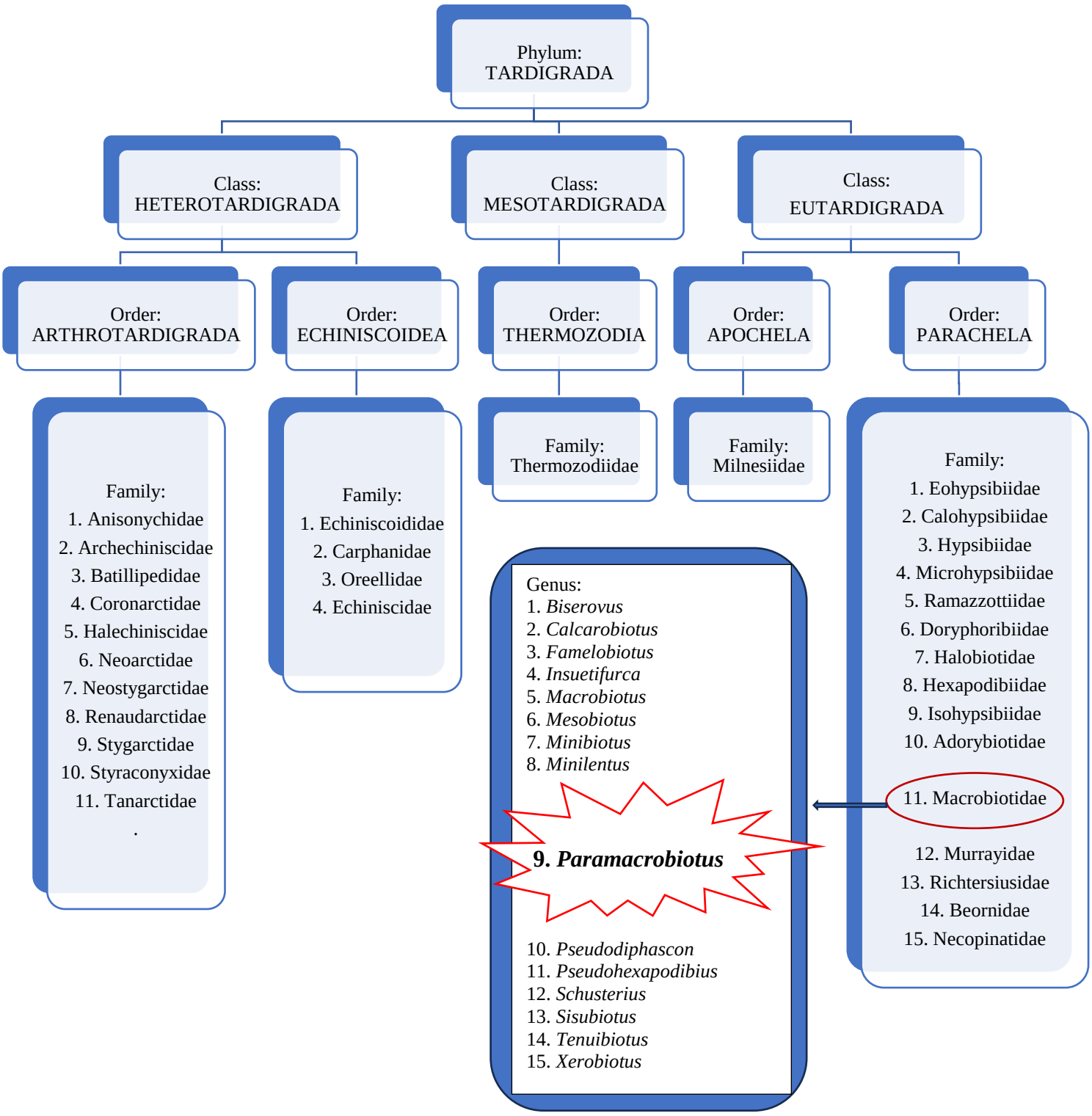

Figure 1. Taxonomic position of genus *Paramacrobiotus* within phylum Tardigrada based on Degma & Guidetti (2009) (but, not all genera and subgenera are listed).

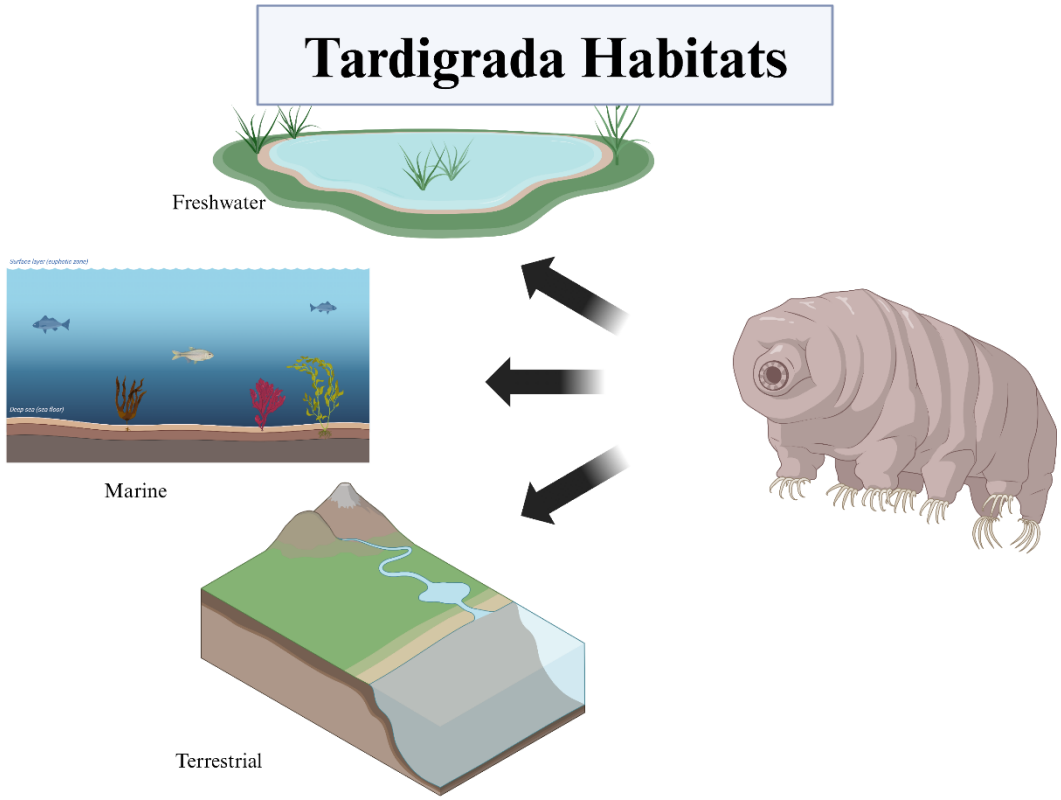

Figure 2. Habitats where tardigrades can be found (Created using BioRender.com).

Integrative taxonomy

In the past, species were described using a traditional taxonomy approach which was solely based on morphology and morphometrics and therefore came with its limits (Dayrat, 2005). For this reason, taxonomists have embraced the integrative taxonomy approach (Figure 3), which is based on both morphological and morphometric data, as well as, phylogenetic analysis using DNA barcodes (Figure 4). In the case of tardigrades, four DNA markers, three nuclear (18S rRNA, 28S rRNA, ITS-2) and one mitochondrial (COI) are used to characterize the species genetically. The mitochondrial marker COI alone can be enough to identify and distinguish taxa at the species level (Cesari et al., 2009), but for higher taxonomic units, more conserved 18S rRNA and 28S rRNA markers concatenated with both COI and ITS or either one of them are more accurate. For morphology and morphometrics, Phase Contrast or Nomarski Contrast Microscopy and Scanning Electron Microscopy are used to study the morphology of both eggs and adults, as well as, to measure various characteristics of both eggs and adults.

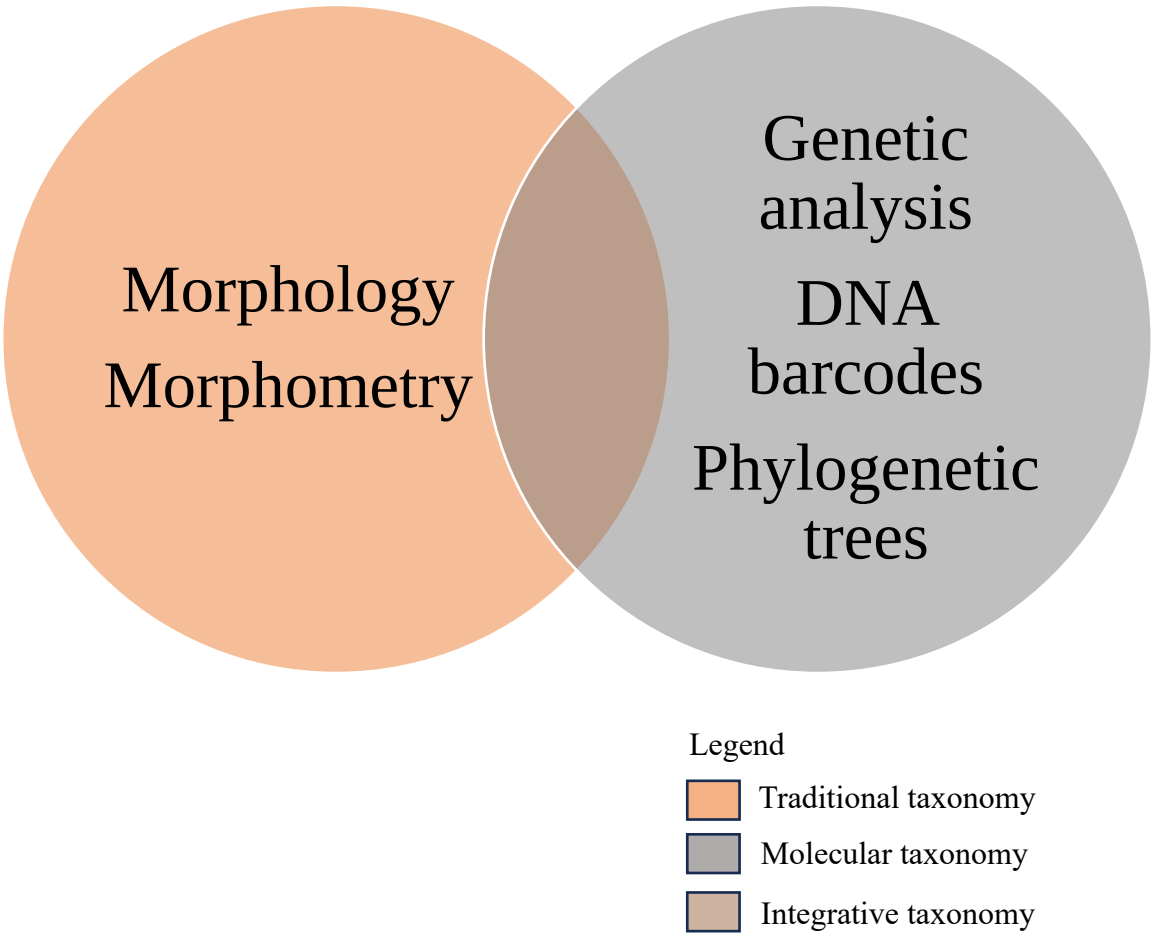

Figure 3. Schematic representation of the integrative taxonomy approach.

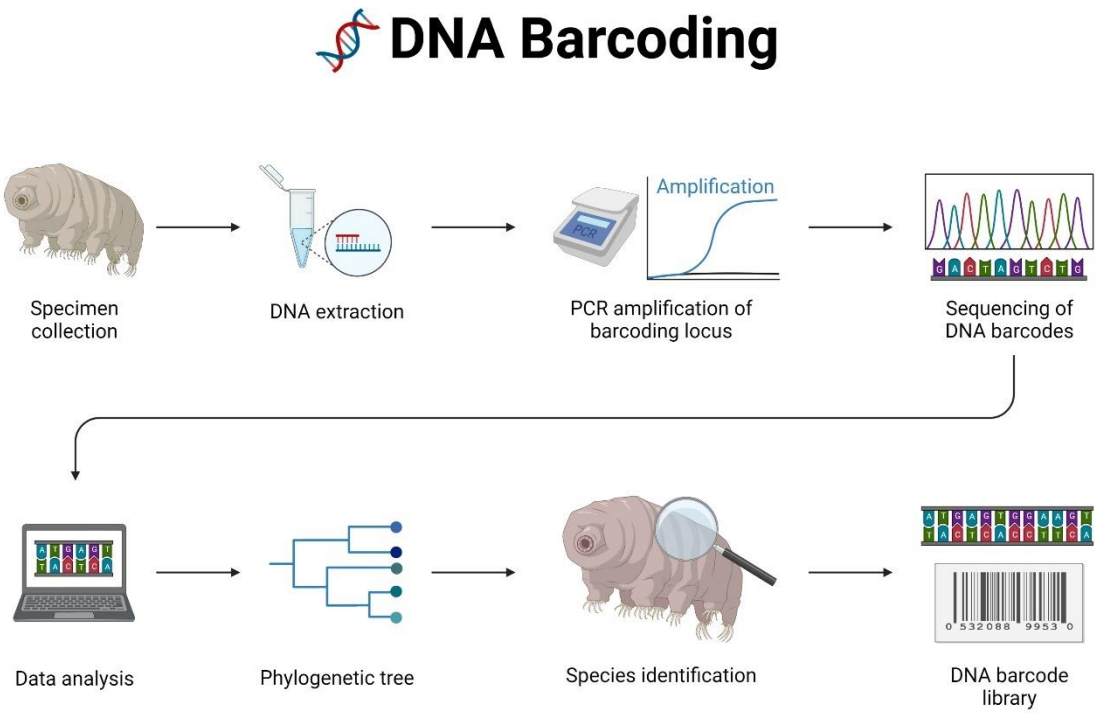

Figure 4. Schematic representation of the DNA barcoding methodology for tardigrades (Created using BioRender.com).

"Everything is Everywhere" (EiE) hypothesis

Finlay (2002) proposed in the 'everything is everywhere' concept that species below 1µm have the potential to be distributed everywhere and that the environment will determine if they can survive. Although some morpho-species (species determined by morphological criteria only) may be found throughout the world, their gene pools may or may not be disjunct (Baas-Becking, 1934; Beijerinck, 1913; Finlay, 2002; Finlay & Clarke, 1999). Many researchers proposed or hypothesized tardigrade cosmopolitism (e.g. Ramazzotti & Maucci, 1983; Nielsen, 2012). However, few previous investigations, based exclusively on classical taxonomy and indirectly testing the 'EiE' hypothesis (Guil, 2011; Guil et al., 2009; Pilato and Binda 2001), did not support the cosmopolitan distribution of tardigrades. Furthermore, recent genetic results suggested that certain tardigrade species' ranges may be significantly smaller than previously thought. These findings show that previous conclusions on the distribution of tardigrades, which were all based entirely on classical taxonomy, were most likely incorrect. However, as of today, there are four tardigrade species known from more than one

zoogeographic realm namely *Echiniscus testudo* (Doyère, 1840), *Milnesium inceptum* (Morek , Suzuki, Schill, Georgiev, Yankova, Marley & Michalczyk, 2019), *Pam. fairbanksi* (Schill, Förster, Dandekar & Wolf, 2010) and *Pam. gadabouti* (Kayastha , Stec, Mioduchowska and Kaczmarek, 2023). Genetic findings confirmed the distribution of these species in more than one zoogeographic realm. So, cosmopolitan distribution might not generally be true for all tardigrades, but it is true at least for some tardigrade species.

Environmental niche modelling (ENM)

The notion of ecological niches was developed by Joseph Grinnell (Grinnell, 1917), and he was the first who investigated links between ecological niches and species distribution. His concept, which was later adapted into contemporary jargon, was that a species' ecological niche is the set of conditions in which the species may sustain generations despite requiring immigration from other localities (Grinnell, 1917). The ENMs are similar to species distribution models and are experimental or analytical estimates of a species' ecological niche (Sillero et al., 2021). They are statistical approaches or theoretically generated response surfaces that describe and predict species distributions by relating physiological or distribution data to environmental variables (Franklin, 2014; Guisan et al., 2017; Peterson et al., 2011; Sillero, 2011; Sillero et al., 2021). The ENMs are generated using a variety of methods, using georeferenced occurrence databases (i.e. sample localities that include latitude and longitude data) and data pertaining to the environment in the form of GIS (Geographic Information System) datasets (Elith et al., 2006). Some ENM-specific modelling algorithms, such as Maximum Entropy (Phillips et al., 2006, 2017) or Ecological Niche Factor Analysis (ENFA) (Hirzel et al., 2002), are only available in dedicated statistical software. They are increasingly used in studying bioinvasions, conservation biology, bioresponse to climate change, disease transmission in space, and various aspects of ecology and evolutionary studies (GengPing et al., 2013). There has been a significant increase in the use of ENM in recent years. The ENMs are used to explain populations' or species' ecological tolerances. Furthermore, the simultaneous advancements in phylogeography resulting from the increased spatial data accessibility and methodological breakthroughs in species distribution modelling, led to widening the area of problems explored and delivering of unique insights (Alvarado-Serrano & Knowles, 2014). The ENMs also help predict the invasion and cosmopolitanism or wide distribution of various species (Ba et al., 2010; Iniesta et al., 2020). Only two studies using

ENM have been performed using tardigrades to date. Gąsiorek et al. (2019) used ENM for the first time to model statistical predictions of tardigrades' geographical distribution. The other study that used ENM to provide proof of cosmopolitanism for two widely distributed *Paramacrobiotus* species was published by Kayastha et al., 2023).

Stressors

Stress is a complex force that is essential to life's evolution. Organisms change in response to stress, and stressful environments can constitute selection limits (Kültz, 2020). Species are frequently subjected to an array of stresses, both natural and generated by human (Sih et al., 2004). Increase in pollution as a consequence of anthropogenic activities are causing biodiversity loss, affected ecosystems, and habitat degradation (Ojekunle & Sodipe, 2020). Various pollutants including toxic heavy metals and chemicals are released into the environment resulting in environmental problems. Nonetheless, temperature is another stressor used extensively to understand the effects of global climate change on various biological systems (e.g. Doney et al., 2012; Morón Lugo et al., 2020). Invertebrates are at risk when exposed to both toxic chemicals and higher temperatures; see, e.g., the effect of temperature on metabolic energy balance in marine invertebrates (Newell & Branch, 1980), temperature rises affect invertebrate population and slowing down decomposition (Figueroa et al., 2021), a large-scale ecotoxicology/health stressor trial for mussel embryos (Young et al., 2023), toxicology of sodium chloride-based road salt formulations in juvenile aquatic invertebrates (Harwood et al., 2023) and so on. Tardigrades are known for their ability to undergo cryptobiosis and were subjected to different kinds of stressors, and have also been used as a model system to study the effect of temperature, both high-temperature tolerance and freeze tolerance (e.g. Hengherr, Worland, Reuner, Brümmer, et al., 2009; Hengherr, Worland, Reuner, Brummer, et al., 2009; Neves et al., 2020, 2022; Rebecchi et al., 2009). Furthermore, the effects of toxic chemicals have also been studied on the tardigrades (Czerneková et al., 2017; Hygum et al., 2017; Ojekunle & Sodipe, 2020; Wieczorkiewicz et al., 2023). One of the studies performed was to check the tolerance of magnesium perchlorate on various invertebrates including tardigrades (Kayastha et al., 2023) since presence of different concentrations of perchlorates (ClO_4^-), reaching a mean of 0.6 wt% in the Martian regolith (a blanket of unconsolidated, loose, heterogeneous surface deposits overlaying solid rock), are regarded as a severe obstacle for terrestrial life forms (Glavin et al., 2013; Hecht et al., 2009;

Kounaves et al., 2010, 2014; Leshin et al., 2013; Martin et al., 2020; Ming et al., 2014; Sutter et al., 2017). On Earth, perchlorate is produced both naturally and artificially (Brown & Gu, 2006; Isobe et al., 2013; Vega et al., 2018) and is a pollutant that can remain in groundwater and soil and is regularly identified at human-health-relevant quantities in various ecosystems (Acevedo-Barrios et al., 2022). However, perchlorate concentrations on Mars much exceed those observed on Earth (Calderón et al., 2014; Ericksen, 1983) which is why the study was performed using range of magnesium perchlorate solutions higher than that found in Martian regolith.

The **main aims of my PhD thesis** are:

1. To describe new *Paramacrobiotus* species using an integrative taxonomy approach.

2. To study distribution patterns of parthenogenetic *Paramacrobiotus* species and test the 'everything is everywhere' hypothesis.

3. To study the influence of different stress factors on the ultrastructure, survivability and heat shock proteins expression in the species of the genus *Paramacrobiotus*.

4. To verify the available data on barcodes and construct a phylogeny, biogeography, distribution, microbiome, reproduction, feeding behaviour, life history and cryptobiotic ability of the species from the genus *Paramacrobiotus*.

5. To prepare a new diagnostic key for *Paramacrobiotus* based on the morphological and morphometric characters of adults and eggs.

The first aim of my thesis is accomplished in studies presented in publication 1. Further, the second aim is fulfilled in the investigation performed and reported in publication 2. Similarly, the third aim of the thesis is achieved in the results produced in publications 3 and 4. Lastly, the fourth and fifth aim of the thesis is fulfilled in publication 5.

In the first paper (Kayastha et al., 2023), which is part of my PhD thesis, I am presenting the description of a species of tardigrade from the genus *Paramacrobiotus* new to science, identified using integrative taxonomy – *Pam. gadabouti* Kayastha, Stec, Mioduchowska and Kaczmarek 2023. The new species belongs to the *Pam. richtersi* group due to the presence of microplacoid in the pharynx and *richtersi*-type of eggs. *Paramacrobiotus*. *gadabouti* shares the most similarity with *Pam. alekseevi* (Tumanov, 2005) (Tumanov 2005), *Pam. filipi*

Dudziak, Stec and Michalczyk 2020 (Stec et al., 2020) and *Pam. garynahi* (Kaczmarek, Michalczyk and Diduszko 2005) (Kaczmarek et al., 2005), but differs from them mostly in egg morphology and adult morphometrics (Table 1). For genotyping, four barcodes were sequenced i.e. three nuclear (18S rRNA, 28S rRNA, ITS-2) and one mitochondrial (COI). The description of the new species increased the number of known *Paramacrobiotus* species to forty-five. Additionally, this new species' reproduction mode was determined to be parthenogenetic (Figure 5). This fact supports that parthenogenetic species of *Paramacrobiotus* are widespread (Paper 1).

Table 1. Shortened differential diagnosis of *Pam. gadabouti* based on morphometrical characters.

Characters	***Pam. gadabouti***	***Pam. alekseevi***
Pores inside egg areoles	Present	Absent
pt value of second macroplacoid	Higher	Lower
Microplacoid length	Longer	Shorter
Characters	***Pam. gadabouti***	***Pam. filipi***
Dorsal cuticle granulation	Absent	Present
Second macroplacoid	Longer	Shorter
pt value of macroplacoid row	Higher	Lower
Placoid row	Longer	Shorter
Full egg diameter	Larger	Smaller
Characters	***Pam. gadabouti***	***Pam. garynahi***
Medioventral tooth in the third band of teeth in the oral cavity	Divided	Not divided
Eggs chorion ornamentation	*richtersi* type	*areolatus* type
pt value of macroplacoid and placoid rows	Higher	Lower
Eggs bare and full diameter	Smaller	Larger

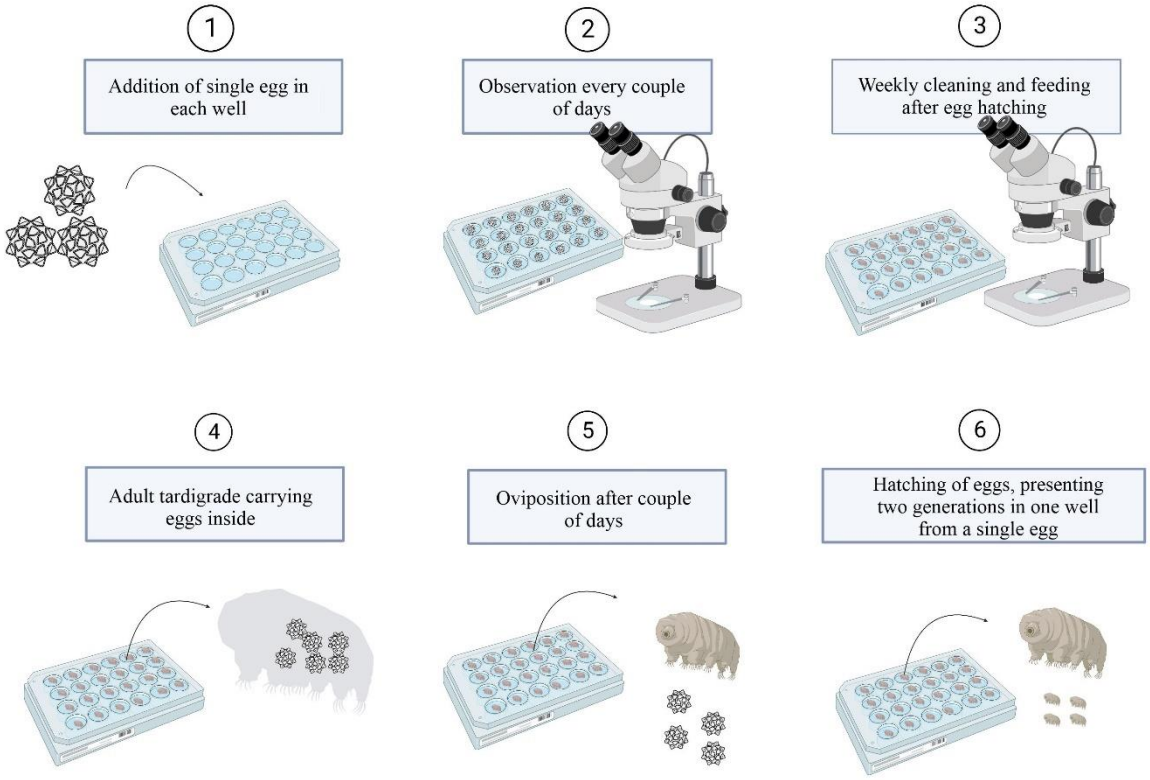

Figure 4. Workflow used to determine of the parthenogenetic reproduction mode in *Pam. gadabouti* (Created using BioRender.com).

In the second paper, part of my PhD thesis, I am presenting the distribution patterns of two parthenogenetic *Pramacrobiotus* species i.e. *Pam. fairbanksi* and *Pam. gadabouti*. I analysed nine populations of the *Pam. fairbanksi* from various localities. Five known from literature and new finding from Albania, Canada, Madeira and Mongolia (Kayastha et al., 2023). The distribution pattern obtained using ecological niche modelling strengthens the claim for the cosmopolitanism of the species. Ecological niche modelling using maximum entropy approach was performed using MaxEnt. MERRAclim dataset (Vega et al., 2017) was used for bioclimatic data as it contains dataset for Antarctica, including one of the localities where *Pam. fairbanksi* was found. Similarly, five populations of the *Pam. gadabouti* has been analysed and the distribution pattern obtained using the ecological niche model shows its wide distribution globally, favouring the areas with Mediterranean climates. The wide distribution of these two parthenogenetic *Paramacrobiotus* species confirms that the hypothesis 'everything is everywhere' is correct, at least for some tardigrades. Furthermore, the morphological and genetic variability of *Pam. fairbanksi* was studied. Analysis of variance (ANOVA) test with

post hoc comparison of pairs of measurements, applying Bonferroni correction was used to statistically analyse single characters and R script provided in Stec et al. (2021) was used to execute Principal Component Analysis (PCA). Statistically significant morphometric differences were observed in the specimens from the populations from different localities. Furthermore, the analysed species showed higher haplotype diversity in COI compared to other barcodes, but the overall variability remains very low (Paper 2).

In the third paper which is part of my PhD thesis, I tested the influence of magnesium perchlorate on the survivability in the *Pam. experimentalis* Kaczmarek, Mioduchowska, Poprawa and Roszkowska, 2020 (Kaczmarek et al., 2020). A three different solutions of magnesium perchlorate were used, i.e. 0.25%, 0.50% and 1.00% (mean of 0.6 wt % found in Martian regolith) for two different time periods, i.e. 24 and 72h (Figure 5). The study showed that 33.3% of the tardigrades were active after 24h in 0.25% solution, 16.7% after 24h in 0.50% solution and 0% after 24h in 1.00% solution. However, 93.3%, 76.7% and 86.7% of specimens exposed to 0.25%, 0.50% and 1.00% solutions returned to activity when placed back in culture medium for 24h. Furthermore, 30.0% of the specimens were active after 72h in 0.25% solution, 26.7% after 72h in 0.50% solution and 0.00% after 72h in 1.00% solution. Later it was found that 83.3%, 86.7% and 10.0% of specimens exposed to 0.25%, 0.50% and 1.00% solutions returned to activity when placed back in culture medium for 24h. Additionally, median deactivation time (i.e. >50% of the specimens showed no activity) was calculated. The median deactivation time was the same for specimens exposed to 0.25% and 0.50% magnesium perchlorate solutions (13.5-24h) and significantly lower for specimens exposed to 1.00% magnesium perchlorate solutions (1.5-2.5h) (Paper 3).

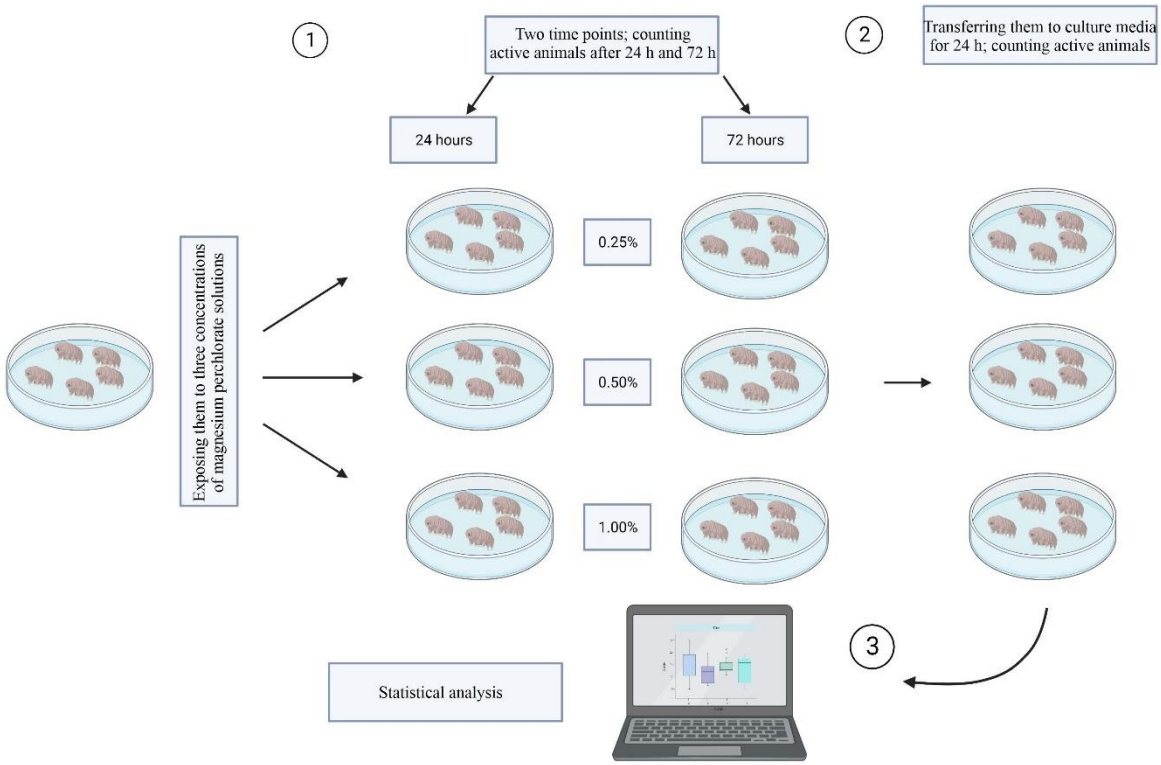

Figure 5. Protocol for testing *Paramacrobiotus experimentalis* tolerance against exposure to magnesium perchlorate solution (Created using BioRender.com).

In the fourth paper, part of my PhD thesis, I tested how increased temperature affects storage cells ultrastructure and heat shock proteins levels in active specimens and in anhydrobiotic tuns of *Pam. experimentalis*.

All active specimens' experiments were carried out in 1.5 ml Eppendorf tubes with 10 specimens placed in the culture medium. For 5 hours, the Eppendorf tubes were placed on a heat block with an open cover set to 20 °C, 35 °C, 37 °C, 40 °C, and 42 °C. Specimens were then transferred to Petri dishes and subjected to ultrastructural and biochemical investigations (Figure 6). For anhydrobiotic tuns, specimens were first subjected to anhydrobiosis and then exposed to 20 °C, 35 °C, 37 °C, 40 °C, and 42 °C for 5 hours and then subjected to ultrastructural investigations (Figure 7).

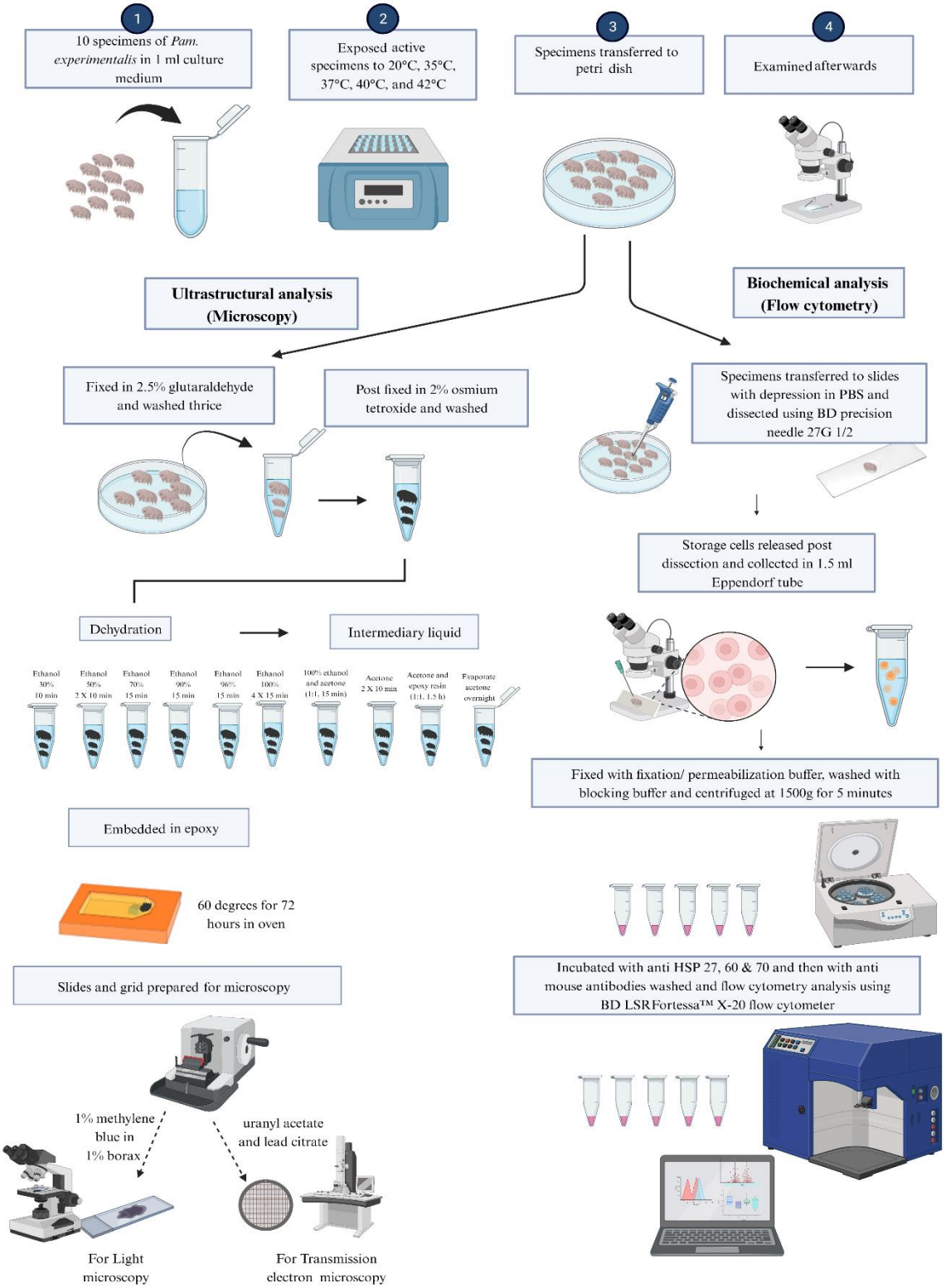

Figure 6. Protocol for testing effects of heat stress on active specimens of the *Paramacrobiotus experimentalis* (Created using BioRender.com).

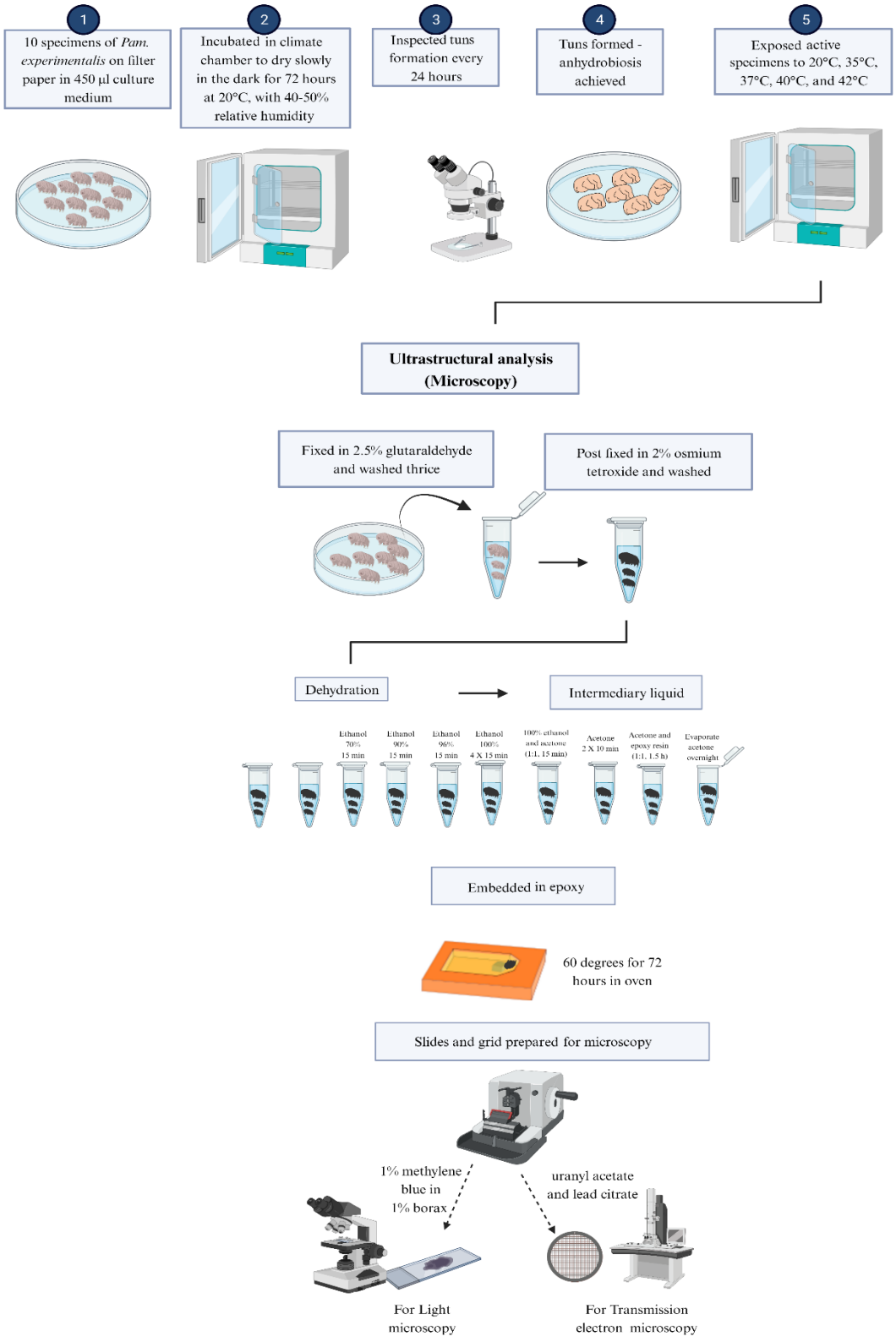

Figure 7. Protocol for anhydrobiosis and testing effects of heat stress on anhydrobiotic tuns of the *Paramacrobiotus experimentalis* (Created using BioRender.com)

When active animals were exposed to 35 °C, 37 °C, 40 °C, and 42 °C, various changes were observed (see below for details). Active specimens incubated at 35 °C already showed the first modifications in the ultrastructure of storage cells. Mitochondria showed signs of degradation and lost their cristae. Most of the mitochondria in the storage cells of active animals that were exposed to 37 °C deteriorated and lost their crests. Numerous autophagic structures also emerged. Individuals incubated at 40 °C showed similar, but significantly more pronounced changes. Additionally, at 42 °C, the cytoplasm of the storage cells became electron-lucent, the cell membrane ruptured, and necrotic symptoms were visible in degenerated cells and organs. In anhydrobiotic tuns, there were no differences in the ultrastructure of storage cells between the control group (20 °C) and the cells treated to 35 °C, 37 °C, 40 °C. However, in the tuns storage cells incubated at 42 °C, the karyolymph grew denser and the mitochondrial electron-dense substance accumulated. Furthermore, the levels of three distinct heat shock proteins (Hsp27 (sHsp), Hsp60, and Hsp70) were measured in active specimens of *Pam. experimentalis* exposed to 20 °C, 37 °C, and 42 °C. It was found that heat stress leads to upregulation of expression of all studied HSPs (Paper 4).

In the fifth paper, part of my PhD thesis, all the information regarding the genus *Pramacrobiotus* till date was reviewed using available literature. It was deemed necessary to compile all the data, such as the geographical distribution of all species, feeding behaviour, life history, microbiome community, *Wolbachia* endosymbiont identification, reproduction, phylogeny, morphological and molecular taxonomy and cryptobiotic ability, to give them a proper overview. The genus *Paramacrobiotus* consists of 45 species till date, among which 13 belong to the *areolatus* group and 32 to the *richtersi* group, and the species are both bisexual and unisexual. The genus is truly cosmopolitan, as species are present throughout the world. The species are sometimes omnivorous (but most often carnivorous), consuming cyanobacteria, algae, fungi, rotifers and tardigrades. In our analysis of COI barcode sequences, speciation events that resulted in polytomies within the phylogeny of the genus *Paramacrobiotus* were observed (Kayastha et al., 2023). Furthermore, only few species' lifespan is known till date including *Pam. fairbanski*, *Pam. kenianus* (Schill, Förster, Dandekar & Wolf, 2010), *Pam. metropolitanus* (Sugiura Matsumoto & Kunieda, 2022), *Pam. richtersi* (Murray, 1911) and *Pam. tonollii* (Ramazzotti, 1956). Also, only *Pam. metropolitanus* whole genome is available among species of the genus *Paramacrobiotus*. Similarly, the microbiomes of a few species have been studied to date. Proteobacteria and Bacteroidetes were found in the

microbial community of *Pam. areolatus* (Murray, 1907). Two unique patterns in the diversity detected between tardigrades and their substrates demonstrate that tardigrades had much less microbial variety than their substrates (Vecchi et al., 2018). Also, microbiome analysis on two populations of *Pam. experimentalis* from Madagascar and their laboratory culture environment were conducted where Proteobacteria, Firmicutes and Bacteroides were the most abundant phyla (Kaczmarek et al., 2020). Also, Rickettsiales endosymbionts were identified as possible endosymbionts. Moreover, *Wolbachia* endosymbiont identification was performed by Mioduchowska et al., 2021. Proteobacteria, Firmicutes, and Actinobacteria were most common, but the purpose was to study *Wolbachia* endosymbiont and both Rickettsiales and *Wolbachia* were detected in adult *Paramacrobiotus* sp. Moreover, few studies regarding cryptobiosis in the species of *Paramacrobiotus* have also been conducted. To better understand the energy aspect of anhydrobiosis, Reuner et al., 2010 studied how starvation and anhydrobiosis alter the size and number of storage cells in *Pam. tonollii*. Antioxidant defence (the ability to combat reactive oxygen species (ROS)) in *Pam. richtersi* in both active and dehydrated stages was studied by Rizzo et al. (2010). Giovannini et al., 2022 studied the formation of reactive oxygen species and the participation of bioprotectants during anhydrobiosis in *Pam. spatialis* Guidetti, Cesari, Bertolani, Altiero & Rebecchi, 2019 where they concluded that ROS production corresponds to the time spent in anhydrobiosis. Furthermore, Roszkowska et al. (2023) have investigated how long several tardigrades, including *Pam. experimentalis*, can survive in anhydrobiotic conditions. All such data were summarized in my review. Additionally, a new diagnostic key to the genus *Paramacrobiotus* is provided based on the morphological and morphometric characters of adults and eggs (Paper 5).

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Paper I

Kayastha, P., Stec, D., Sługocki, Ł. *et al.* Integrative taxonomy reveals new, widely distributed tardigrade species of the genus *Paramacrobiotus* (Eutardigrada: Macrobiotidae). *Sci Rep* **13**, 2196 (2023). https://doi.org/10.1038/s41598-023-28714-w

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Integrative taxonomy reveals new, widely distributed tardigrade species of the genus *Paramacrobiotus* (Eutardigrada: Macrobiotidae)

Pushpalata Kayastha[1]✉, Daniel Stec[2], Łukasz Sługocki[3], Magdalena Gawlak[4], Monika Mioduchowska[5] & Łukasz Kaczmarek[1]

In a moss sample collected in Ribeiro Frio, Madeira, *Paramacrobiotus gadabouti* sp. nov. was found and described using the integrative taxonomy approach. The new species is described based on morphological and morphometric data from both phase-contrast light microscopy (PCM), as well as scanning electron microscopy (SEM). Moreover, four DNA markers, three nuclear (18S rRNA, 28S rRNA, ITS-2) and one mitochondrial (COI) markers, were used to elucidate the phylogenetic position of the new species within the family Macrobiotidae. The new species has a microplacoid that placed it within *Parmacrobiotus richtersi* group and exhibit *richtersi*-type eggs having processes terminated with cap-like structures. *Paramacrobiotus gadabouti* sp. nov. is most similar to *Pam. alekseevi*, *Pam. filipi* and *Pam. garynahi*, but differs from them mainly in details of egg morphology and morphometrics. Unlike other species from this group, which were confirmed as bisexual and showed limited distribution, *Paramacrobiotus gadabouti* sp. nov. is yet another parthenogenetic species with a wide distribution, demonstrating that at least some tardigrades confirm to the hypothesis of 'everything is everywhere'.

The Phylum Tardigrada currently consists of over 1400 species[1–4] that inhabit terrestrial and aquatic (freshwater and marine) environments throughout the world[5–7]. Knowledge of terrestrial tardigrades of Madeira, Portugal is rather poor as to date, only 33 species (22 Eutardigrada and 11 Heterotardigrada taxa) have been reported from this region[8–10].

The genus *Paramacrobiotus* Guidetti et al.[11], currently comprises 43 formally named species[4]. It was formally erected in 2009 based on morphological and genetic analyses[11]. Morphologically two distinct species groups are present in the genus, one exhibiting a microplacoid within the pharynx, i.e. *richtersi* group and the second one without microplacoid, i.e. *areolatus* group. This phenotypic difference led Kaczmarek et al.[12] to propose these two groups to constitute separate subgenera for which specific names were clarified by Marley et al.[13]. However, their erection was subseqently questioned independently based on two phylogenetic analyses[14,15]. Within the genus *Paramacrobiotus* bisexual and unisexual species/populations have been observed and reported in the past (e.g. in populations of *Pam. richtersi* (Murray, 1911)[16] from Italy (bisexual and unisexual); according to modern taxonomy they probably constitute distinct species), *Pam. areolatus* (Murray, 1907)[17] from Italy (bisexual) and Svalbard (unisexual), *Pam. tonolli* (Ramazzotti, 1956)[18] (bisexual) from USA, *Pam. fairbanksi* Schill, Förster, Dandekar and Wolf, 2010[19] (unisexual) from Antarctic, Italy, Poland, Spain and USA, *Pam. kenianus* Schill, Förster, Dandekar and Wolf, 2010[19] (unisexual) from Kenya and *Pam. palaui* (unisexual) Schill, Förster, Dandekar and Wolf, 2010[19] from Micronesia, *Pam. depressus* Guidetti, Cesari, Bertolani, Altiero and Rebecchi, 2019[14] (bisexual)

[1]Department of Animal Taxonomy and Ecology, Faculty of Biology, Adam Mickiewicz University in Poznań, Poznań, Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland. [2]Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31-016 Kraków, Poland. [3]Department of Hydrobiology, Institute of Biology, University of Szczecin, Szczecin, Poland. [4]The Institute of Plant Protection-National Research Institute, Węgorka 20, 60-318 Poznań, Poland. [5]Department of Evolutionary Genetics and Biosystematics, Faculty of Biology, University of Gdańsk, Gdańsk, Wita Stwosza 59, 80-308 Gdańsk, Poland. ✉email: pushpalata.kayastha@gmail.com

from Italy, *Pam. celsus* Guidetti, Cesari, Bertolani, Altiero and Rebecchi, 2019[14] (bisexual) from Italy, *Pam. spatialis* Guidetti, Cesari, Bertolani, Altiero and Rebecchi, 2019[14] (bisexual) from Italy, *Pam. arduus* Guidetti, Cesari, Bertolani, Altiero and Rebecchi, 2019[14] (bisexual) from Italy, *Pam. experimentalis* Kaczmarek, Mioduchowska, Poprawa and Roszkowska, 2020[20] (bisexual) from Madagascar[15,19–29]. Importantly, Guidetti et al.[14] also concluded that unisexual species like *Pam. fairbanksi* have a wider geographical range compared to bisexual *Paramacrobiotus* taxa. Subsequetly, Stec et al.[15] corroborated this hypothesis additionally suggesting that the wide distribution of some taxa of the genus may be caused by human-mediated dispersion, since most of these populations were found in populated areas with trade and touristes.

In the present paper, we provide a description of a new parthenogenetic and widespread *Paramacrobiotus* species based on its population discovered in Madeira. The study was framed within an integrative taxonomy with detailed morphological and genetic analyses. We also conducted species molecular delimitation analyses based on all COI sequences of the genus *Paramacrobiotus* available in GenBank. Finally, we reconstructed the multilocus phylogeny of superclade II of the family Macrobiotidae (sensu Stec et al.[29]) to elucidate the phylogenetic position of the new species.

Material and methods

Sample processing. The moss sample was collected in Ribeiro Frio, Madeira (32°44′36.7"N, 16°54′28.0"W) in September 2019. The sample was packed in paper envelope, dried at a temperature of *ca.* 25 °C and delivered to the laboratory at the Faculty of Biology, Adam Mickiewicz University in Poznań, Poland. Tardigrades were extracted from the samples and studied following the protocol of Stec et al.[30].

Tardigrade culture. Specimens of a new species have been cultured continuously since February 2022. The cultures were maintained in plastic Petri dishes containing a mixture of ddH_2O and "Żywiec Zdrój" spring water (3:1). To aid tardigrades locomotion, each Petri dish bottom was scratched with fine sandpaper. The culture was maintained in an environmental chamber (model POL ST1 BASIC) at 18 °C and fed once per week on rotifers (2 ml of culture of *Lecane inermis* (Bryce 1892))[31]. Once per week, the medium was exchanged using a sterile plastic pipette to avoid contamination. To establish the type of reproduction in the new species, 50 eggs were collected and incubated in a glass cube and inspected daily. Upon hatching, each juvenile was transferred to a single well of 24 well plates with scratched bottom. The isloated individuals were then observed and fed every week.

Genotyping. Prior to DNA extraction, each tardigrade specimen was examined in vivo under PCM (400 × magnification). In order to obtain voucher specimens, genomic DNA was extracted from individual animals following a Chelex 100 resin (BioRad) extraction method[32] with modifications according to Stec et al.[33].

Two conservative nuclear ribosomal subunit genes were sequenced, i.e. 18S rRNA, 28S rRNA as well as nuclear ITS-2 (internal transcribed spacer 2) and mitochondrial COI (cytochrome C oxidase subunit I) barcode sequences. Fragments of the nuclear genes were amplified using the following primers: 18S_Tar_Ff1 (5′-AGG CGAAACCGCGAATGGCTC-3′) and 18S_Tar_Rr1 (5′-GCCGCAGGCTCCACTCCTGG-3′; Stec et al.[34]) for the 18S rRNA gene fragment; 28SF0001 (5′-ACCCvCynAATTTAAGCATAT-3′) and 28SR0990 (5′-CCTTGG TCCGTGTTTCAAGAC-3′; Mironov et al.[35]) for the 28S rRNA gene fragment; ITS–3 (5′- GCATCGATGAAG AACGCAGC.-3′) and ITS-4 (5′- TCCTCCGCTTATTGATATGC-3′; White et al.[36]) for the ITS-2 gene fragment. In turn, the COI molecular marker was amplified using universal primers: HCO2198 (5′-TAAACTTCAGGG TGACCAAAAAATCA-3′) and LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG–3′; Folmer et al.[37]). All PCR reactions were performed in 20 µl volume containing 0.8 × JumpStart Taq ReadyMix (1 U of JumpStart Taq DNA polymerase, 4 mM Tris–HCl (pH 8.3), 20 mM KCl, 0.6 mM $MgCl_2$, 0.08 mM of dNTP; Sigma-Aldrich), 0.4 µM of proper forward and reverse primers and *ca.* 1 ng of DNA. The PCR cycling profiles to amplify the 28S rRNA, ITS-2 and COI sequences were performed according to the protocols described in Kaczmarek et al.[20]. In turn, 18S rRNA sequences were amplified according to the protocol described in Stec et al.[34]. The reactions were performed in a BiometraTProfessional thermocycler. The PCR products were cleaned up by exonuclease I (20 U/µl, Thermo Scientific) and alkaline phosphatase FastAP (1 U/µl, Thermo Scientific). The Sanger sequencing method was carried out in both directions using the BigDyeTM terminator cycle sequencing and ABI Prism 3130xl genetic analyser (Life Technologies). In case ITS-2 gene fragment poor sequencing results have been obtained. Finally, this molecular marker was not applied in the analysis.

Phylogenetic analysis and molecular species delimitation. Phylogenetic analyses were performed in order to establish phyletic position of the new species and reconstruct the relationships within Macrobiotidae clade II (sensu Stec et al.[29]). The data set was compiled from taxa/specimens for which DNA sequences of at least two (out of all four commonly used 18S rRNA, 28S rRNA, ITS-2, COI) molecular markers are available and suitable for concatenation (Table 1). The DNA sequences of *Macrobiotus rybaki* Vecchi & Stec, 2021[38] and *Sisusbiotus spectabilis* Thulin, 1928[39], and *Mesobiotus datanlanicus* Stec, 2019[40] were used as the outgroup. The sequences were aligned using the AUTO method (for COI and ITS2) and the Q-INS-I method (for ribosomal markers: 18S rRNA and 28S rRNA) of MAFFT version 7[41,42] and manually checked against non-conservative alignments in BioEdit. Then, the aligned sequences were trimmed to: 994 (18S rRNA), 811 (28S rRNA), 487 (ITS-2), 658 (COI) bp and concatenated using SequenceMatrix[43]. Before partitioning, the concatenated alignment was divided into six data blocks constituting three separate blocks of ribosomal markers and three separate blocks of three codon positions in COI data set. Using PartitionFinder[44] under the Akaike Information Criterion (AIC), the best scheme of partitioning and substitution models were chosen for Bayesian phylogenetic analysis. Before running phylogenetic analysis, we also performed a substitution saturation test with DAMBE for two variable DNA fragments that were used in our analyses, namly COI and ITS2[45,46]. Bayesian inference (BI) marginal posterior prob-

Taxon	18S rRNA	28S rRNA	COI	ITS–2	Source
Paramacrobiotus gadabouti sp. nov. MD50.1	OP394210		OP394113		This study
Paramacrobiotus gadabouti sp. nov. MD50.2	OP394211	OP394209			This study
Paramacrobiotus gadabouti sp. nov. MD50.4	OP394212		OP394114		This study
Macrobiotus rybaki[37]	MW588029	MW588034	MW593931	MW588022	37
Mesobiotus datanlanicus[39]	MK584659	MK584658	MK578905	MK584657	39
Minibiotus furcatus[51]	FJ435746	FJ435760	FJ435802		26
Minibiotus gumersindoi[52]	FJ435748	FJ435761	FJ435803		26
Minibiotus intermedius[53]	ON005189	ON005195	ON005160		54
Minibiotus ioculator[33]	MT023998	MT024041	MT023412	MT024000	33
Minibiotus pentannulatus 1[55]	MT023999	MT024042	MT023413	MT024001	33
Minibiotus pentannulatus 2[55]	MT023999	MT024043	MT023414	MT024001	33
Minibiotus sp.	OK663227	OK663238		OK663216	56
Paramacrobiotus aff. *richtersi* AU	MH664932	MH664949	MH675999	MH666081	15
Paramacrobiotus aff. *richtersi* BR 1	MH664934	MH664952	MH676000	MH666082	15
Paramacrobiotus aff. *richtersi* BR 2			MH676001		15
Paramacrobiotus aff. *richtersi* BR 3			MH676002		15
Paramacrobiotus aff. *richtersi* FR 1	MH664935	MH664953	MH676003	MH666083	15
Paramacrobiotus aff. *richtersi* FR 2			MH676004		15
Paramacrobiotus aff. *richtersi* HU 1	MH664936	MH664954	MH676005	MH666084	15
Paramacrobiotus aff. *richtersi* HU 2			MH676006		15
Paramacrobiotus aff. *richtersi* MG 1	MH664938	MH664956	MH676008	MH666086	15
Paramacrobiotus aff. *richtersi* MG 2				MH666087	15
Paramacrobiotus aff. *richtersi* NO	MH664939	MH664957	MH676009	MH666088	15
Paramacrobiotus aff. *richtersi* NZ	MH664940	MH664958	MH676010	MH666089	15
Paramacrobiotus aff. *richtersi* PT 1	MH664944	MH664961	MH676014	MH666093	15
Paramacrobiotus aff. *richtersi* PT 2			MH676015		15
Paramacrobiotus aff. *richtersi* TN	MH664945	MH664962	MH676016	MH666094	15
Paramacrobiotus aff. *richtersi* TZ	MH664933	MH664951	MH676017	MH666095	15
Paramacrobiotus arduus[14]	MK041032		MK041020		14
Paramacrobiotus areolatus[17]	MH664931	MH664948	MH675998	MH666080	15
Paramacrobiotus celsus[14]	MK041031		MK041019		14
Paramacrobiotus cf. *klymenki* IT	MH664937	MH664955	MH676007	MH666085	15
Paramacrobiotus cf. *klymenki* PT	MH664943	MH664960	MH676013	MH666092	15
Paramacrobiotus depressus[14]	MK041030		MK041015		14
Paramacrobiotus experimentalis[20]	MN073468	MN073465	MN097837	MN073464	20
Paramacrobiotus fairbanksi PL[19]	MH664941	MH664950	MH676011	MH666090	15
Paramacrobiotus filipi 1[57]	MT261913	MT261904	MT260372		57
Paramacrobiotus filipi 2[57]			MT260373		57
Paramacrobiotus lachowskae[58]	MF568532	MF568533	MF568534	MF568535	58
Paramacrobiotus metropolitanus[59]	LC637243	LC649795	LC637242	LC649794	59
Paramacrobiotus richtersi[16]	MK041023		MK040994		14
Paramacrobiotus richtersi S38 1[16]	OK663224	OK663236	OK662995	OK663213	56
Paramacrobiotus spatialis[14]	MK041024		MK040996		14
Paramacrobiotus spatialis S107 1[14]	OK663225	OK663236	OK662996	OK663214	56
Paramacrobiotus tonolli US[18]	MH664946	MH664963	MH676018	MH666096	15
Sisubiotus spectabilis[38]	MN888371	MN888357	MN888322	MN888331	37
Tenuibiotus cf. *ciprianoi*	MN888376	MN888361	MN888328	MN888348	37
Tenuibiotus danilovi[60]	MN888377	MN888362	MN888329	MN888349	37
Tenuibiotus tenuiformis[60]	MN888378	MN888363	MN888330	MN888350	37
Tenuibiotus voronkovi[60]	KX810045	KX810049	KX810042	KX810046	61
Tenuibiotus zandrae[62]	MN443040	MN443035	MN444827	MN443038	62

Table 1. Sequences used for phylogenetic analysis. Bold font indicates sequences obtained in this study.

abilities were calculated for the concatenated (18S rRNA+28S rRNA+ITS-2+COI) data set using MrBayes v3.2[47]. Random starting trees were used and the analysis was run for ten million generations, sampling the Markov chain every 1000 generations. An average standard deviation of split frequencies of < 0.01 was used as a guide to ensure the two independent analyses had converged. The program Tracer v1.6[48] was then used to ensure Markov chains had reached stationarity, and to determine the correct 'burn-in' for the analysis which was the first 10% of generations. The effective sample size (ESS) values were greater than 200 and the consensus tree was obtained after summarising the resulting topologies and discarding the 'burn-in'. Maximum-likelihood (ML) tree was computed using RAxML v8.0.19[49]. Strength of support for internal nodes of ML construction was measured using 1,000 rapid bootstrap replicates. The consensus tree was viewed and visualised by FigTree v.1.4.3 available from http://tree.bio.ed.ac.uk/software/figtree. The best evolutionary models of sequence evolution selected for BI and ML analyses, as well as the results of saturation test are given in supplementary materials (SM.01). Networks of haplotypes of the new species were prepared using PopARTver.1.7 (http://popart.otago.ac.nz) with the implementation of Median-Joining method[50] For this purpose, we used all COI and ITS-2 sequences od speciemens of the new species that were present in our phylogenetic analyses (N = 5 for ITS-2 and N = 8 for COI).

Using the COI data set comprising all *Paramacrobiotus* sequences of this marker avilabe in GenBank (80 sequences), we performed two genetic species delimitation analyses. According to the recommendation by Fontaneto et al.[63] one of them was a tree-based method, the Poisson Tree Processes (bPTP[64]), whereas the second one was a distance-based method, the Assemble Species by Automatic Partitioning (ASAP[65]). For the bPTP analysis, we computed a ML tree using RAxML v8.0.19[49] also with prior search of the best model and partition scheme using PartionFinder2[66] (SM.01). The calculations were conducted on the bPTP webserver (http://species.h-its.org/ptp), with 500,000 MCMC generations, thinning the set to 100, burning at 10% and performing a search for ML and Bayesian solutions. For ASAP analysis we used the COI alignment as input data. The analyses were run on the respective server (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) with default settings. All COI sequences used for the analyses and their outputs are given within the supplementary materials (SM.02).

Microscopy and imaging. In total 33 animals, 5 exuvium, 2 simplex and 24 eggs were mounted on microscope slides in the Hoyer's medium, and then examined under Olympus BX41 Phase-contrast light Microscope (PCM) associated with Olympus SC50 digital camera (Olympus Corporation, Shinjuku-ku, Japan). Thirty animals and 10 eggs were prepared for Scanning Electron Microscope (SEM) observation according to the protocol in Roszkowska et al.[67] and examined under high vacuum in Hitachi S3000N SEM (Hitachi, Japan). All figures were assembled in Corel Photo-Paint 2017. For deep structures that could not be fully focused on a single photograph, a series of 2–10 images were taken every *ca.* 0.5 µm and then manually assembled into a single deep-focus image in Corel Photo-Paint 2017.

Morphometrics and morphological nomenclature. All measurements are given in micrometers [µm]. Structures were measured only if their orientation was suitable. Body length was measured from the anterior extremity to the end of the body, excluding the hind legs. The types of bucco-pharyngeal apparatuses and claws were classified according to Pilato and Binda[68]. All measurements and terminology of adults and eggs of *Paramacrobiotus* were prepared according to Kaczmarek and Michalczyk[69] and Kaczmarek et al.[12]. Terminology describing the oral cavity armature (OCA) follows Michalczyk and Kaczmarek[70]. The macroplacoid length sequence was indicated according to Kaczmarek et al.[71]. Morphological states of the cuticular bars on legs follow Kiosya et al.[72]. The *pt* ratio is the ratio of the length of a given structure to the length of the buccal tube expressed as a percentage[73]. Morphometric data were handled using the "Parachela" ver. 1.8 template available from the Tardigrada Register[74]. Row morphometric data for the new species are given in Supplementry Materials (SM.03). Tardigrade taxonomy follows Bertolani et al.[75] and Stec et al.[29]. Genus abbreviations follow Perry et al.[76].

Comparative material. For comparison with the new species, holotype and/or paratypes of *Pam. derkai* (Degma, Michalczyk and Kaczmarek, 2008)[77], *Pam. experimentalis*, *Pam. fairbanksi* Schill, Förster, Dandekar and Wolf, 2010[19], *Pam. filipi* Dudziak, Stec and Michalczyk, 2020[57], *Pam. garynahi* (Kaczmarek, Michalczyk and Diduszko, 2005)[78], *Pam. huziori* (Michalczyk and Kaczmarek, 2006)[79], *Pam. intii* Kaczmarek, Cytan, Zawierucha, Diduszko and Michalczyk, 2014[71], *Pam. lachowskae* Stec, Roszkowska, Kaczmarek and Michalczyk, 2018[58], *Pam. magdalenae* (Michalczyk and Kaczmarek, 2006)[80], *Pam. sklodowskae* (Michalczyk, Kaczmarek and Węglarska, 2006[81]) and *Pam. spinosus* Kaczmarek, Gawlak, Bartels, Nelson and Roszkowska, 2017[12] were examined. Moreover, for species identification, the key in Kaczmarek et al.[12] and original descriptions were also used (i.e.[15,78,82]).

Results

Phylogeny and species delimitation. Both phylogenetic analyzes resulted with trees of similar topology and mostly well-supported nodes in which *Paramacrobiotus* and *Tenuibiots* are monophyletic genera, whereas *Minibiotus* was recovered paraphyletic (Fig. 1). Monophyly was not confirmed for *Pam. richtersi* and *Pam. areolatus* morpho-groups since representatives of the latter form a paraphyletic group caused by *Pam. lachowskae* which cluster together with the former morpho-group (Fig. 1). The sequences of the new species obtained in this study clastered together with *Paramacrobiotus* aff. *richtersi* populations from France, Portugal, Australia and Tunisia previously reported by Stec et al.[15], forming a monophyletic clade staying in sister relationship with *Paramacrobiotus* aff. *richtersi* population from Hungary. Haplotype networks showed higher haplotype diversity in case of COI than in ITS-2 marker, with same COI haplotype shered sometimes with populations from very distinct localities (Fig. 2). Molecular species delimitation analyzes recovered 22 and 29 putative species for ASAP and bPTP methods, respectively, with all valid nominal taxa delineated coherently as distinct entities (SM.02). Importantly, 9 ASAP and 12 bPTP entities were delimited from COI sequences without assignment to any nomi-

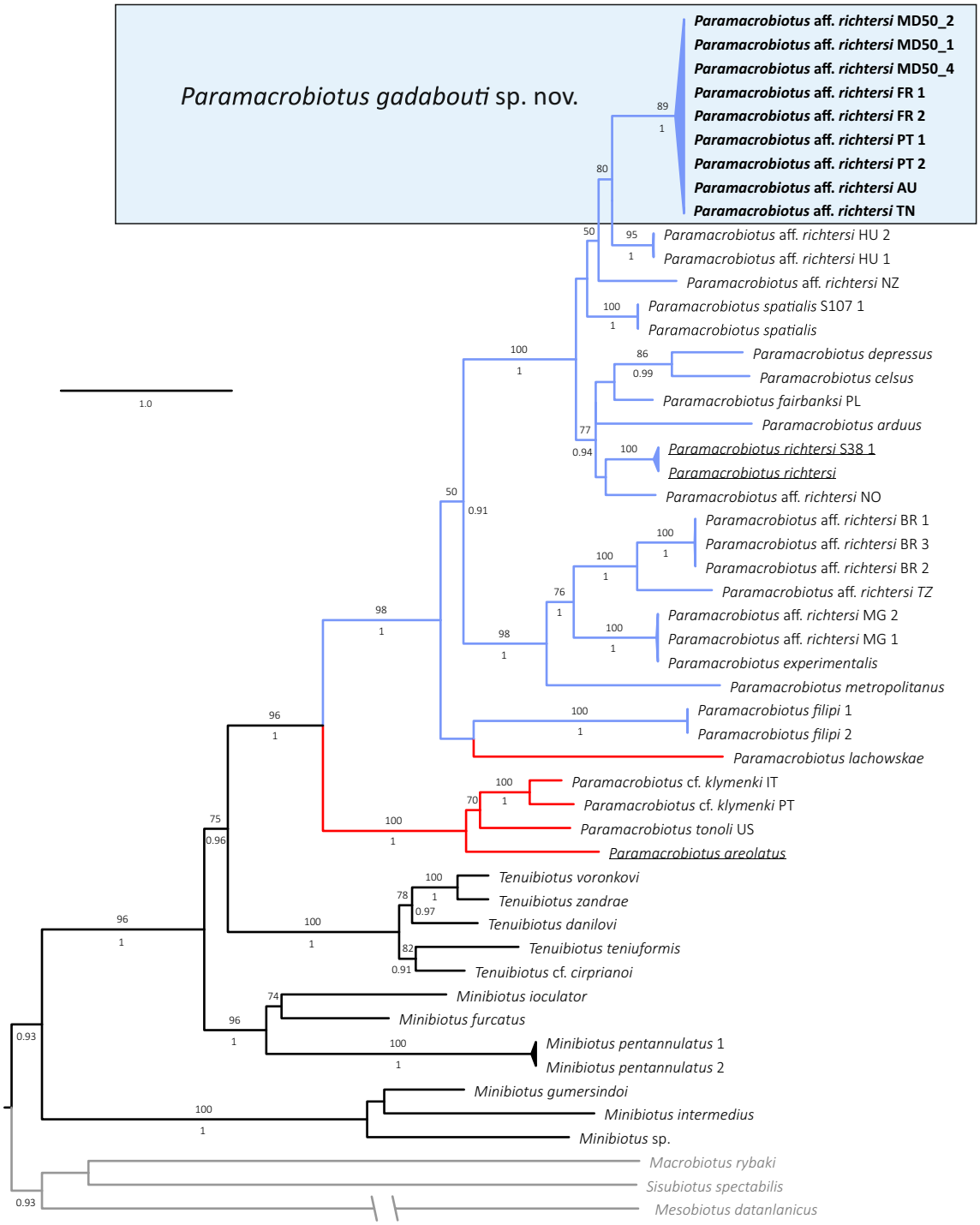

Figure 1. Maximum likelihood (ML) phylogeny constructed from concatenated sequences of the family Macrobiotidae (18S rRNA + 28S rRNA + ITS-2 + COI; Table 1). Numbers above branches indicate bootstrap support values, while Bayesian posterior probabilities (pp) are given below branches. Bootstrap < 50 and pp < 0.90 are not shown. Taxa of the *Pam. richtersi* and *Pam. areolatus* complex are indicated by blue and red branches, respectively. The outgroup is indicated in gray font. The scale bar represents substitutions per position.

nal *Paramacrobiotus* species. Single locus delimitations confirmed the results from multilocus phylogeny, recognizing the newly studied population and *Paramacrobiotus* aff. *richtersi* populations from France, Portugal, Australia and Tunisia as a single species (Fig. 1; SM.02) which is formally described below.

Taxonomic Account.

Phylum: Tardigrada (Doyère, 1840)[83]

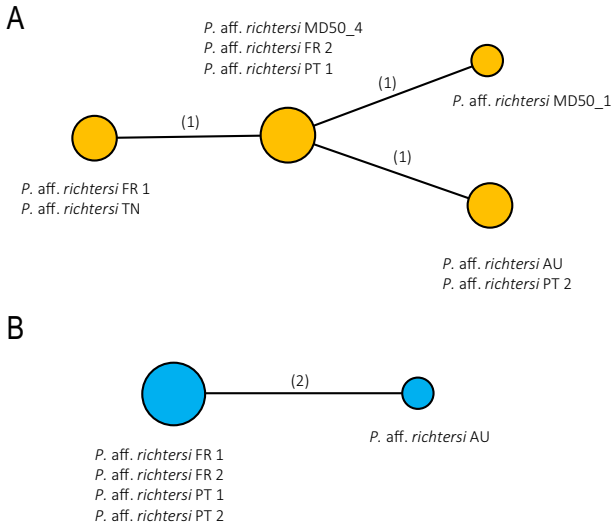

Figure 2. Haplotype Median Joining networks for mitochondrial (COI) and nuclear (ITS-2) markers of *P. gadabouti* sp. nov: (**A**)—COI; (**B**)—ITS-2. Haplotypes are represented by coloured circles. The size of circles is proportional to the number sequences/specimens of each particular haplotype. Sequence/specimen names correspond with names presented in phylogenetic tree in Fig. 1. Numbers in brackets indicate the numbers of mutations between the haplotypes.

Class: Eutardigrada (Richters, 1926)[84]
Order: Macrobiotoidea (Thulin, 1928)[39]
Family: Macrobiotidae (Thulin, 1928)[39]
Genus: *Paramacrobiotus* (Guidetti et al., 2009)[11]

Paramacrobiotus gadabouti sp. nov. Kayastha, Stec, Mioduchowska and Kaczmarek.
(Figs. 2, 3, 4 5, 6 and 7; Tables 2 and 3).

Type Material. Holotype (slide M50/4 (+ 6 paratypes (3 animals + 2 exuvium + 1 simplex) on the same slide)) and 101 paratypes (29 animals + 3 exuvium + 1 simplex + 24 eggs; slides: M50/*, where the asterisk can be substituted by any of the following numbers: 1–3, 5–20), 4 exoskeleton after DNA extraction (M50.1/S, M50.2/S, M50.3/S and 50.4/S) and 30 animals + 10 eggs on one SEM stub.

Description (measurements and statistics in Table 2). *Animals:* Body colour transparent/white, eyes absent in living specimens (Fig. 3A–B). Except for granulation on legs I–IV (Fig. 4A–D), cuticle is smooth, i.e. without

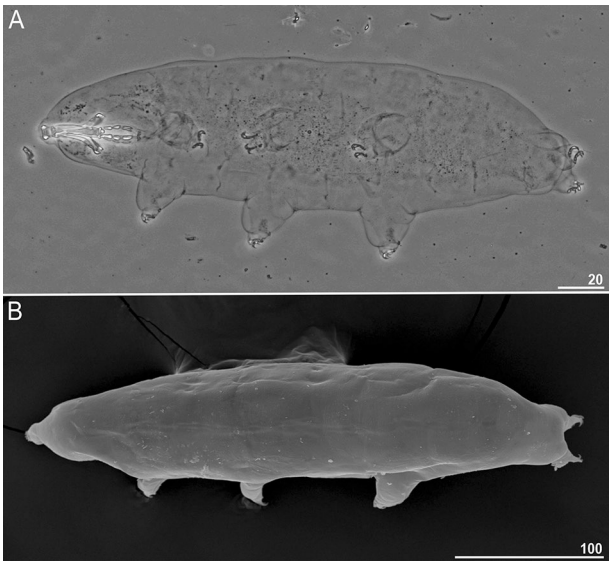

Figure 3. *Paramacrobiotus gadabouti* sp. nov.: (**A**)—ventral-dorsal projection (holotype, PCM); (**B**)—dorso-ventral projection (paratype, SEM). Scale bars in μm.

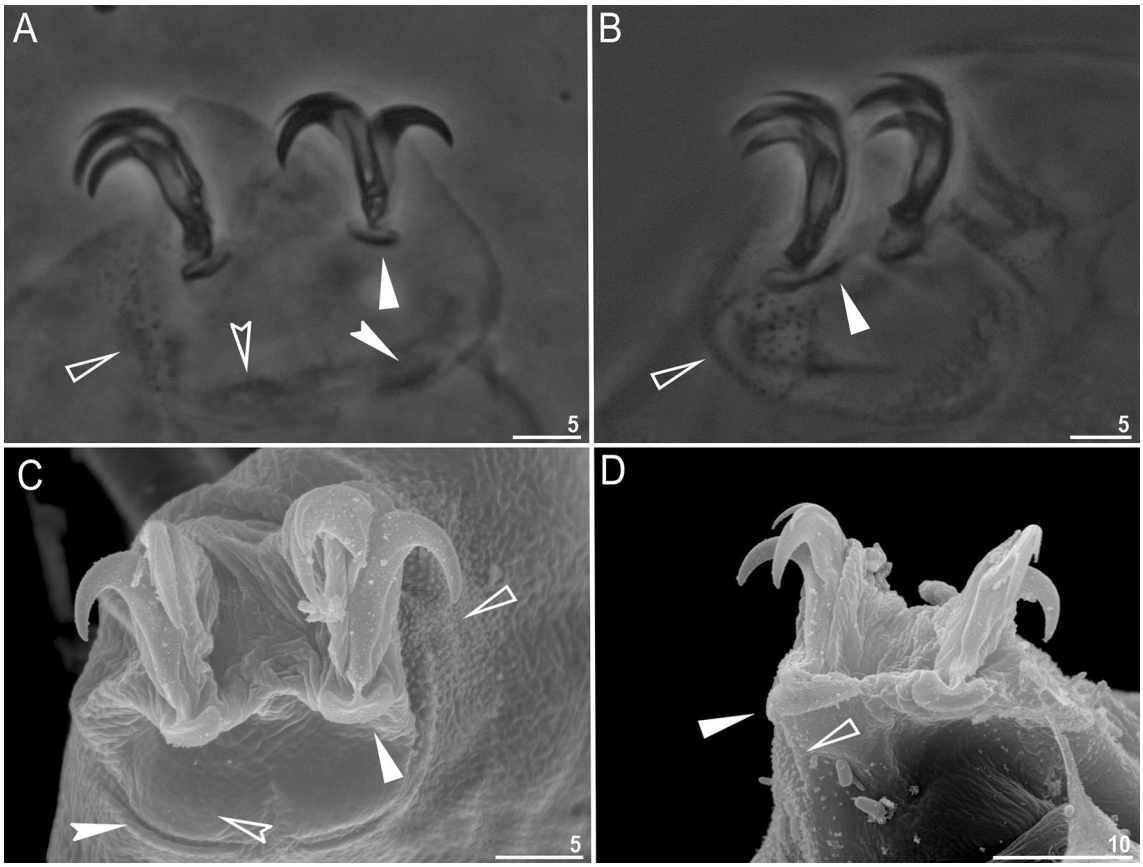

Figure 4. *Paramacrobiotus gadabouti* sp. nov.: (**A**)—claws II (paratype, PCM); (**B**)—claws IV (paratype, PCM); (**C**)—claws II (paratype, SEM); (**D**)—claws IV (paratype, SEM). Filled unindented arrowhead represents smooth lunulae, empty unindented arrowhead represents granulation, empty indented arrowheads represent single continuous bar and filled indented arrowheads represent doubled muscle attachments. Scale bars in μm.

gibbosities, papillae, pores, spines or sculpturing. The leg granulation is present on the external surface of legs I–III and on lateral and dorsal surfaces of the hind legs (Fig. 4A–D). Claws of the *hufelandi* type, stout (Fig. 4A–D). Primary branches with distinct accessory points. Smooth lunulae present under all claws (Fig. 4A–D, filled unindented arrowhead). Single continuous cuticular bar constricted in the middle and paired muscle attachments below claws I–III present (Fig. 4A–D, empty indented arrowhead and filled indented arrowhead).

Bucco-pharyngeal apparatus of the *Macrobiotus* type (Fig. 5A–C), with ventral lamina and ten peribuccal lamellae (Fig. 6A). Mouth antero-ventral. The OCA is composed of three bands of teeth (similar on dorsal and ventral sides) (Figs. 5D–E and 6A–C). The first band of teeth consists of small cones (granules in PCM) situated at the anterior portion of the oral cavity, and just behind the base of the peribuccal lamellae (4–5 rows) (Figs. 5D, 6B, filled arrow). The second band of teeth positioned in the rear of the oral cavity between the ring fold and the third band of teeth (Figs. 5D, 6B, empty arrow) and composed of larger cones (small ridges parallel to the main axis of the buccal tube in PCM), arranged in one row that runs around the oral cavity wall (Figs. 5D–E and 6B, filled unindented arrowhead). The third band of teeth positioned just before the buccal tube opening and composed of dorsal and ventral portion (Figs. 5D–E and 6B–C). The dorsal portion of the third band comprises three, distinctly separated, long and thin ridges (Fig. 5D and 6C). Similarlty, the ventral portion is composed of three distinct teeth with two ventro-lateral onces in shape of ridges and one medio-ventral tooth being often divided into 2–3 smaller granular teeth (Fig. 5E). Additional teeth absent (Figs. 5D–E and 6A–C). Pharyngeal bulb spherical, with triangular apophyses and three rod-shaped macroplacoids. Macroplacoid length sequence $2 < 1 < 3$ (Fig. 5A–C). The first macroplacoid without constrictions, but distinctly narrower anteriorly. The second macroplacoid of uniform width and without constrictions. The third macroplacoid with a sub-terminal constriction (Fig. 5A–B; empty unindented arrowhead). Microplacoid present, triangular in shape (Fig. 5A–B).

Eggs: Laid freely, white, spherical exhibiting ornamentations of the *richtersi* type (Fig. 7A–B). Processes in the shape of rounded or truncated cones (Fig. 7A–F). Top endings of the processes with cap like structures (well visible in PCM in the process midsection and always well visible in SEM) (Fig. 7D–F). The surface of cap-like structrues is mostly rough with small granules and wrinkles that can be visible on its surface but only in SEM (Fig. 7D, F). Labyrinthine layer between process walls visible under PCM as a clear reticular pattern (Fig. 7C). Reticular pattern composed of regular and elongated mesh with straight or slightly sinuous margins. Egg shells areolated with a single ring of 10–12 areolae around each process (Fig. 7C–D). Internal surface of areolae clearly sculptured in PCM and pores that are visible only in SEM (Fig. 7C–D).

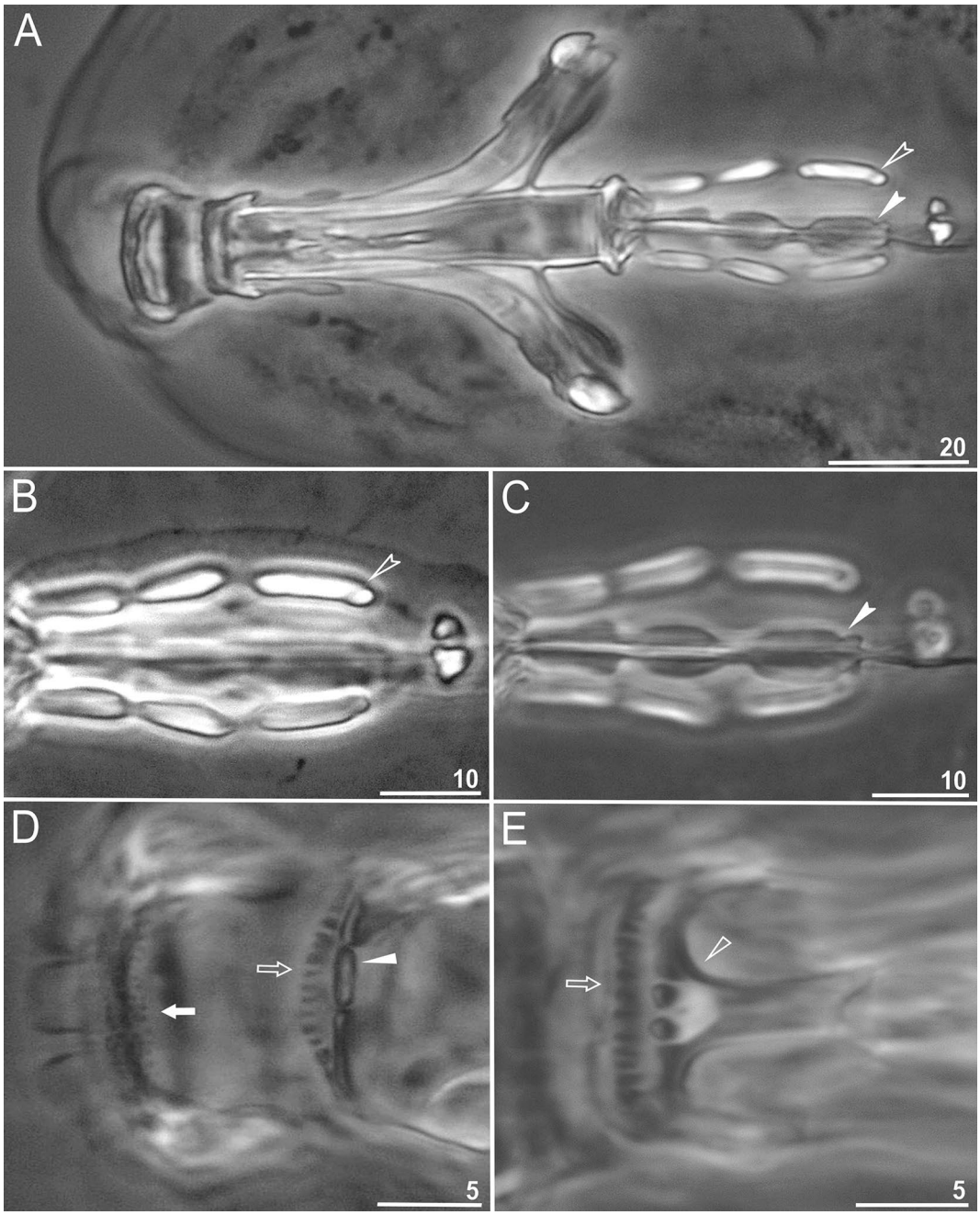

Figure 5. *Paramacrobiotus gadabouti* sp. nov.: (**A**)—bucco-pharyngeal apparatus (dorso–ventral projection) general view (paratype); (**B**)—placoid morphology in dorsal view (paratype); (**C**)—ventral placoids (paratype); (**D**)—oral cavity armature (paratype, PCM) seen from the dorsal side; (**E**)—oral cavity armature (paratype, PCM) seen from the ventral side. Empty indented arrowhead represents third macroplacoid with sub-terminal constriction, filled indented arrowhead represents third macroplacoid with central constriction in ventral side, filled arrow represents first band of teeth, empty arrow represents second band of teeth, filled unindented arrowhead represents third band of teeth from dorsal side and empty unindented arrowhead represents third band of teeth from ventral side. Scale bars in μm.

Reproduction: In the experimental setting with isolated individuals of the new species, eggs laying was observed in all matured animals. These eggs hatched into juveniles. Thus, we conclude the reproduction in *Pam gadabouti* sp. nov. to be parthenogenetic.

Type Locality: Portugal, Madeira Island, 32°44′36.7"N, 16°54′28.0"W, 647 m asl, Ribeiro Frio, moss from rock and rock wall, 23 Sptember 2019, coll. Łukasz Sługocki, Ricardo Araújo and J. J. Gonçalves Silva.

Additional Localities: (1) Portugal, Madeira Island, 32°49′06″N, 16°59′19″W, 299 m asl, Ponta Delgada, moss from rock, 21 February 2018, coll. Łukasz Michalczyk; (2) Australia, Western Australia State, 31°57′16″S,

CHARACTER	N	RANGE		MEAN		SD		Holotype	
		µm	*pt*	µm	*pt*	µm	*pt*	µm	*pt*
Body length	20	435–783		588		95		594	
Buccopharyngeal tube									
Buccal tube length	20	44.7–64.5		54.1		4.5		50.0	
Stylet support insertion point	20	36.2–49.8	*76.1–81.0*	42.4	*78.3*	3.4	*1.2*	39.7	*79.4*
Buccal tube external width	20	9.1–15.4	*20.0–27.9*	12.9	*23.7*	1.7	*1.9*	11.9	*23.7*
Buccal tube internal width	20	6.4–10.8	*14.2–19.6*	9.2	*17.0*	1.2	*1.3*	8.9	*17.7*
Ventral lamina length	17	23.9–38.6	*53.4–60.6*	30.8	*57.4*	3.6	*2.2*	28.4	*56.7*
Placoid lengths									
Macroplacoid 1	20	7.7–12.6	*17.1–21.8*	10.4	*19.1*	1.4	*1.3*	8.8	*17.6*
Macroplacoid 2	20	7.0–11.3	*15.4–19.8*	9.6	*17.7*	1.3	*1.3*	8.3	*16.5*
Macroplacoid 3	20	8.4–14.9	*18.7–26.4*	12.5	*22.9*	1.7	*1.8*	11.4	*22.8*
Microplacoid	20	3.3–5.1	*6.7–9.3*	4.2	*7.9*	0.5	*0.8*	4.7	*9.3*
Macroplacoid row	20	27.4–41.9	*60.7–69.8*	35.1	*64.7*	3.9	*2.8*	32.3	*64.6*
Placoid row	20	34.9–53.6	*77.7–87.4*	44.8	*82.6*	4.8	*3.3*	41.8	*83.5*
Claw I heights									
External primary branch	19	11.3–20.5	*24.0–31.8*	15.1	*27.6*	2.0	*1.9*	14.2	*28.4*
External secondary branch	19	9.2–17.1	*19.2–26.5*	11.8	*21.6*	1.6	*1.5*	11.6	*23.2*
Internal primary branch	19	11.7–19.1	*23.3–29.6*	14.2	*26.2*	1.7	*1.7*	13.8	*27.6*
Internal secondary branch	19	9.2–15.2	*16.9–23.6*	11.3	*20.8*	1.4	*1.6*	10.4	*20.8*
Claw II heights									
External primary branch	19	11.8–23.9	*24.5–37.1*	15.5	*28.4*	2.5	*2.8*	14.7	*29.3*
External secondary branch	19	8.1–16.2	*13.9–25.5*	11.9	*21.9*	2.1	*3.2*	11.4	*22.7*
Internal primary branch	19	11.8–20.0	*23.6–31.0*	14.5	*26.5*	1.9	*1.8*	11.8	*23.6*
Internal secondary branch	19	9.6–15.4	*19.7–24.2*	11.8	*21.7*	1.5	*1.4*	10.7	*21.4*
Claw III heights									
External primary branch	19	12.9–21.6	*26.8–33.5*	15.9	*29.3*	1.9	*1.5*	14.3	*28.6*
External secondary branch	19	9.7–15.2	*18.5–27.2*	12.3	*22.6*	1.5	*2.0*	11.1	*22.2*
Internal primary branch	19	12.1–20.3	*24.0–31.5*	14.7	*27.1*	1.8	*1.8*	14.0	*27.9*
Internal secondary branch	19	10.1–17.1	*18.6–26.5*	11.8	*21.6*	1.6	*2.0*	11.4	*22.7*
Claw IV heights									
Anterior primary branch	19	11.7–22.8	*26.2–35.4*	16.7	*30.7*	2.1	*2.2*	15.7	*31.4*
Anterior secondary branch	19	8.7–19.2	*17.7–29.8*	13.0	*23.8*	2.3	*2.9*	11.4	*22.7*
Posterior primary branch	19	13.4–23.1	*27.3–35.8*	16.3	*30.0*	2.2	*2.2*	14.3	*28.6*
Posterior secondary branch	19	10.7–17.0	*21.6–29.0*	12.9	*23.9*	1.4	*2.0*	12.6	*25.1*

Table 2. Measurements [in µm] and *pt* values of selected morphological structures of individuals of *Paramacrobiotus gadabouti* sp. nov. mounted in Hoyer's medium (N—number of specimens/structures measured; RANGE refers to the smallest and the largest structure among all measured specimens; SD—standard deviation, *pt*—ratio of the length of a given structure to the length of the buccal tube expressed as a percentage). *pt* values are in italic.

CHARACTER	N	RANGE	MEAN	SD
Egg bare diameter	17	64.3–91.7	78.2	6.8
Egg full diameter	17	104.8–125.3	112.7	7.2
Process height	50	12.1–23.7	17.5	2.2
Process base width	50	15.0–25.5	19.2	2.3
Process base/height ratio	50	91%–135%	110%	12%
Inter-process distance	50	2.5–6.1	3.8	0.8
Number of processes on the egg circumference	17	11–13	12.1	0.9

Table 3. Measurements [in µm] of selected morphological structures of eggs of *Paramacrobiotus gadabouti* sp. nov. mounted in Hoyer's medium (N—number of specimens/structures measured; RANGE refers to the smallest and the largest structure among all measured eggs; SD—standard deviation).

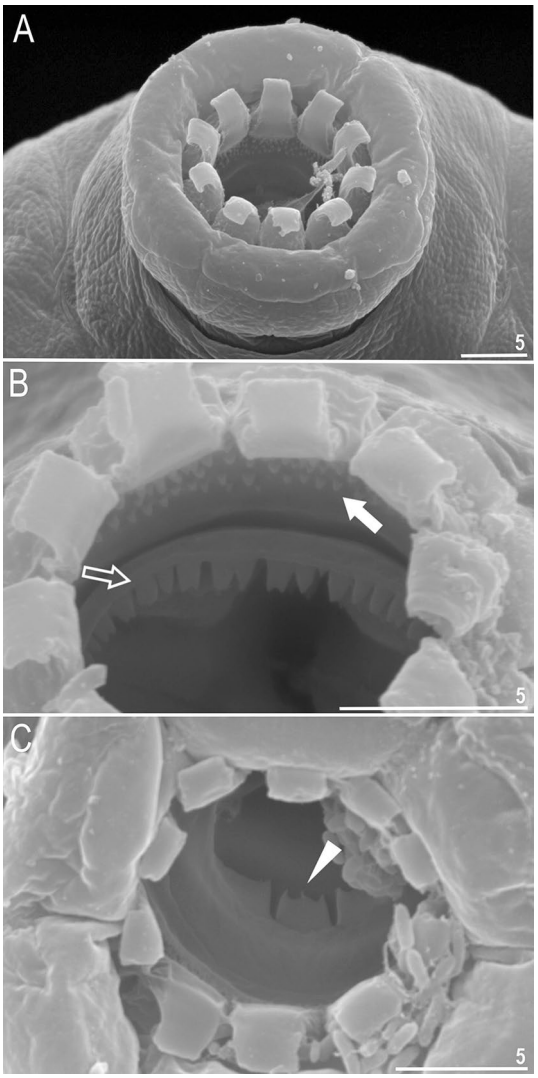

Figure 6. *Paramacrobiotus gadabouti* sp. nov.: (**A**)—mouth with ten peribuccal lamellae (paratype, SEM); (**B**)—oral cavity armature with first and second band of teeth (paratype, SEM); (**C**)—oral cavity armature with third band of teeth (paratype, SEM) from dorsal side. Filled arrow represents first band of teeth, empty arrow represents second band of teeth and filled unindented arrowhead represents third band of teeth. Scale bars in μm.

115°50′40″E, 56 m asl, Perth, Kings Park, moss from tree, 22 March 2015, coll. Łukasz Michalczyk; (3) France, Île-de-France Region, 48°51′35.5″N, 2°23′40″E, 80 m asl, Paris, Père Lachaise Cemetery, moss from grave, 23 May 2016, coll. Witold Morek; (4) Tunisia, Beni M'tir, Jendouba Governorate, 36°73′92″N, 8°72′99″E, 516 m asl, moss from soil in a forest, 12 June 2015, coll. Jamila Ben Marnissi. All these additional localities have been previously reported in Stec et al.[15].

Etymology: The name *'gadabouti'* refers to the new species ubiquity; from Eng. 'gadabout': someone who restlessly moves from place to place seeking amusement or the companionship of others.

Type depositories: Holotype (M50/4 (+6 paratypes (3 animals + 2 exuvium + 1 simplex) on the same slide)) and 97 paratypes (slides: M50/*, where the asterisk can be substituted by any of the following numbers: 1–3, 5–20, 1/S, 2/S, 3/S and 4/S) were deposited at the Department of Animal Taxonomy and Ecology, Institute of Environmental Biology, Adam Mickiewicz University in Poznań, Uniwersytetu Poznańskiego 6, 61–614 Poznań, Poland and four paratypes (slides: M50/7 and M50/13 (3 animals and 1 egg)) were deposited at Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31–016, Kraków, Poland.

Discussion

Differential diagnosis of the new species. *Paramacrobiotus gadabouti* sp. nov. by having a microplacoid in the pharynx and eggs ornamentation of the *richtersi* type with egg processes ended with cap-like structures is similar to *Pam. alekseevi*[82] (Tumanov 2005), *Pam. filipi*[57] Dudziak, Stec and Michalczyk 2020 and *Pam. garynahi*[78] (Kaczmarek, Michalczyk and Diduszko 2005). The new species differs specifically from:

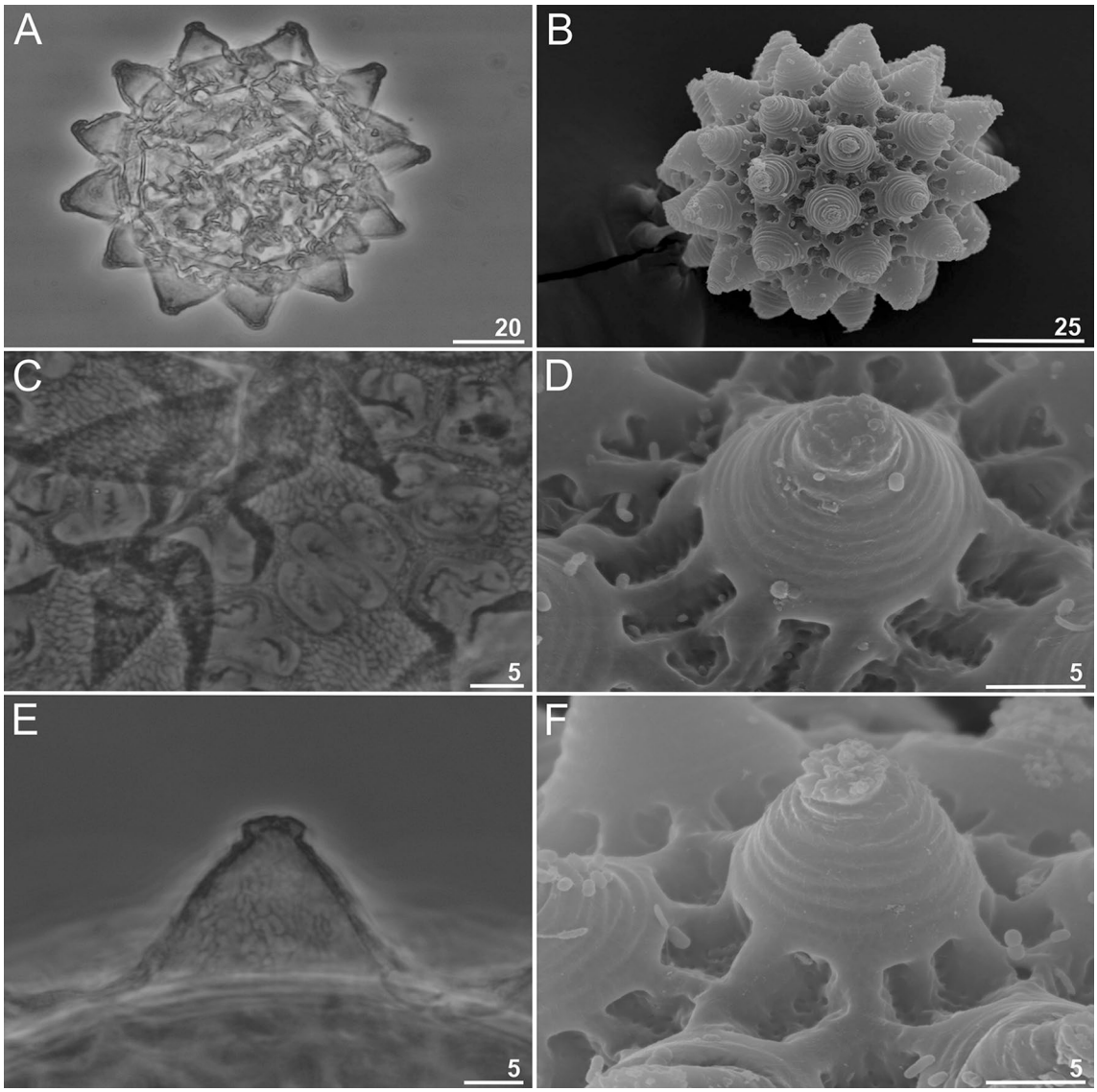

Figure 7. *Paramacrobiotus gadabouti* sp. nov.: eggs: (**A**, **B**)—egg chorion (paratype, PCM and SEM respectively); (**C**, **D**)—the surface between egg processes (paratype, PCM and SEM respectively); (**E**, **F**)—egg processes (paratype, PCM and SEM respectively). Scale bars in µm.

1 *Paramacrobiotus alekseevi*, known only from type locality in Thailand[82], by: the presence of pores inside egg areoles, a higher *pt* value of second macroplacoid (*15.4–19.8* in the new species vs *9.8–14.5* in *Pam. alekseevi*) and a longer microplacoid (3.3–5.1 µm [*pt* = *6.7–9.3*] in the new species vs 1.9–3.0 µm [*pt* = *4.0–6.2*] in *Pam. alekseevi*). *Remarks:* The comparison was made based on the *Pam. alekseevi* original description[77] as well as the amended description by Stec et al.[71].

2 *Paramacrobiotus filipi*, known only from type locality in Malaysian part of Borneo[57], by: the absence of dorsal cuticle granulation, a longer second macroplacoid (7.0–11.3 µm [*pt* = *15.4–19.8*] in the new species vs 2.4–6.2 µm [*pt* = *8.0–13.8*] in *Pam. filipi*), a higher *pt* value of macroplacoid row (*60.7–69.8* in the new species vs *44.4–58.6* in *Pam. filipi*), a longer placoid row (34.9–53.6 µm [*pt* = *77.7–87.4*] in the new species vs 17.4–34.5 µm [*pt* = *52.9–73.6*] in *Pam. filipi*) and a larger full egg diameter (104.8–125.3 µm in the new species vs 99.0–104.5 µm in *Pam. filipi*).

3 *Paramacrobiotus garynahi*, known only from type locality in Baikal Region (Russia)[78], by: medioventral tooth in the third band of teeth in the oral cavity divided, eggs chorion ornamentation of the *richtersi* type, i.e. areoles with pores inside (*areolatus* type with areoles wrinkled inside in *Pam. garynahi*), a higher *pt* value of macroplacoid and placoid rows (*60.7–69.8* and *77.7–87.4*, respectively, in the new species vs *44.4–56.9* and *55.3–70.3*, respectively, in *Pam. garynahi*) and a smaller eggs bare and full diameter (64.3–91.7 µm and 104.8–125.3 µm, respectively, in new thespecies vs 96.0–132.0 µm and 142.0–180.0 µm, respectively, in *Pam. garynahi*).

Diversity and distribution of *Paramacrobiotus* taxa. Studies on the genus *Paramacrobiotus* become easier due to several revisions and redescriptions of *Paramacrobiotus* species which have been recently published (e.g.[12,14,15]). The barrier for tardigrade diversity studies is currently being broken down especially by an integrative approach implemented into taxonomic and faunistic research. The tight link between genetic data and the exact specimen/species name and its morphology provided by authors of integrative studies is and will be crucial to understand species diversity in the genus *Paramacrobiotus*. Similarly, to Stec et al.[15], our molecular analyzes showed 9–10 taxa without a certain assignment to any nominal *Paramacrobiotus* species. They may constitute already known species that were described in the past based on morphology only and for which genetic data are lacking or they constitute new for science species awaiting their formal descriptions. Although the results indicate considerable diversity that is still hidden within the genus, it should be also noted that in our study more putative species were delimited by tree-based methods compared with distance-based methods. However, this finding is in line with recent studies on tardigrades, but also studies on other animal groups[85–87]. Based on the research which examined numerous *Paramacrobiotus* populations[14,15], we can notice that many species in this genus (especially in the *Pam. richtersi* group) are extremely similar to each other often exhibiting a considerable intraspecific variation in egg chorion morphology. This makes many of these taxa as sutiable candidates to be considered as cryptic or pseudocryptic species[14,15]. Therefore, it seems very likely that future taxonomic studies on the genus *Paramacrobiotus* would be able to formally name many newly discovered evolutionary lineages only by rigorous tests of distinct species hypotheses with integrative methods.

Over the years, species of *Paramacrobiotus* have been recorded in various geographic regions. Nominal species of the genus have been found in six continents (Table 4). Additionally, there are many unconfirmed taxa from *Pam. richtersi* and *Pam. areolatus* group which are known from numerous localities around the world (see e.g.[14,15,26,88–92]). Importantly, verification of these records is now extremely hard and, in many cases, not possible because of the lack of genetic data and original material. The majority of the *Paramacrobiotus* species are known only from their type localities or from very restricted geographic ranges. However, some of them are reported from slightly wider geographical areas, like: *Pam. danielae* from Ecuador and Peru, and *Pam. sklodowskae* from Cyprus and Tunisia. There are also much wider distributed species, like, for example *Pam. centesimus,* known from Brazil and Ecuador, *Pam. gerlachae* from Costa Rica and the Seychelles, *Pam. tonolli* known from Canada and many states in USA or *Pam. vanescens* reported from the Democratic Republic of the Congo, the Republic of Guinea-Bissau, the Republic of Zambia and Tanzania[15,88–91]. However, the most widely distributed species in the genus *Paramacrobiotus*, which should be considered as truly cosmopolitan, is the parthenogenetic *Pam. fairbanksi* reported alredy from Antarctica, Italy, Poland, Spain and USA (Alaska)[28]. Furthermore, parthenogenetic *Pam. gadabouti* sp. nov. described here has been confirmed in our study to be present in Australia, France, Portugal and Tunisia (see Figs. 1 and 2). This, all together with *Pam. fairbanksi*, corroborate that at least some tardigrade species conform to "everything is everywhere" hypothesis. In contrast, other species from the *Pam. richtersi* group which are bisexual, in most cases the range seems to be limited and restricted e.g. *Pam. experimentalis* reported only from Madagascar, *Pam. metropolitanus* from Japan, *Pam. celsus*, *Pam. depressus* and *Pam.*

Geographic region	Total number of species	Type localities and species
Australia and New Zealand	2	a) Australia: *Pam. peteri*[93]; b) New Zealand: *Pam. hapukuensis*[94]
Central America	2	a) Costa Rica: *Pam. Huziori*[80] and *Pam. Magdalenae*[80]
North America	3	a) USA: *Pam. fairbanksi*[19], *Pam. halei*[95] and *Pam. tonollii*[18]
Africa	7	a) Kenya: *Pam. kenianus*[19]; b) Madagascar: *Pam. experimentalis*[20]; c) Republic of Guinea-Bissau: *Pam. priviterae*[96]; d) Seychelles: *Pam. corgatensis*[97] *Pam. danielisae*[98] and *Pam. gerlachae*[99]; e) Tanzania: *Pam. vanescens*[100]
Asia	7	a) India: *Pam. chieregoi*[101]; b) Japan: *Pam. metropolitanus*[59]; c) Malaysia: *Pam. filipi*[57]; d) New Guinea: *Pam. wauensis*[102]; e) Palau: *Pam. palaui*[19]; f) Sri Lanka: *Pam. savai*[103]; g) Thailand: *Pam. alekseevi*[82]
South America	8	a) Brazil: *Pam. centesimus*[104]; b) Colombia: *Pam. derkai*[77], *Pam. lachowskae*[58], and *Pam. sagani*[105]; c) Ecuador: *Pam. danielae*[96] and *Pam. spinosus*[12]; d) Peru: *Pam. intii*[71]; e) Uruguay: *Pam. rioplatensis*[106]
Europe	15	a) Austria: *Pam. submorulatus*[107]; b) Belarus: *Pam. klymenki*[108]; c) Cyprus: *Pam. sklodowskae*[81]; d) Hungary: *Pam. csotiensis*[107]; e) Greece: *Pam. beotiae*[109]; f) Ireland: *Pam. richtersi*[16]; g) Italy: *Pam. arduus*[14], *Pam. celsus*[14], *Pam. depressus*[14], *Pam. pius*[110] and *Pam. spatialis*[14] h) Norway: *Pam. areolatus*[17] i) Russia: *Pam. garynahi*[77], *Pam. lorenae*[111] and *Pam. walteri*[112]

Table 4. Type localities of all known *Paramacrobiotus* species.

spatialis reported only from Italian locations, but type species of the genus *Pam. richtersi* is reported from Ireland and Finland[14,15,20,56,59]. Importantly when comparing haplotype networks presented for *Paramacrobiotus* taxa in Guidetti et al.[14] and haplotype network provided in our study (Fig. 2) one may see that the divergence between haplotypes in bisexual species (*Pam. richtersi*, *Pam. celsus*, *Pam. arduus*, *Pam. depressus* and *Pam. spatialis*) seems to be higher than divergence between haplotypes in parthenoegenetic species (*Pam. fairbanksi*, *Pam. gadabouti*). Unfortunately, it is premature to conclude if this result could be considered an actual biological pattern or if it simply reflects biases in the analysed data sets, that might be potentially caused by not very large number of sequences analysed per each studied species/population.

Paramacrobiotus gadabouti sp. nov. is the fourth tardigrade species known from more than one zoogeographic realm and the third known from both the Palaearctic and the Australasian realms. The first two being *Echiniscus testudo*[83] (Doyère 1840) and *Milnesium inceptum*[113] Morek, Suzuki, Schill, Georgiev, Yankova, Marley and Michalczyk 2019. This is all in line with the recent study on global distribution of the *Minesium* populations which demonstrated that most of the species have limited distribution; however, some others can be considered cosmopolitan[114]. These examples also confirm the hypothesis presented by Guidetti et al.[14] that parthenogenetic tardigrades should have a wider distribution due to the adventage in inhabiting new places caused by asexual reproduction. On the other hand, it must be noted that most records of these four disuscussed parthenogenetic species (*Ech. testudo*, *Mil. inceptum*, *Pam. fairbanksi*, *Pam. gadabouti* sp. nov.) come from highly populated and often touristic places. Therefore, it is also likely that their wide distribution range was additionally enhanced by human-mediated dispersion[15] or other vectors such as wind, mammals, birds and animals as evidence has been brought to light regarding the dispersal of tardigrades via these various other vectors[115–117].

Data availability

The datasets generated and/or analysed during the current study are available in the GenBank repository, ACCESSION NUMBER OP394113–OP394114, OP394209–OP394212. The data of all sequences are available for public access.

Received: 25 October 2022; Accepted: 23 January 2023
Published online: 07 February 2023

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Acknowledgements

Studies have been partially conducted in the framework of activities of BARg (Biodiversity and Astrobiology Research group). The PK is scholarship holder of Passport to the future—Interdisciplinary doctoral studies at the Faculty of Biology, Adam Mickiewicz University, Poznań POWR.03.02.00-00-I006/17 and her study was also supported by Minigrant Edition II, contract number POWER6/2021/2ed, Adam Mickiewicz University, POWR.03.02.00-00- I006/17. The work of MM was supported by National Science Centre, Poland, Grant No. 2021/43/D/NZ8/00344 and Grant No. 1220/146/2021 from the Small Grants Programme of the University of Gdansk (i.e., UGrants-first competition). The authors also wish to thank Cambridge Proofreading LLC (http://proofreading.org) for their linguistic assistance.

Author contributions

Conceptualization, P.K. and Ł.K.; data curation, P.K.; sample collection, Ł.S.; formal analysis, P.K., D.S., M.M. and Ł.K.; investigation, P.K., D.S., Ł.S., M.M., M.G. and Ł.K.; methodology, P.K., D.S., M.M. and Ł.K.; supervision, Ł.K.; validation, P.K., D.S., M.M. and Ł.K.; visualization, P.K. and M.G.; writing—original draft, P.K., D.S., M.M. and Ł.K.; writing—review and editing, All authors reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-023-28714-w.

Correspondence and requests for materials should be addressed to P.K.

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Supplementary Information 1.

Super Clade II Phylogeny

Mr Bayes

Best partitioning scheme
Scheme Name : start_scheme
Scheme lnL : -23012.6773071
Scheme AIC : 46331.3546143
Number of params : 153
Number of sites : 2950
Number of subsets : 6

Subset	Best Model	# sites	subset id	Partition names
1	GTR+I+G	994	2889c2fb1bf9f0caa22cc409057d23b1	18S
2	GTR+I+G	811	bfd3f838ea65ae9d114a3ad078eff508	28S
3	GTR+G	220	8a568435a1eacd075784355505a11263	COI_pos2
4	SYM+I+G	219	4678c624c3e9ba568220c1842f1eb93d	COI_pos3
5	GTR+I+G	219	2a109f3301b5e0638b9230c437f23295	COI_pos1
6	K80+I+G	487	6a2d17c31cf1c70a30d027d73033e9b8	ITS2

Maximum Likelihood:

Best partitioning scheme
Scheme Name : start_scheme
Scheme lnL : -23009.5776978
Scheme AIC : 46347.1553955
Number of params : 164
Number of sites : 2950
Number of subsets : 6

Subset	Best Model	# sites	subset id	Partition names
1	GTR+I+G	994	2889c2fb1bf9f0caa22cc409057d23b1	18S
2	GTR+I+G	811	bfd3f838ea65ae9d114a3ad078eff508	28S
3	GTR+I+G	220	8a568435a1eacd075784355505a11263	COI_pos2
4	GTR+I+G	219	4678c624c3e9ba568220c1842f1eb93d	COI_pos3
5	GTR+I+G	219	2a109f3301b5e0638b9230c437f23295	COI_pos1
6	GTR+I+G	487	6a2d17c31cf1c70a30d027d73033e9b8	ITS2

Scheme Description in PartitionFinder format
Scheme_start_scheme = (18S) (28S) (COI_pos2) (COI_pos3) (COI_pos1) (ITS2);

COI Paramacrobiotus Phylogeny

Maximum Likelihood:

Best partitioning scheme
Scheme Name : start_scheme
Scheme lnL : -7977.70068359
Scheme AICc : 16502.7892982
Number of params : 193
Number of sites : 658
Number of subsets : 3

Subset | Best Model | # sites | subset id | Partition names

1 | GTR+I+G | 220 | 787c222c3fd320008b879c06120a62c3 | Gene1_pos1

2 | GTR+I+G | 219 | fa4964d2cabb811ad402226154a49982 | Gene1_pos2

3 | GTR+I+G | 219 | 221348b558ce91aa7bdc154b4945821e | Gene1_pos3

Scheme Description in PartitionFinder format
Scheme_start_scheme = (Gene1_pos1) (Gene1_pos2) (Gene1_pos3);

Saturation test with DAMBE

ITS-2 alignment

```
Test of substitution saturation (Xia et al. 2003; Xia and Lemey 2009)

Analysis performed on all sites.

Testing whether the observed Iss is significantly lower than Iss.c.
  IssSym is Iss.c assuming a symmetrical topology.
  IssAsym is Iss.c assuming an asymmetrical topology.

NumOTU       Iss  Iss.cSym       T    DF        P Iss.cAsym       T    DF        P
================================================================================
=======
     4     0,626     0,795   5,150   486   0,0000     0,760   4,106   486   0,0000
     8     0,698     0,750   1,178   486   0,2396     0,639   1,325   486   0,1857
    16     0,782     0,718   1,121   486   0,2629     0,509   4,801   486   0,0000
    32     0,870     0,701   2,437   486   0,0152     0,376   7,121   486   0,0000
Note: two-tailed t-tests are used.

================================================================================
=======
Interpretation of results:
               Significant Difference
               ----------------------
               Yes                 No
```

```
-----------------------------------------------------
Iss < Iss.c    Little             Substantial
saturation         saturation
-----------------------------------------------------
Iss > Iss.c    Useless            Very poor
sequences          for phylogenetics
-----------------------------------------------------------------
```

COI alignment

```
Test of substitution saturation (Xia et al. 2003; Xia and Lemey 2009)

Analysis performed on all sites.

Testing whether the observed Iss is significantly lower than Iss.c.
  IssSym is Iss.c assuming a symmetrical topology.
  IssAsym is Iss.c assuming an asymmetrical topology.
```

NumOTU	Iss	Iss.cSym	T	DF	P	Iss.cAsym	T	DF	P
4	0,370	0,805	18,372	657	0,0000	0,774	17,058	657	0,0000
8	0,385	0,766	13,335	657	0,0000	0,656	9,496	657	0,0000
16	0,407	0,744	10,319	657	0,0000	0,535	3,896	657	0,0001
32	0,428	0,718	7,937	657	0,0000	0,392	0,987	657	0,3239

```
Note: two-tailed t-tests are used.

Interpretation of results:
               Significant Difference
               ----------------------
               Yes                 No
-----------------------------------------------------
Iss < Iss.c    Little             Substantial
saturation         saturation
-----------------------------------------------------
Iss > Iss.c    Useless            Very poor
sequences          for phylogenetics
-----------------------------------------------------------------
```

Supplementary Information 2.

	ASAP	bPTP_BI	bPTP_ML
OP394113_MD50.1	1	15	15
OP394114_MD.50.4	1	15	15
MH676014_Paramacrobiotus_sp._PT.048	1	15	15
MH676004_Paramacrobiotus_sp._FR.077	1	15	15
MH676015_Paramacrobiotus_sp._PT.048	1	15	15
MH675999_Paramacrobiotus_sp._AU.044	1	15	15
MH676016_Paramacrobiotus_sp._TN.014	1	15	15
MH676003_Paramacrobiotus_sp._FR.077	1	15	15
OK662996_Paramacrobiotus_spatialis	2	11	11
MK041001_Paramacrobiotus_spatialis	2	11	11
MK040998_Paramacrobiotus_spatialis	2	11	11
MK040997_Paramacrobiotus_spatialis	2	11	11
MK040996_Paramacrobiotus_spatialis	2	11	11
MK040995_Paramacrobiotus_spatialis	2	11	11
MK041000_Paramacrobiotus_spatialis	2	11	11
MK040999_Paramacrobiotus_spatialis	2	11	11
OK662995_Paramacrobiotus_richtersi	3	8	8
MK040994_Paramacrobiotus_richtersi	3	8	8
MK040993_Paramacrobiotus_richtersi	3	8	8
MK040992_Paramacrobiotus_richtersi	3	8	8
LC637242_Paramacrobiotus_metropolitanus	4	2	2
LC649796_Paramacrobiotus_metropolitanus	4	2	2
MT731035_Paramacrobiotus_sp._ZY-2020	5	7	7
MN964282_Paramacrobiotus_fairbanksi	6	9	9
MN964281_Paramacrobiotus_fairbanksi	6	9	9
MN961616_Paramacrobiotus_fairbanksi	6	9	9
MH676011_Paramacrobiotus_fairbanksi	6	9	9
KU513421_Paramacrobiotus_cf._richtersi_DS-2016	6	9	9
MH676012_Paramacrobiotus_fairbanksi	6	9	9

MK041011_Paramacrobiotus_fairbanksi	6	9	9
MK041010_Paramacrobiotus_fairbanksi	6	9	9
MK041009_Paramacrobiotus_fairbanksi	6	9	9
MK041008_Paramacrobiotus_fairbanksi	6	9	9
MK041007_Paramacrobiotus_fairbanksi	6	9	9
MK041006_Paramacrobiotus_fairbanksi	6	9	9
MK041003_Paramacrobiotus_fairbanksi	6	9	9
EU244597_Paramacrobiotus_sp._richtersi_group_1	6	9	9
AY598779_Paramacrobiotus_richtersi	6	9	9
FJ435808_Paramacrobiotus_richtersi_group_sp._NG-2008	6	9	9
AY598778_Paramacrobiotus_richtersi	6	9	9
MK041004_Paramacrobiotus_fairbanksi	6	9	9
MK041005_Paramacrobiotus_fairbanksi	6	9	9
FJ435809_Paramacrobiotus_richtersi_group_sp._NG-2008	6	9	9
MT260373_Paramacrobiotus_filipi	7	1	1
MT260372_Paramacrobiotus_filipi	7	1	1
MH676018_Paramacrobiotus_tonollii	8	12	12
MH676017_Paramacrobiotus_sp.	9	21	21
EU244599_Paramacrobiotus_sp._richtersi_group_3	9	24	24
EU244598_Paramacrobiotus_sp._richtersi_group_2	9	25	25
MH676013_Paramacrobiotus_sp.	10	19	19
MH676007_Paramacrobiotus_sp.	10	20	20
MH676010_Paramacrobiotus_sp.	11	14	14
MH676009_Paramacrobiotus_sp.	12	18	18
MH676008_Paramacrobiotus_sp.	13	4	4
MN097836_Paramacrobiotus_experimentalis	13	4	4
MN097837_Paramacrobiotus_experimentalis	13	4	4
MH676006_Paramacrobiotus_sp.	14	16	16
MH676005_Paramacrobiotus_sp.	14	16	16
MH676002_Paramacrobiotus_sp.	15	13	13
MH676001_Paramacrobiotus_sp.	15	13	13
MH676000_Paramacrobiotus_sp.	15	13	13
MH675998_Paramacrobiotus_areolatus	16	6	6

MK041022_Paramacrobiotus_aff._arduus_Ca1_Guidetti_et_al._2019	17	28	28
MK041021_Paramacrobiotus_arduus	17	27	27
MK041020_Paramacrobiotus_arduus	17	27	27
MK041019_Paramacrobiotus_celsus	18	17	17
MK041018_Paramacrobiotus_celsus	18	17	17
MK041017_Paramacrobiotus_celsus	18	17	17
MK041016_Paramacrobiotus_depressus	19	22	22
MK041015_Paramacrobiotus_depressus	19	23	23
MK041014_Paramacrobiotus_depressus	19	28	28
MK041012_Paramacrobiotus_depressus	19	28	28
MK041013_Paramacrobiotus_depressus	19	29	29
MK041002_Paramacrobiotus_aff._spatialis_Ca1_Guidetti_et_al._2019	20	10	10
MF568534_Paramacrobiotus_lachowskae	21	5	5
KF788257_Paramacrobiotus_richtersi_group_sp._1_MAC-2014	22	3	3
KF788256_Paramacrobiotus_richtersi_group_sp._1_MAC-2014	22	3	3
KF788255_Paramacrobiotus_richtersi_group_sp._1_MAC-2014	22	3	3
KF788254_Paramacrobiotus_richtersi_group_sp._1_MAC-2014	22	3	3
KF788253_Paramacrobiotus_richtersi_group_sp._1_MAC-2014	22	3	3
KF788252_Paramacrobiotus_richtersi_group_sp._1_MAC-2014	22	3	3
KF788251_Paramacrobiotus_richtersi_group_sp._1_MAC-2014	22	3	3
Total number of species	**22**	**29**	**29**

SEQ_Name nucleotides

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AATTGACTTGTCCCTTTAATAATTGGAGCCCCTGATATAGCATTTCCTCGAATAAATAAT
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OP394114_MD.50.4

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Supplementary Information 3.

https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-023-28714-w/MediaObjects/41598_2023_28714_MOESM3_ESM.xlsx

Paper II

Kayastha, P., Szydło, W., Mioduchowska, M. and Kaczmarek, Ł. Morphological and genetic variability in cosmopolitan tardigrade species - *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf, 2010. (In review Scientific Reports) https://doi.org/10.21203/rs.3.rs-2736709/v1

Morphological and genetic variability in cosmopolitan tardigrade species - Paramacrobiotus fairbanksi Schill, Förster, Dandekar & Wolf, 2010

Pushpalata Kayastha (pushpalata.kayastha@gmail.com)
Adam Mickiewicz University, Poznań
Wiktoria Szydło
Adam Mickiewicz University, Poznań
Monika Mioduchowska
University of Gdańsk
Łukasz Kaczmarek
Adam Mickiewicz University, Poznań

Article

Keywords:

Posted Date: July 17th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2736709/v2

Additional Declarations: No competing interests reported.

Morphological and genetic variability in cosmopolitan tardigrade species - *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf, 2010

Pushpalata Kayastha[1*], Wiktoria Szydło[2,3], Monika Mioduchowska[4] and Łukasz Kaczmarek[1]

[1]Department of Animal Taxonomy and Ecology, Faculty of Biology, Adam Mickiewicz University, Poznań, Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland; kaczmar@amu.edu.pl

[2]Center for Advanced Technology, Adam Mickiewicz University in Poznań, Uniwersytetu Poznańskiego 10, 61-614 Poznań, Poland; wiktoria.szydlo@amu.edu.pl

[3]Population Ecology Lab, Faculty of Biology, Adam Mickiewicz University, Poznań, Poland

[4]Department of Evolutionary Genetics and Biosystematics, Faculty of Biology, University of Gdańsk, Poland; e-mail: monika.mioduchowska@ug.edu.pl

*Correspondence: pushpalata.kayastha@gmail.com

Abstract: *Paramacrobiotus fairbanksi* was described from Alaska (USA) based on integrative taxonomy and later reported from various geographical locations making it a true cosmopolitan species. The 'Everything is Everywhere' (EiE) hypothesis assumes that microscopic organisms have unique features that help them to inhabit many different environments, meaning they can be considered cosmopolitan. In the present work we report four new populations of *Pam. fairbanksi* from the Northern Hemisphere which suggests that the 'EiE' hypothesis is true, at least for some tardigrade species. We also compared all known populations of *Pam. fairbanksi* at the genetic and morphological levels. The p-distances between COI haplotypes of all sequenced *Pam. fairbanksi* populations from Albania, Antarctica, Canada, Italy, Madeira, Mongolia, Spain, USA and Poland ranged from 0.002% to 0.005%. In total, twelve haplotypes (H1-H12) of COI gene fragments were identified. We also report statistically significant morphometrical differences of species even though they were cultured and bred in the same laboratory conditions, and propose epigenetic factor as a main cause rather than temperature, predation risk and food availability. Furthermore, we also discuss differences in the potential distribution of two *Paramacrobiotus* species.

Keywords: cosmopolitism, dispersal, 'Everything is Everywhere' hypothesis, Tardigrada, water bears, zoogeography

Introduction

The Phylum Tardigrada currently consists of *ca*. 1,500 species[1] that inhabit terrestrial and aquatic environments throughout the world[2]. Currently there are 33 families, 159 genera, 1464 species and 21 additional subspecies within this phylum[1]. *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf, 2010[3] was described from Alaska (USA) and reported from the Antarctic, Italy, Poland and Spain[4] (reported as *Macrobiotus richtersi* Murray, 1911[5])[6,7,8,9]. It is a large-size (up to 800 µm) parthenogenetic *Paramacrobiotus* found mostly in mosses and can be shortly characterized by white or transparent cuticle without pores, three bands of teeth in the oral cavity, three macroplacoids and microplacoid in pharynx (*richtersi* group), smooth lunules under all claws, granulation on all legs, and eggs with reticulated conical processes without caps or spines. *Pam. fairbanksi* is a triploid species[8] inhabiting various locations throughout the globe. The species is an omnivore, i.e., it feeds on algae, cyanobacteria, fungi, nematodes and rotifer[10]. However, dietary preferences have been observed to differ between juveniles and adults (juveniles prefer green alga and adults favour rotifers and nematodes[10]).

The 'Everything is Everywhere' hypothesis, which was proposed at the beginning of the 20th century[11,12] suggests that microorganisms and small invertebrates should have a cosmopolitan distribution. Microscopic organisms are often considered cosmopolitan species, as, the presence of specific adaptations allows them to inhabit most environments. These adaptations include a) the possibility of easy passive dispersion (by wind, rivers, sea currents, other animals, etc.), b) the presence of very resistant spore stages (which include cysts, eggs or cryptobiotic individuals) that help to survive extreme conditions, and c) the presence of asexual or parthenogenetic reproduction, allowing for rapid increase in the number of individuals[12,13,14,15,16]. Cosmopolitism was strongly suggested for many tardigrade species in the past, however, the suggestion was later undermined (e.g. ref [17,18,19]). At present, we have strong and compelling evidence of a wide distribution of some tardigrade taxa, which means that we return to the concept of cosmopolitism of at least some species of tardigrades (e.g. ref[8,9,20,21,22]) which can support the hypothesis 'Everything is Everywhere' (EiE) for tardigrades. According to Gąsiorek et al[21], "a species may be termed as cosmopolitan if it was recorded in more than one zoogeographic realm". There are four tardigrade species known from more than one zoogeographic realm, i.e., *Echiniscus testudo*[23] (Doyère, 1840), *Milnesium inceptum*[24] Morek, Suzuki, Schill, Georgiev, Yankova, Marley & Michalczyk, 2019, *Pam. gadabouti*[22] Kayastha, Stec, Mioduchowska and Kaczmarek. 2023 and *Pam. fairbanksi*. Furthermore, two parthenogenetic species from the genus *Paramacrobiotus*, i.e., *Pam.*

fairbanksi and *Pam. gadabouti*, are contenders as they show a wide distribution that supports the hypothesis EiE.

In the present paper we compare different populations of *Pam. fairbanksi* from all known localities of this species in Albania, the Antarctic, Canada, Italy, Mongolia, Poland, Portugal (Madeira) and the USA. We also discuss genetic and morphological differences between them and consider the general distribution of *Pam. fairbanksi*.

Materials and Methods

Sample processing

Four moss samples from trees and rocks were collected in 2018 (Mongolia) and 2019 (Albania, Canada and Madeira) (for details, see Table 1, Figure 1). The samples were packed in paper envelopes, dried at room temperature and delivered to the laboratory at the Faculty of Biology, Adam Mickiewicz University in Poznań, Poland. Tardigrades were extracted from the samples and studied following the protocol of Stec et al.[25]. The moss samples (Alb, CN8, M85 and MN01) were dried post extractions and were deposited at the Department of Animal Taxonomy and Ecology, Institute of Environmental Biology, Adam Mickiewicz University in Poznań, Uniwersytetu Poznańskiego 6, 61–614 Poznań, Poland.

Additionally, we used morphometric and genetic data of *Pam. fairbanksi* populations from the Antarctic, Italy, Spain, the USA and Poland[9].

Table 1. Studied populations of *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf, 2010[3] (see also Figure 1).

Sample No	Coordinates	Locality and sample description	Remarks
1	41°19’36”N, 19°49’08”E; 112 m asl	Albania, Tirana County, Tirana, near Bunk’Art 2; moss on tree	Present study
2	*ca.* 67°39′S, 46°09′E; 0 m asl	Antarctic, near Vechernia Mt Base; moss (*Ceratodon purpureus*)	Kaczmarek et al.[9]
3	51°24’21”N, 116°14’27”W; 1900 m asl	Canada, Alberta, Banff National Park, near east end of the Louise Lake; moss on stone	Present study
4	*ca.* 44°26’N, 10°51’E; 510 m asl	Riccò, Modena Province, Italy; beech leaf litter	Kaczmarek et al.[9]

5	32°44’37.3”N, 16°54’14.4”W; 710 m asl	Portugal, Madeira, Ribera de Brava; moss on rock	Present study
6	47°49’57.0”N, 107°31’26.8”E; 1 432 m asl	Mongolia, Töv Province; moss on rocky hill	Roszkowska et al.[26]
7	50°03’44”N, 19°57’26”E; 205 m asl	Poland, Lesser Poland Province, Kraków, Jagiellonian University Botanical Garden, Kopernika 27 street; moss on tree	Kaczmarek et al.[9]
8	40°52′42″N, 03°50′45″W; asl	Spain, Madrid; litter, oaks	Guil and Giribet[6]
9	*ca.* 64°50’N, 147°43’E; 135 m asl	USA, Alaska, Fairbanks; moss	Kaczmarek et al.[9]

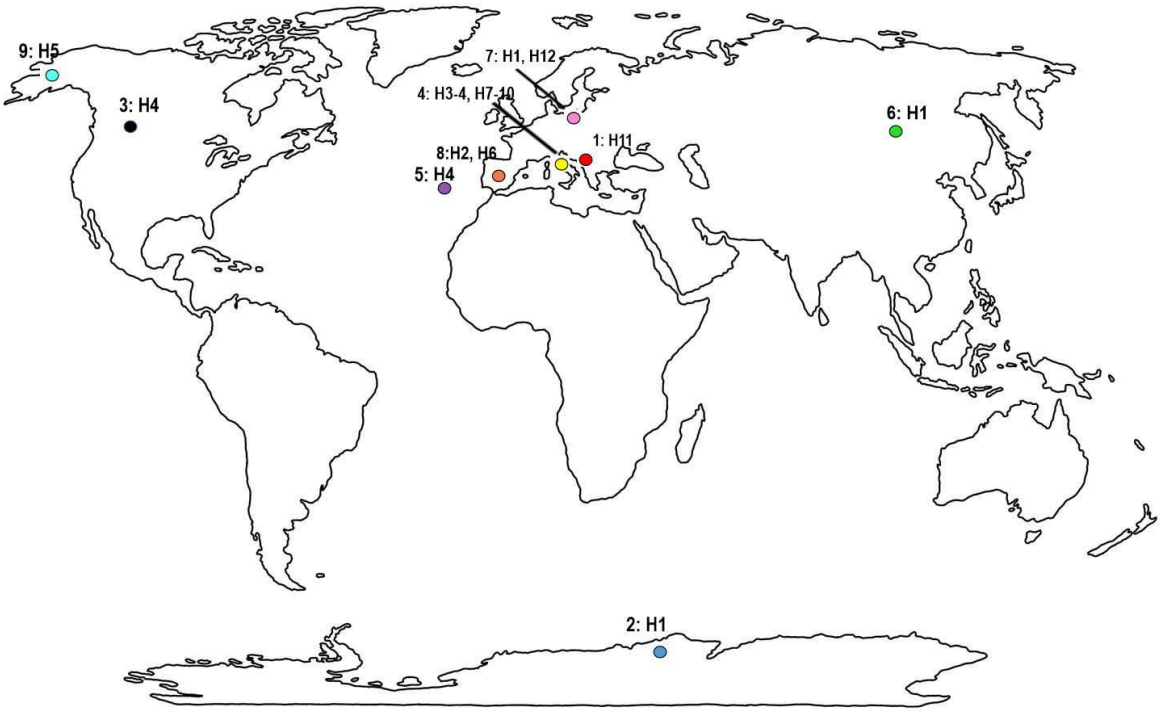

Figure 1. A World map with indicated sample number from Table 1 along with haplotypes of *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf 2010[3] found in different localities (see also Figure 8). The world map is from https://www.wpmap.org/blank-world-map-with-antarctica/blank-world-map-jpg/ and the figure was prepared in Corel Photo-Paint 2021.

Culture procedure

Specimens of the populations from Albania, Canada, Madeira and Mongolia were cultured in the Department of Animal Taxonomy and Ecology (Faculty of Biology, Adam Mickiewicz University in Poznań) according to the protocol described by Roszkowska et al.[26]. In summary, tardigrades were cultured in small Petri dishes in spring water mixed with distilled water (1:3) with the rotifers and nematodes added as food *ad libitum*. All cultures were kept in the environmental chamber at a temperature of 18°C and in darkness.

Microscopy, morphometrics and morphological nomenclature

Specimens were extracted from cultures and prepared for light microscopy analysis. They were mounted on microscope slides in a small drop of Hoyer's medium and secured with a cover slip[27,28]. Slides were then placed in an incubator and dried for two days at *ca.* 60°C. Dried slides were sealed with transparent nail polish and examined under an Olympus BX41.

All measurements are given in micrometers [µm]. Structures were measured only if their orientation was suitable. Body length was measured from the anterior extremity to the end of the body, excluding the hind legs. Buccal tubes, claws and eggs were measured according to Kaczmarek & Michalczyk[29]. Macroplacoid length sequence is given according to Kaczmarek et al.[30]. The *pt* ratio is the ratio of the length of a given structure to the length of the buccal tube, expressed as a percentage[31]. The *pt* values always provided in italics. Morphometric data were handled using the "Parachela" ver. 1.8 template available from the Tardigrada Register[32]. Tardigrade taxonomy follows Bertolani et al.[33]. Genus abbreviations follow Perry et al.[34].

Genotyping

Before genomic DNA extraction, each specimen of *Pam. fairbanksi* was identified *in vivo* using light microscopy (LM). To obtain voucher specimens, DNA extractions were made from individuals using a Chelex® 100 resin (Bio-Rad) extraction method provided by Casquet et al.[35] with modifications described in Stec et al.[25]. We sequenced three molecular markers, which differ in effective mutation rates: two nuclear fragments (18S rRNA and 28S rRNA) and one mitochondrial fragment (COI). All DNA fragments were amplified according to the protocols described in Kaczmarek et al.[9], with primers listed in Table 2. Alkaline phosphatase FastAP (1 U/µl, Thermo Scientific) and exonuclease I (20 U/µl, Thermo Scientific) were used to clean the PCR products. Sequencing in both directions was carried out using the BigDyeTM terminator cycle sequencing method and ABI Prism 3130xl Genetic Analyzer (Life Technologies).

Table 2. Primers with their original references used for sequencing of three molecular markers of *Paramacrobiotus fairbanksi*.

DNA molecular marker	Primer name and direction	Primer sequence (5'-3')	Source
COI	LCO1490 (forward)	GGTCAACAAATCATAAAGATATTGG	36
	HCO2198 (reverse)	TAAACTTCAGGGTGACCAAAAAATCA	
18S rRNA	SSU01_F (forward)	AACCTGGTTGATCCTGCCAGT	37
	SSU82_R (reverse)	TGATCCTTCTGCAGGTTCACCTAC	
28S rRNA	28SF0001 (forward)	ACCCvCynAATTTAAGCATAT	38
	28SR0990 (reverse)	CCTTGGTCCGTGTTTCAAGAC	

Molecular data analysis

The amplified nuclear and mitochondrial barcode sequences were edited using the BIOEDIT software[39]. Comparison of obtained molecular markers with those deposited in GenBank and homology search were performed using BLAST application (Basic Local Alignment Search Tool[40]). The COI haplotypes were generated using the DnaSP v5.10.01 program[41] and were translated into amino acid sequences using the EMBOSS-TRANSEQ application[42] to check for internal stop codons and indels. Then all sequences obtained in our study, and the sequences downloaded from the GenBank database as originating from *Pam. fairbanksi*, were aligned with CLUSTALW using default settings. Alignment sequences were trimmed to 689, 572 and 574 bp for 28S rRNA, 18S rRNA and COI barcodes, respectively. The calculation for the uncorrected pairwise distances (p-distances) was performed for COI sequences using the MEGA X[43].

All obtained sequences have been deposited in GenBank (for the accession numbers please see Table 3). The slides prepared from exoskeleton/voucher after DNA extraction of *Pam. fairbanksi* were deposited at the Department of Animal Taxonomy and Ecology, Institute of Environmental Biology, Adam Mickiewicz University in Poznań, Uniwersytetu Poznańskiego 6, 61–614 Poznań, Poland.

Table 3. GenBank accession numbers of sequences obtained in the present study along with the slide numbers of voucher specimens.

Populations of *Paramacrobiotus fairbanksi*	GenBank accession number; bp long DNA molecules			Voucher numbers
	COI mtDNA	18S rRNA	28S rRNA	
Albania	ON911917-18; 623-678	ON872386; 1480	ON872380-81; 805	Alb2/S, Alb3/S, Alb4/S
Canada	ON911919;	ON872387;	ON872382;	CN8.2/S

	625	1480	793	
Madeira	ON911920-21;	ON872388;	ON872383;	M85.11/S,
	678-679	1547	744	M85.12/S
Mongolia	ON911922-23;	ON872389;	ON872384-85;	MNO101/S,
	687-689	917	694-711	MNO103/S

Reconstruction of genetic relationships among COI haplotypes and genealogical connections was carried out using the NETWORK 4.6.1.3 software (www.fluxuxengineering.com). The median-joining algorithm (MJ)[44] and substitution rates with the weight of 3 for transitions and 1 for transversions (transition: transversion ratio (ti:tv)) were applied. The star contraction pre-processing was generated to delete all superfluous median vectors and links. Additionally, the maximum parsimony post-processing was calculated. In turn, signatures of population expansion, equilibrium or decline in *Pam. fairbanksi* were inferred from the neutrality tests calculation (Tajima *D*[45] and Fu *Fs*[46], respectively) computed in the DnaSP v5.10.01 program and Arlequin v.3.5. software[47]. Analyses were performed with 1 000 replicates.

Statistical analysis

We used the Analysis of Variance (ANOVA) test with post hoc comparison of pairs of measurements, applying Bonferroni correction to statistically analyze the differences in morphometrics between different populations of *Pam. fairbanksi*. Measurements of the body and buccal tube length (BL and BTL, respectively) were used as the dependent and the populations as grouping variables. Normal distribution in residuals was checked using the Shapiro test. Other morphometric traits, i.e., stylet support insertion points (SSIP), external width of buccal tube (BTEW) and placoids (M1 – macroplacoid 1, M2 – macroplacoid 2, M3 – macroplacoid 3, Mi – microplacoid, MR – macroplacoid row, PR – placoid row) were also analysed. All the analyses were performed in R 4.1.3[49]. The level of statistical significance was considered at $p < 0.05$. In the case of post hoc tests, only statistically significant results were presented. Principal Component Analysis (PCA) was performed using the R script from Stec et al.[48]. The analysis was performed for data from eggs and animals. For animals, both absolute values (raw measurements in μm) (BLm, BTLm, SSIPm, BTEWm, M1m, M2m, M3m, Mim, MRm and PRm) and relative *pt* values (BL*pt*, SSIP*pt*, BTEW*pt*, M1*pt*, M2*pt*, M3*pt*, Mi*pt*, MR*pt* and PR*pt*) were used. For eggs, absolute values (raw measurements in μm) were used. All analyses were carried out using the R software program[49]. The "imputePCA" function of the R package "missMDA ver. 1.17" was used to impute missing data in the animal data set using the PCA imputation technique[50]. Cross-validation (function "estim_ncpPCA") was used

to determine the number of components utilized to impute the missing data. The PCA function of the software "FactoMineR ver. 2.3"[51] was used to perform PCAs on the scaled data. The software "ggplot2 ver. 3.3.2", "plyr ver. 1.8.6", and "gridExtra ver. 2.3" were used to depict PCAs[52,53]. The presence of a structure in the PCA data was tested using a randomization approach on the eigenvalues and statistics according to Björklund[54] and an in-house R script developed by MV in Stec et al.[48]. PERMANOVA was done on the PCs with the R packages "vegan ver. 2.5.6" and "pairwiseAdonis ver. 0.3"[55], with the species hypothesis generated by phylogenetic techniques as the independent variable. Using the Benjamini-Hochberg correction, the -level for multiple post hoc comparisons was adjusted independently for adult and egg[56]. In total, 106 tardigrade specimens (16 Albanian, 16 Antarctic, 17 Canadian, 15 Madeiran, 14 Mongolian, 15 Polish, 4 Italian and 9 Alaskan) were measured and later used in the analyses for animals. Furthermore, differences in egg morphology between populations were studied and tested using ANOVA. Egg bare diameter (EBD), full diameter (EFD) and processes height (PH) were characters for the populations used as the dependent variable to determine compared groups and Bonferroni corrections. In total, 100 tardigrade eggs (15 Albanian, 16 Antarctic, 15 Canadian, 15 Madeiran, 6 Mongolian, 15 Polish and 18 Alaskan) were measured and used in the analyses. All the analyses were performed in R 4.1.0. The level of statistical significance was considered at $p < 0.05$. Only statistically significant results were presented for post hoc tests.

Potential distribution of cosmopolitan Pam. fairbanksi and Pam. gadabouti

A map of the known distribution of *Pam. fairbanksi* populations was assembled in Corel Photo-Paint 2021.

An ecological niche modelling (ENM) approach was used to predict the current potential distribution of *Pam. fairbanksi* and *Pam. gadabouti*. The ENM was performed with the use of the Maxent algorithm, ver. 3.4.4.[48]. MaxEnt performs the model with the fewest possible occurrence data and takes presence-only (PO) data. The model generates models of habitat appropriateness by handling continuous and categorical variables using regularization parameters[57,58]. The raster package in R was used to extract climatic raster values, and for ENM evaluation, version 0.3.1 of ENMevaluate in R was used. The bioclimatic variables available in MERRAclim Dataset 19 were used as environmental variables for Maxent modelling. We used MERRAclim Dataset because it provides a global set of satellite-based bioclimatic variables that includes Antarctica, which is one of the locations for *Pam. fairbanksi*. The 19 global bioclimatic datasets from the 2000s at 5 arcminutes resolution (mean value) [59] consist of

temperature layers (BIO1-BIO11) and humidity layers (BIO12-BIO19). The temperature layers are in degrees Celsius multiplied by 10 and the humidity layers are in kg of water/kg of air multiplied by 100000[59]. The receiver operating characteristic (ROC) plot's area under curve (AUC) was used to assess the model's accuracy[57]. AUC describes the relationship between the proportion of correctly-anticipated presences and the proportion of absences of mistakenly-projected species in the model[60]. The AUC gauges the effectiveness of the model with a value between 0 and 1. Furthermore, AUC values > 0.9 indicate excellent accuracy, 0.7 to 0.9 indicate good accuracy, and values below 0.7 indicate low accuracy[57,61,62]. The jackknife test was used to estimate the model's variable relevance. The localities for *Pam. fairbanksi* are from Table 1 and *Pam. gadabouti* from Kayastha et al.[22]. The coordinate list is provided in SM.01 and the R script for ENM in SM.02.

3. Results

Morphometric comparison of different Pam. fairbanksi populations

No significant differences were shown by the ANOVA test performed on BL between the studied populations (df = 7; F = 7.832; p = 0.902; N = 106; Table 7, Table 9, Table 11, Table 13; Figure 2A). However, significant differences were found on BTL between different populations (df = 7; F = 5.633; p = 0.010; N = 106; Table 7, Table 9, Table 11, Table 13), where the buccal tube of the specimens from Mongolia was significantly longer than in specimens from the Albanian and Canadian populations (p = 0.002 and p = 0.005 respectively; Figure 2B). The buccal tube of the specimens from Madeira was significantly longer than in specimens from the Polish population (p = 0.003; Figure 2B). Analysis for SSIP length showed significant differences as well (df = 7; F = 4.812; p = 0.016; N = 106; Figure 2C), with the specimens in the Polish population having significantly lower SSIP than in specimens from the Antarctic (p = 0.048), Madeiran (p = 0.038), and Mongolian (p = 0.023) populations. Analysis of M2 length showed significant differences as well (df = 7; F = 8.48; p = 0.020; N = 106; Table 7, Table 9, Table 11, Table 13; Figure 2D).

The ANOVA test showed, however, no statistical significance for *pt* of BL between populations (df = 7; F = 8.056; p = 0.678; N = 106; Table 7, Table 9, Table 11, Table 13; Figure 3A). The ANOVA test for *pt* values of the SSIP showed no statistically significant differences between studied populations (df = 7; F = 20.81; p = 0.112; N = 106; Table 7, Table 9, Table 11, Table 13; Figure 3A) whereas the ANOVA test for *pt* values of the BTEW showed differences between studied populations (df = 7; F = 9.87; p = 0.0.001; N = 106; Table 7, Table 9, Table

11, Table 13; Figure 3B). The *pt* values specimens from the Italian population were higher than the Canadian population (p=0.042) and the Polish population (p=0.004), while *pt* values of specimens from the Madeiran population were higher than the Polish population (p=0.003) and *pt* values of specimens from the Alaskan population were higher than the Madeiran population (p=0.018) and the Mongolian population (p=0.0001) (Figure 3B). The ANOVA test for *pt* values of the M1(df = 7; F = 8.38; p = 0.0.007; N = 106; Table 7, Table 9, Table 11, Table 13; Figure 3C)and M3 (df = 7; F = 14.53; p = 0.001; N = 106; Table 7, Table 9, Table 11, Table 13; Figure 3D) showed differences between studied populations.

The ANOVA performed on EFD measurements of eggs (df = 6; F = 22.92; p = 0.002; N = 100) showed significant differences between all the populations (Table 8, Table 10, Table 12, Table 14). Eggs in the Polish population were significantly smaller than those from Antarctica (p = 0.068) and Canada (p = 0.003), and, eggs from the Alaskan population were clearly smaller than those from Canada (p = 0.008) (Figure 4A). Analysis of EBD values, however, showed no statistically important differences between eggs in different populations (df = 6; F = 9.192; p = 0.249; n = 100; Table 8, Table 10, Table 12, Table 14; Figure 4B). There were also no statistically important differences between the studied populations (Table 8, Table 10, Table 12, Table 14) in the size of egg processes (PH) (df = 6; F = 24.42; p = 0.260; n = 100; Figure 4C).

The randomization test in PCA demonstrated that only the first two PCs explained greater variation than was anticipated by the null model (no data structure) for both animal and egg datasets (SM.11). As a result, only the initial two PCs were maintained and used for additional investigation and interpretation. Furthermore, the ψ and ϕ statistics of the PCA were significantly distinct from what they anticipated under the null assumption (animals: ψ=60.72 p<0.001, ϕ=0.82 p<0.001; animals *pt*: ψ=13.14 p<0.001, ϕ=0.43 p<0.001; eggs: ψ=17.62 p<0.001, ϕ=0.50 p<0.001). The first two components of the PCA of animals' absolute measured value (Figure 5) explained 90% of the overall variation (83.7% for PC1 and 6.7% for PC2) and for animals' *pt* indices (Figure 6) explained 65% of the overall variation (46.3% for PC1 and 18.7% for PC2). PCA of egg measurements (Figure 7) described 68% of the total variance with the first two components (52.5% for PC1 and 15.5% for PC2). PERMANOVA revealed that species identity has a substantial overall effect on PCs (p<0.001, Table 4, Table 5, Table 6). Raw morphometric data for all the populations in the present study are given in the Supplementary Materials (SM.03-SM.06). R script for single characters as well as measurement files for both adults and eggs, are provided in the Supplementary Materials (SM.07-SM.09).

All the test results from R are provided in Supplementary Materials (SM.10). Results of PCA randomization tests in the Supplementary Materials (SM.11).

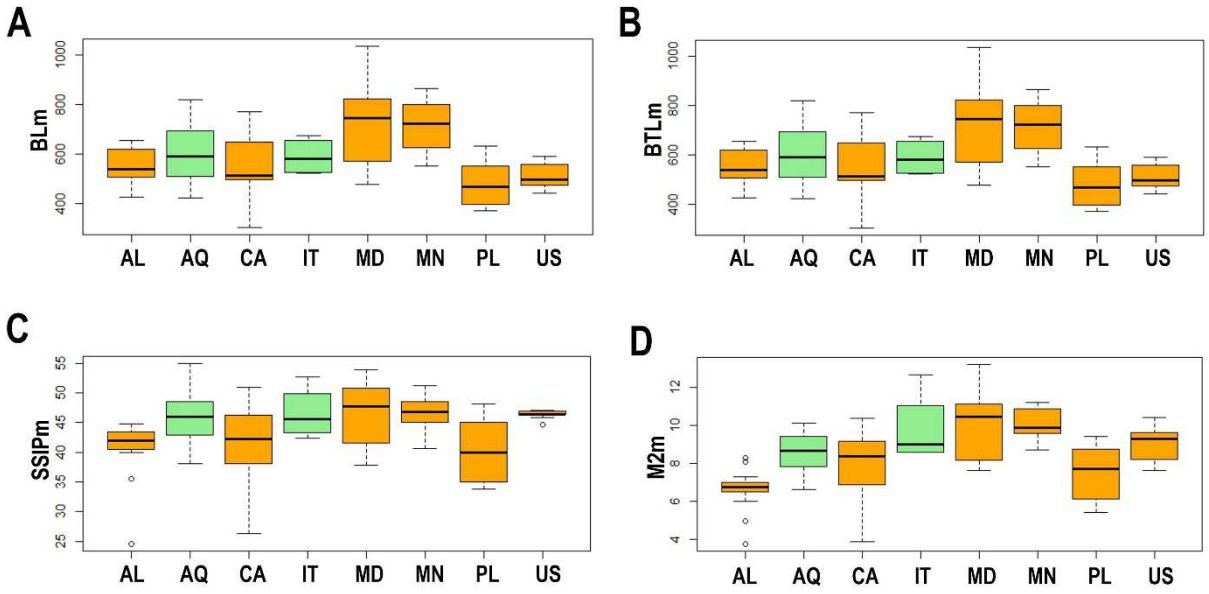

Figure 2. A – Differences in the body length (BLm); B – differences in the buccal tube length (BTLm); C – differences in the stylet support insertion point (SSIPm); D – differences in the Macroplacoid 2 length (M2m). The studied populations of *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf 2010 are AL – Albania; AQ – Antarctic; CA – Canada; IT – Italy; MD – Madeira; MN – Mongolia; PL – Poland; US – USA. Minimum, maximum, median, first quartile and third quartile for each population are presented. All measurements are in micrometres [µm]. Orange boxplots represent cultured population and green boxplots represent wild population.

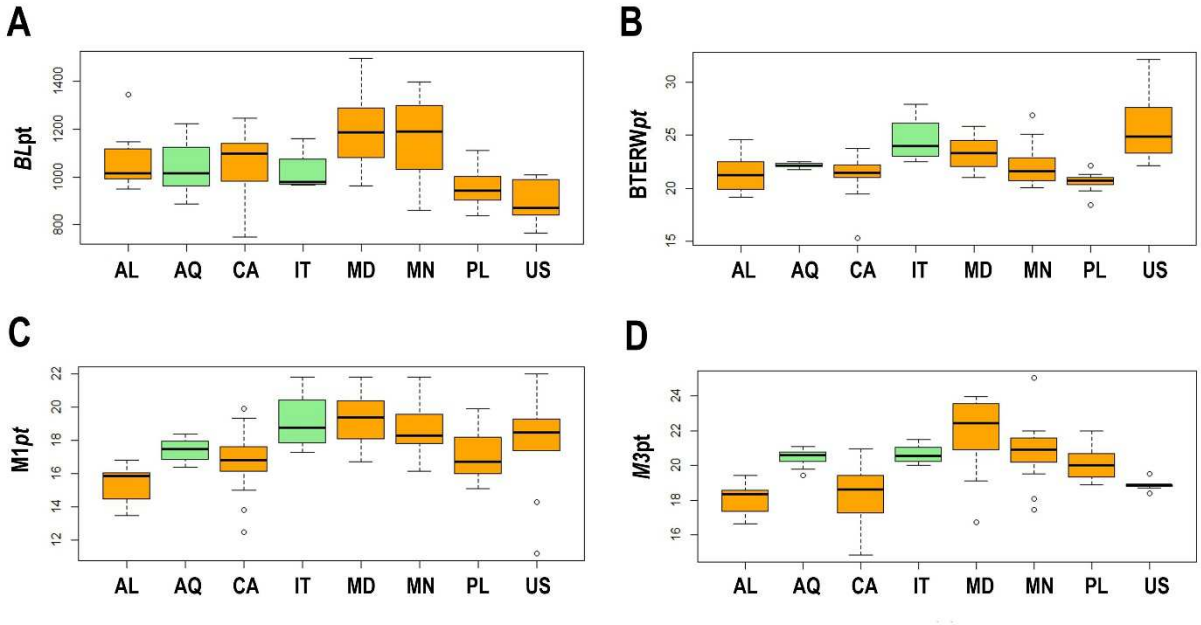

Figure 3. A – Differences in the *pt* body length (BL*pt*); B – differences in the *pt* of external width of buccal tube (BTEW*pt*); C – differences in the *pt* of Macroplacoid 1 (M1*pt*); D – differences in the *pt* of Macroplacoid 3 (M3*pt*). The studied populations of *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf 2010 are AL – Albania; AQ – Antarctic; CA – Canada; IT – Italy; MD – Madeira; MN – Mongolia; PL – Poland; US – USA. Minimum, maximum, median, first quartile and third quartile for each population are presented. Orange boxplots represent cultured population and green boxplots represent wild population.

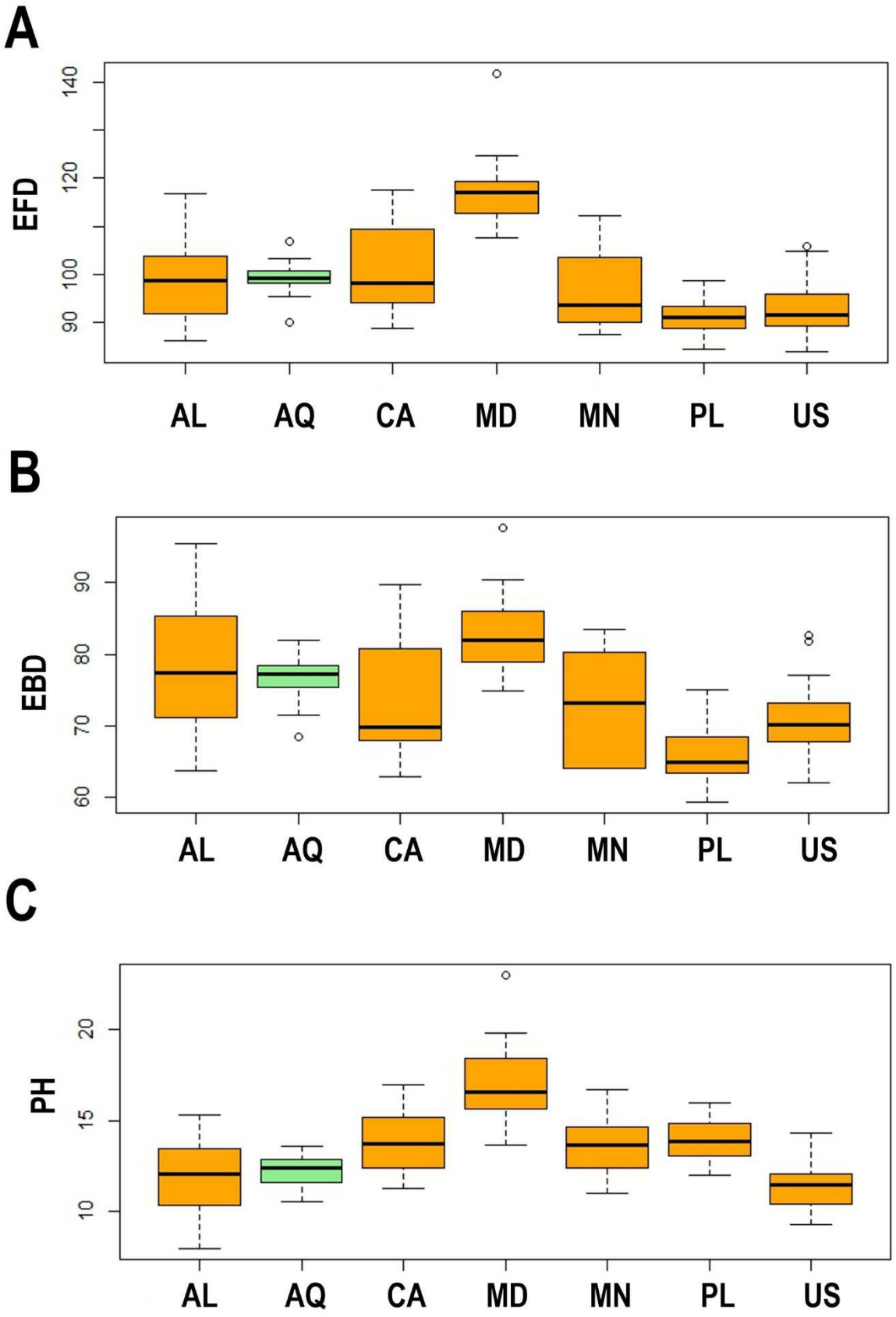
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Figure 4. A – Differences in the egg full diameter (EFD); B – differences in the egg bare diameter (EBD); C – differences in the egg processes height (PH). The studied populations of *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf 2010 are AL – Albania; AQ – Antarctic; CA – Canada; MD – Madeira; MN – Mongolia; PL – Poland; US – USA. Minimum, maximum, median, first quartile and third quartile for each population are presented. All measurements are in micrometres [µm]. Orange boxplots represent cultured population and green boxplots represent wild population.

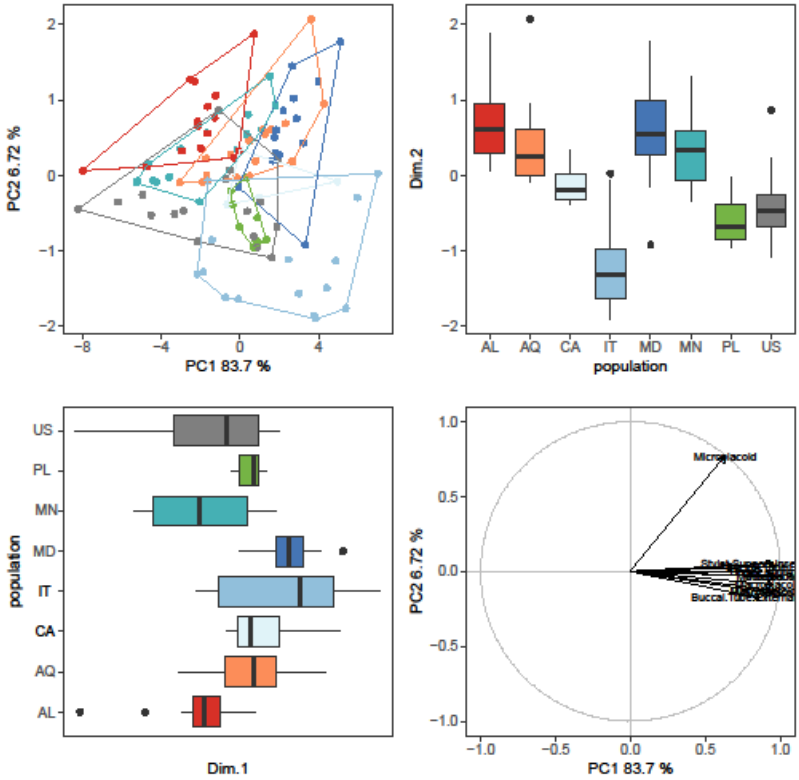


Figure 5. Results of PCA for animal measurements, 1st and 2nd Principal Components. Score scatterplots presented in top-left quadrants; boxplots of single component scores presented in top-right and bottom-left quadrants and loading plot presented in bottom-right.

Table 4. Results of PERMANOVA and post hoc pairwise PERMANOVA comparisons for the first two principal components (PC1 and PC2) of animal measured values; significant post hoc p-values adjusted with the Benjamini-Hochberg correction.

***Post hoc* comparisons**	**df**	**SS**	**F**	**R^2**	**P**
Poland vs Italy	1	33.92	4.98	0.23	0.068
Poland vs USA	1	36.99	8.09	0.27	0.019
Poland vs Antarctica	1	49.69	8.77	0.23	0.017
Poland vs Albania	1	0.98	0.17	0.01	0.704

Poland vs Canada	1	5.39	0.65	0.02	0.485
Poland vs Madeira	1	144.31	16.56	0.37	0.003
Poland vs Mongolia	1	143.25	31.67	0.54	0.001
Italy vs USA	1	2.71	1.29	0.11	0.344
Italy vs Antarctica	1	2.50	0.52	0.03	0.502
Italy vs Albania	1	37.72	7.93	0.31	0.019
Italy vs Canada	1	26.61	2.96	0.13	0.145
Italy vs Madeira	1	6.09	0.62	0.04	0.496
Italy vs Mongolia	1	5.94	2.13	0.12	0.207
USA vs Antarctica	1	6.00	1.92	0.08	0.214
USA vs Albania	1	44.90	14.65	0.39	0.002
USA vs Canada	1	23.65	3.64	0.13	0.098
USA vs Madeira	1	18.70	2.72	0.11	0.150
USA vs Mongolia	1	30.82	21.91	0.51	0.001
Antarctica vs Albania	1	55.76	12.46	0.29	0.003
Antarctica vs Canada	1	42.92	6.06	0.16	0.033
Antarctica vs Madeira	1	39.28	5.29	0.15	0.041
Antarctica vs Mongolia	1	27.43	8.23	0.23	0.017
Albania vs Canada	1	11.33	1.61	0.05	0.256
Albania vs Madeira	1	164.10	22.24	0.43	0.001
Albania vs Mongolia	1	153.60	46.73	0.63	0.001
Canada vs Madeira	1	115.28	11.55	0.28	0.005

Canada vs Mongolia	1	133.00	21.73	0.43	0.001
Madeira vs Mongolia	1	23.85	3.72	0.12	0.085

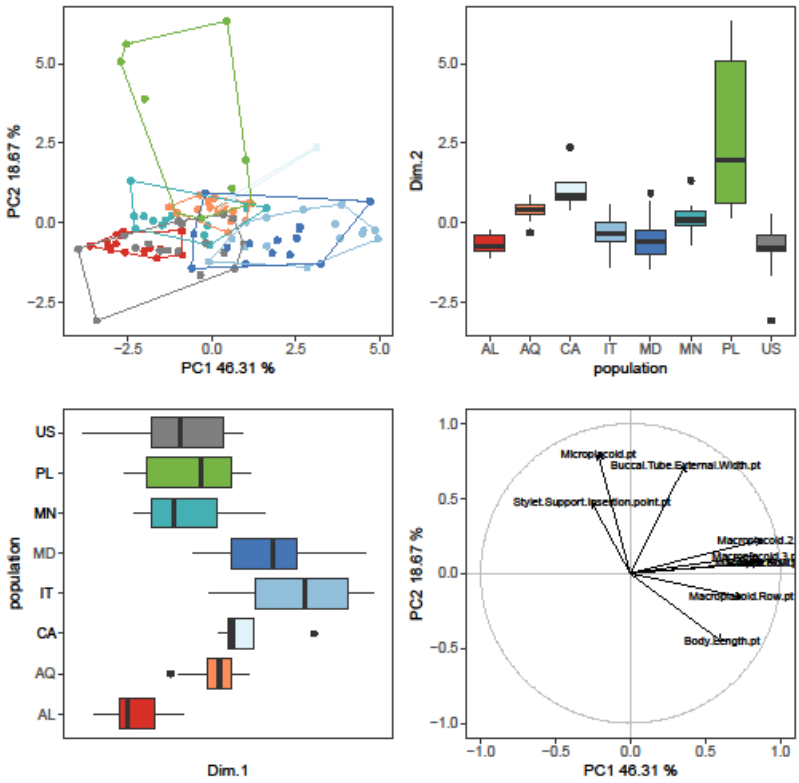


Figure 6. Results of PCA for animal *pt* indices, 1st and 2nd Principal Components. Score scatterplots presented in top-left quadrants; boxplots of single component scores presented in top-right and bottom-left quadrants and loading plot presented in bottom-right.

Table 5. Results of PERMANOVA and post hoc pairwise PERMANOVA comparisons for the first two principal components (PC1 and PC2) of animal *pt* values; significant post hoc p-values adjusted with the Benjamini-Hochberg correction.

***Post hoc* comparisons**	**df**	**SS**	**F**	**R^2**	**P**
Poland vs Italy	1	14.60	7.12	0.30	0.0147
Poland vs USA	1	39.29	9.12	0.29	0.0016
Poland vs Antarctica	1	6.80	5.60	0.16	0.0218
Poland vs Albania	1	23.07	17.70	0.38	0.0002
Poland vs Canada	1	7.41	3.11	0.09	0.0620
Poland vs Madeira	1	96.12	37.76	0.57	0.0002
Poland vs Mongolia	1	45.99	19.38	0.42	0.0002
Italy vs USA	1	15.96	2.33	0.18	0.1088

Italy vs Antarctica	1	5.00	5.75	0.24	0.0214
Italy vs Albania	1	48.07	47.57	0.73	0.0003
Italy vs Canada	1	26.96	9.90	0.34	0.0025
Italy vs Madeira	1	15.25	5.02	0.23	0.0264
Italy vs Mongolia	1	9.39	3.38	0.17	0.0591
USA vs Antarctica	1	36.20	11.01	0.32	0.0013
USA vs Albania	1	85.73	25.23	0.52	0.0002
USA vs Canada	1	76.01	16.33	0.40	0.0002
USA vs Madeira	1	118.93	23.44	0.52	0.0002
USA vs Mongolia	1	88.02	17.70	0.46	0.0002
Antarctica vs Albania	1	55.98	90.28	0.75	0.0002
Antarctica vs Canada	1	22.28	13.23	0.30	0.0002
Antarctica vs Madeira	1	58.07	32.33	0.53	0.0002
Antarctica vs Mongolia	1	23.56	14.70	0.34	0.0003
Albania vs Canada	1	13.26	7.51	0.20	0.0080
Albania vs Madeira	1	199.01	105.68	0.78	0.0002
Albania vs Mongolia	1	116.03	68.54	0.71	0.0002
Canada vs Madeira	1	116.25	39.54	0.57	0.0002
Canada vs Mongolia	1	55.41	19.84	0.41	0.0002
Madeira vs Mongolia	1	9.33	3.12	0.10	0.0783

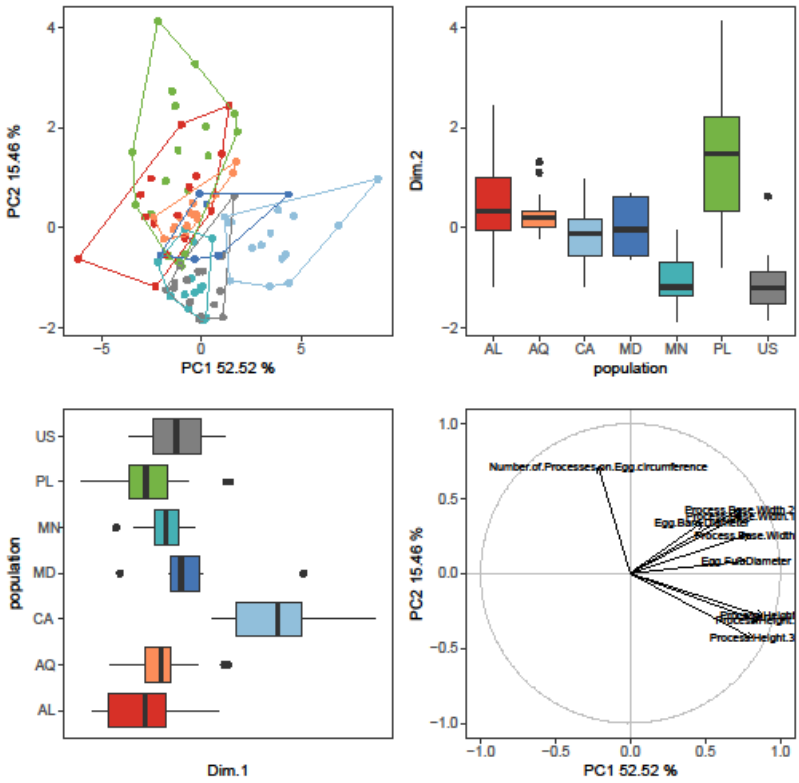


Figure 7. Results of PCA for egg measurements, 1st and 2nd Principal Components. Score scatterplots presented in top-left quadrants; boxplots of single component scores presented in top-right and bottom-left quadrants and loading plot presented in bottom-right.

Table 6. Results of PERMANOVA and post hoc pairwise PERMANOVA comparisons for the first two principal components (PC1 and PC2) of animal *pt* values; significant post hoc p-values adjusted with the Benjamini-Hochberg correction.

***Post hoc* comparisons**	**df**	**SS**	**F**	**R^2**	**P**
Poland vs USA	1	47.08	17.87	0.37	0.0002
Poland vs Antarctica	1	13.96	12.27	0.30	0.0002
Poland vs Albania	1	25.55	9.43	0.25	0.0002
Poland vs Canada	1	1.19	1.04	0.04	0.3524
Poland vs Madeira	1	134.43	47.34	0.63	0.0002
Poland vs Mongolia	1	9.14	4.73	0.20	0.0254

USA vs Antarctica	1	10.77	3.79	0.11	0.0372
USA vs Albania	1	6.23	1.44	0.04	0.2496
USA vs Canada	1	54.20	18.64	0.38	0.0002
USA vs Madeira	1	198.41	44.72	0.59	0.0002
USA vs Mongolia	1	17.89	4.16	0.16	0.0294
Antarctica vs Albania	1	7.47	2.54	0.08	0.1235
Antarctica vs Canada	1	16.51	11.55	0.28	0.0004
Antarctica vs Madeira	1	135.88	44.34	0.60	0.0002
Antarctica vs Mongolia	1	4.74	2.06	0.09	0.1731
Albania vs Canada	1	33.87	11.25	0.29	0.0004
Albania vs Madeira	1	200.18	42.54	0.60	0.0002
Albania vs Mongolia	1	17.57	3.75	0.16	0.0697
Canada vs Madeira	1	111.85	35.60	0.56	0.0002
Canada vs Mongolia	1	7.01	2.95	0.13	0.0893
Madeira vs Mongolia	1	42.85	8.80	0.32	0.0105

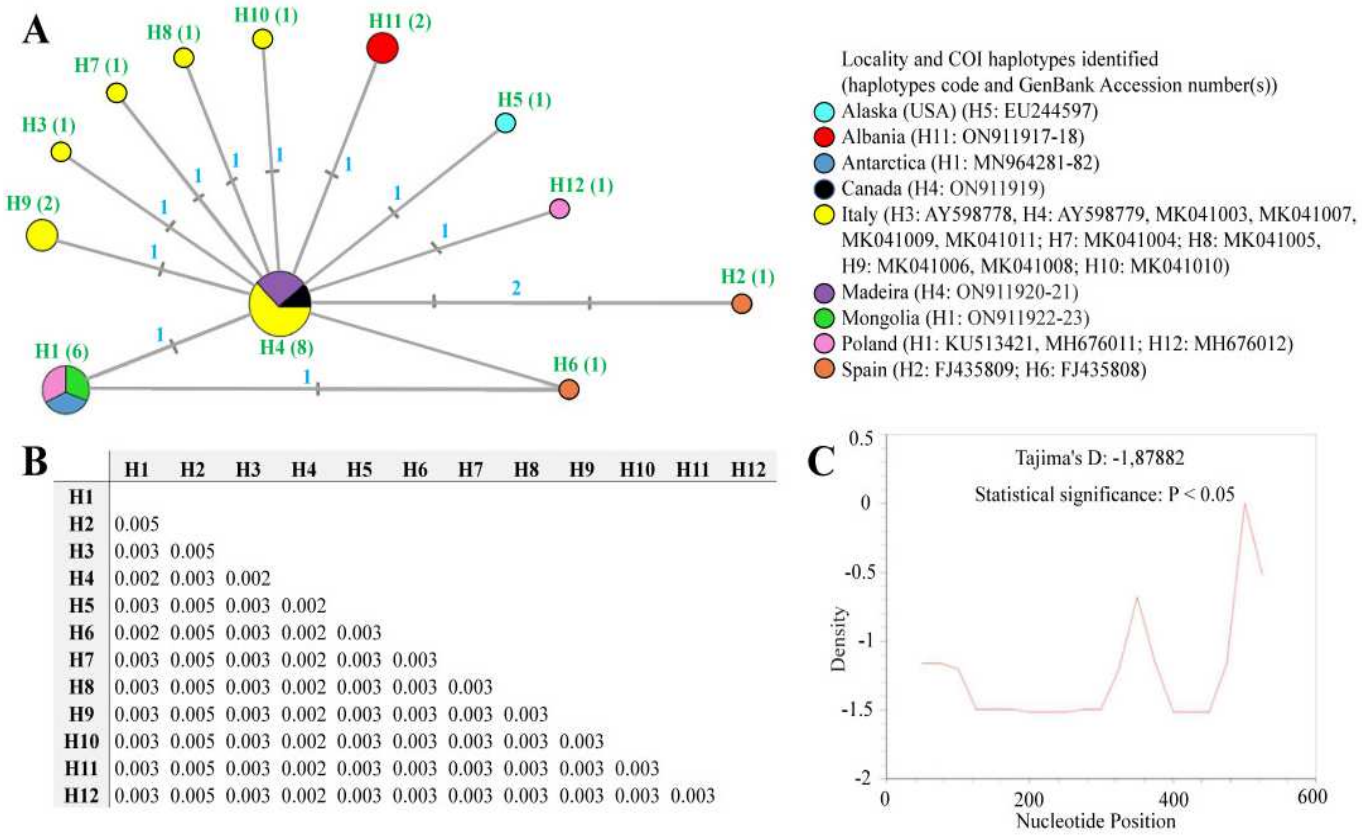
Genetic comparisons and phylogeographical analyses of different populations of the Pam. fairbanksi

The COI sequences of *Pam. fairbanksi* from Albania, Canada, Madeira and Mongolia were 623-689 bp-long, and represented three haplotypes: haplotype H11 was observed in the population from Albania, haplotype H1 was identified in *Pam. fairbanksi* from Mongolia, and haplotype H4 was found in populations from Canada and Madeira (for details see Table 3 and Figure 8A, B). No stop codons, insertions or deletions were observed. The translation was successfully carried out with the –2nd reading frame and the invertebrate mitochondrial codon

table. The p-distances between COI haplotypes of all sequenced *Pam. Fairbanksi* populations deposited in GenBank, i.e., from Antarctica, Italy, Spain, the USA and Poland ranged from 0.002% to 0.005% (an average distance of 0.003%) (Figure 8B). In total, twelve haplotypes (H1-H12) of COI gene fragments were identified after comparing all available COI sequences of *Pam. fairbanksi*. Overall, the median joining COI haplotype network showed a star-like radiation. Interestingly, the most frequent haplotype H4 was present in populations from Italy, Madeira and Canada. This central haplotype H4 was surrounded by ten haplotypes (H1, H3, H5-H12) that differed from it by one mutational step. One haplotype (haplotype H2 from Spain) differed from central haplotype H4 by two mutational steps. In several geographical regions, i.e., the USA, Albania, Italy, Poland and Spain there were regional endemic haplotypes. Surprisingly, the second haplotype that occurred in different localities was haplotype H1 and this haplotype was common for three populations, from Mongolia, Poland and Antarctica.

In turn, the 18S rRNA sequences of *Pam. fairbanksi* from Albania, Canada, Madeira and Mongolia were 917-1547 bp-long (Table 3) and no nucleotide substitution was found (although a single "N" was identified, i.e. software was unable to identify this base). Compared with the data available in GenBank sequences of *Pam. fairbanksi* (sequences were alignment and trimmed to 572 bp), they showed only one nucleotide substitution. A comparison was performed with the sequences from the following geographical localities: Antarctica (GenBank: MN960302[9]), Poland (GenBank: MH664941-42[63]), USA (GenBank: EU038078[64]) and Italy (GenBank: MK041027-29[8]). The 28S rRNA molecular marker was very conservative, and was 694-805 bp-long. No nucleotide substitution was found for all obtained sequences even after comparing (and trimmed to 689 bp) with GenBank sequences from Antarctica (GenBank: MN960306 – MN960307[9]) and Poland (GenBank: MH664950[63]). Nevertheless, one unidentified base was found in the sequence originating from the Polish population.

Demographic expansion was preliminarily tested based on the value of neutrality tests that confirmed a neutral model of observed polymorphism. Negative significant values for Tajima's D were found, indicating a high number of low-frequency polymorphisms in the COI sequences dataset and potential population size expansion (Figure 8C). In turn, values of Fu's FS test statistic for COI data were negative, but non-significant: -0.25702, P = 1.16679 (graphical results not shown).



	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
H1												
H2	0.005											
H3	0.003	0.005										
H4	0.002	0.003	0.002									
H5	0.003	0.005	0.003	0.002								
H6	0.002	0.005	0.003	0.002	0.003							
H7	0.003	0.005	0.003	0.002	0.003	0.003						
H8	0.003	0.005	0.003	0.002	0.003	0.003	0.003					
H9	0.003	0.005	0.003	0.002	0.003	0.003	0.003	0.003				
H10	0.003	0.005	0.003	0.002	0.003	0.003	0.003	0.003	0.003			
H11	0.003	0.005	0.003	0.002	0.003	0.003	0.003	0.003	0.003	0.003		
H12	0.003	0.005	0.003	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.003	

Figure 8. A – Median-joining network based on the COI sequences: haplotypes marked as H1-H12 (the number of sequences is given in parentheses), the size of the circles is proportional to the number of sequences, the mutational steps values are indicated along the lines; B – p-distance value based on the COI barcode sequences; C – Tajima's D neutrality test.

Predictions of the distribution of the two parthenogenetic Paramacrobiotus species

Ecological niche modelling of potential distribution based on available location data was performed for two parthenogenetic species with verified records from various realms, i.e., *Pam. fairbanksi* and *Pam gadabouti*. The study is limited to bioclimatic variables. The stimulated model predicted good accuracy for the overall model with an AUC for *Pam. fairbanksi* of 0.826 and excellent accuracy for the overall model with an AUC for *Pam gadabouti* of 0.924. The suitability for *Pam. fairbanksi* seems moderate (green areas on the map in Figure 9A) to good (yellow areas on the map in Figure 9A) with the most suitable habitats in the northern hemisphere. *Pam. gadabouti* shows maximal suitability around areas with a Mediterranean climate, although it also has wide distribution (Figure 9B).

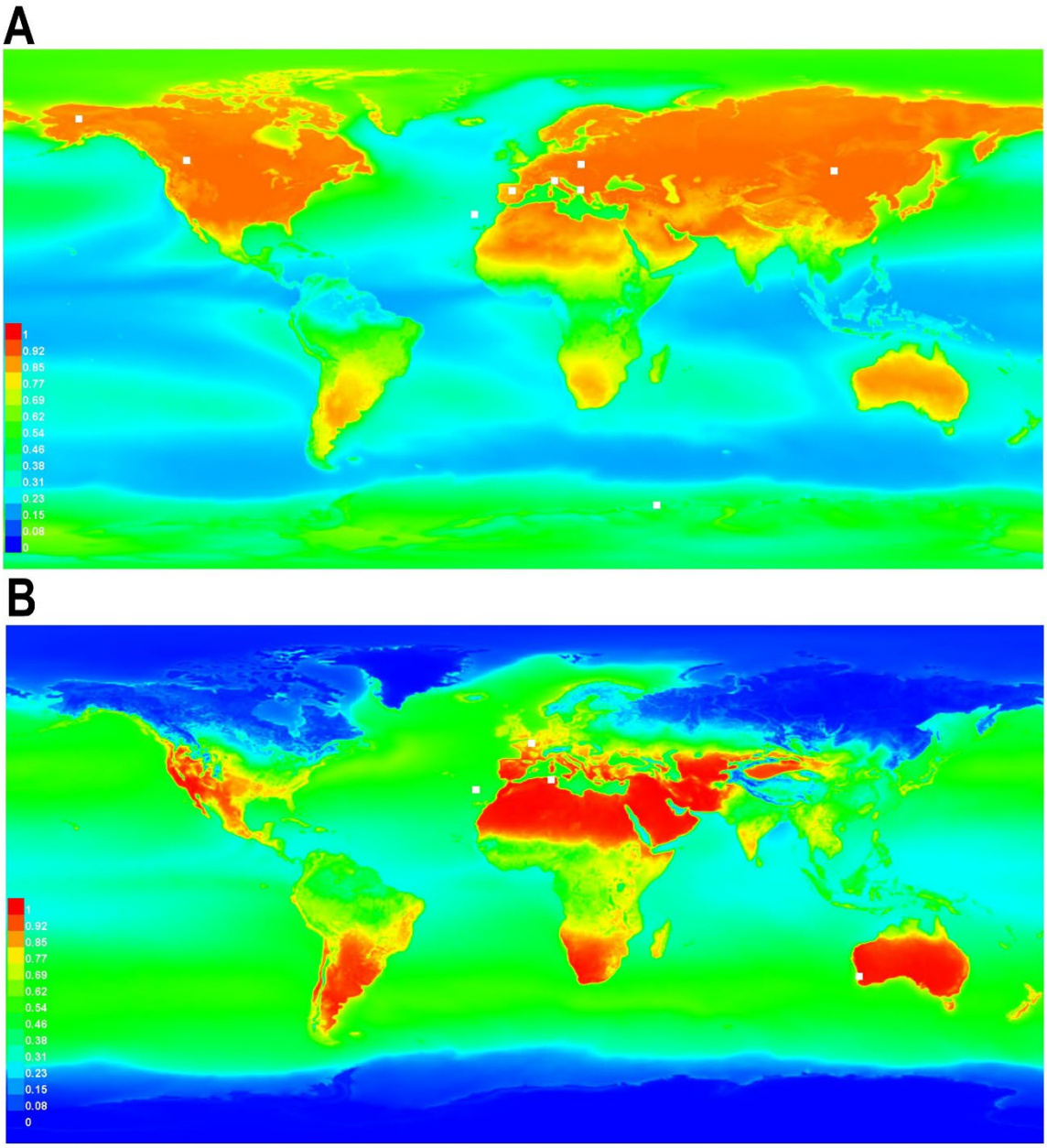


Figure 9. Ecological biogeography of two parthenogenetic *Paramacrobiotus* species with wide distributions – geographic ranges predicted by ecological niche modelling for: (A) *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf 2010[9], (B) *Paramacrobiotus gadabouti* Kayastha, Stec, Mioduchowska and Kaczmarek 2023[21]. Suitability determines whether a given area is characterised by favourable conditions for one of the species (maximal suitability = 1) or by allegedly inhospitable conditions (minimal suitability = 0). Generated using Maxent, ver. 3.4.4 [https://biodiversityinformatics.amnh.org/open_source/maxent/]. Warmer colours show areas with better predicted conditions. White dots show the presence locations used for training.. The maps were generated using MaxEnt software ver. 3.2.0: https://biodiversityinformatics.amnh.org/open_source/maxent/ and assembled in Corel Photo-Paint 2021.

4. Discussion

Morphometric comparison of different populations of the Pam. fairbanksi

Based on morphometric analyses, there is clearly a variation in measurements of morphological features between populations of *Pam. fairbanksi* from different regions of the world. However, the identification of this species is still possible with the morphometric characters alone because of the overlap in measurements of all measured structures. Therefore, it is valid to suggest the correct classification of all the specimens collected from different regions based on their morphology only. Even though the egg processes of Polish and Albanian populations are similar, the EBD of the Polish population are the smallest and those from the Albania are largest. The EFD as well as egg processes of the Madeiran population are largest while those of the Polish population are the smallest. Additionally, body length values of the Polish and USA populations of *Pam. fairbanksi* are smaller compared to the other populations studied.

Kaczmarek et al.[9] suggested that the differences in measurements between different populations of this species are caused by conditions, i.e., specimens from cultures and specimens from wild populations. However, in the present study all measurements were based on specimens from cultured populations, i.e., Albanian, Canadian, Madeiran and Mongolian. Thus, we can suggest that the phenomenon described by Kaczmarek et al.[9] (that dwarfing is caused by suboptimal conditions, high culture densities and inbreeding and that it might be due to the result of ongoing speciation) is unlikely to be true. Similarly, the suggestions that harsh conditions in Antarctica may favor laying larger eggs while in cultures the eggs are smaller because of the lack of such selective pressure[9] seems untrue as the egg size of specimens from Antarctica overlaps with egg sizes of specimens from Albania, Canada and Mongolia, which were sampled from cultured populations in the present study.

Genetic comparison of different populations of the Pam. fairbanksi

Cytochrome oxidase subunit I gene (COI) sequences is one of the most reliable barcodes to investigate genetic variation with phenotypic plasticity since COI is a genetic marker with a high genetic variation compared to multiple other DNA barcodes[66]. Various studies combining COI variation and phenotypic plasticity were conducted throughout different invertebrates' phyla, including tardigrades[9,21,66,67,68,69], proving the marker's accuracy in this group of organisms. The result showed high genetic homogeneity between organisms with wide geographical distribution together with clearly visible morphological differences known as phenotypic plasticity[67].

Furthermore, several studies uncovered data incongruence between mitochondrial and nuclear markers, e.g., for earthworms[70] or corals[71], suggesting that occasionally COI may fail as a barcode marker due to hybridization events. Many studies have already shown that *Wolbachia* (presence shown by Mioduchowska et al[72] in *Pam. fairbanksi*) can increase the speciation rate and can affect COI haplotypes[73]. However, the nuclear markers tested for *Pam. fairbanksi* have been consistent for the studied populations. No molecular markers that correspond with morphological features in tardigrades have been suggested so far. Future studies with higher-resolution markers designed for intrapopulation variation should be performed to determine if any pattern of genetic diversity concordant with morphological variation can be observed.

Based on morphometric and genetic analyses, it is possible that the subtle morphological variation observed in geographically remote populations of *Pam. fairbanksi* can be explained by phenotypic plasticity. This explanation is supported by the fact that populations from Poland and Antarctica share the same haplotype, H1, but vary in EFD measurements of eggs, which is the most variable trait in the analysis. Several distant geographic populations share the same COI haplotype, H1 or H4, yet it does not make them morphologically consistent within the haplotypes. These populations have, as shown in the study, a low evolutionary rate, and the inter-population variation develops under variable conditions experienced in different locations. The exact causes and mechanisms of the phenotypic plasticity in the morphology of adults and eggs of *Pam. fairbanksi* remains unknown, although, it has unsurprisingly been shown, that some physical traits differ in chosen cultured tardigrades depending on the temperature and food abundance[7]. If the morphological variation in *Pam. fairbanksi* is an effect of phenotypic plasticity, it is unclear which factors could cause various morphotype expressions. The specimens from Mongolia, Albania, Canada and Madeira that were measured in our study come from populations cultured in similar laboratory conditions but were started with different counts of founders of various ages, kept in variable densities and with different numbers of generations that had passed culture, so no answer can be proposed at this moment.

Phenotypic plasticity, in morphology and other aspects of phenotype, such as life history traits, is seen as an advantage for thriving in heterogeneous environments (e.g. ref[74]), which tardigrades' habitats clearly are. Furthermore, phenotypic plasticity has been widely observed in other invertebrates like corals (e.g. *Pseudopterogorgia bipinnata* Verrill 1864[75]), scallops (e.g. *Pecten maximus* Linnaeus, 1758[76]), marine invertebrates, gastropods (e.g. *Littorina littorea* Linnaeus, 1758[62]), rotifers (*Keratella tropica* (Apstein, 1907)[77]) and many more. No concordant genetic variation was observed, but a large and discrete differentiation of

morphotypes was present and was always associated with external environmental factors such as temperature, predation risk and food availability[78.79,80,82,82].

Parthenogenesis and wide distribution

The phenomenon where parthenogenetic (asexual) lineages occupy a wider geographical range, but sexual populations are restricted to a limited area, is termed 'geographical parthenogenesis'[83]. Guidetti et al.[8] concluded that the difference in the dispersal potential of tardigrades is associated with the two types of reproduction, i.e., parthenogenetic species show a very wide distribution, inhabiting more continents, while the amphimictic species show a very limited or punctiform distribution. A similar pattern was shown for arthropods where parthenogenesis has been linked with higher dispersal abilities[84] (for example, the freshwater ostracod *Eucypris virens* (Jurine, 1820)[85] and the scorpion species *Liocheles australasiae* (Fabricus 1775) are parthenogenetic for multiple generations in captivity[86,87] and are widely distributed[88,89]. Similar cases are found in many animals and plants (ref[90,91,92,93])). However, Baker et al.[84] also suggested that parthenogenesis indicates morphological variation as a result of epigenetic mechanisms. Furthermore, Mioduchowska et al.[72] provided molecular evidence of the presence of the bacterial endosymbiont *Wolbachia* based on next generation sequencing in tardigrades. *Wolbachia* have an effect on the evolution as well as the ecology of their hosts, and have been found to cause effects including cytoplasmic incompatibility, feminization, male killing, and induced parthenogenesis[94] It has been noted that at the intraspecific level, even individuals from the same population can undergo morphological changes in their characters to diversify within niches available to the species[95]. Similarly, Kihm et al.[88] proposed epigenetic factors as a main cause for variability in tardigrade *Dactylobiotus ovimutans* egg morphology, although the population was cultured under controlled laboratory conditions. Despite being rare, it is known that intraspecific variation is caused by external environmental conditions, epigenetics and seasonality[96]. Therefore, it is also likely that the morphological differences that we observed in the present study might be due to epigenetic factors, as the studied populations were cultured under controlled laboratory conditions.

"Two faces" of cosmopolitism in the Paramacrobiotus

Ecological niche modelling is an important and useful tool that has been used to address issues in many fields of basic and applied ecology[97]. It effectively predicts habitat suitability for rare and poorly studied taxa[98,99]. *Pam. fairbanksi* presence is linked to the presence of

suitable microhabitats, like moss patches, and their life strategy can make them less likely to be affected by general climatic conditions. However, bioclimatic variables used in the study may be a good predictor of the possibility of the occurrence of suitable microhabitats. We investigated the possible distribution of *Pam. fairbanksi* and compared it with other widely distributed species of the genus *Paramacrobiotus*, i.e., *Pam. gadabouti*. *Paramacrobiotus fairbanksi* already reported from various continents exhibit a cosmopolitan distribution covering different types of environments, whereas *Pam. gadabouti*, although also potentially cosmopolitan, has a clear affinity to areas with a Mediterranean climate. Its distribution is poorly known due to lack of sampling in many habitats. Such differences clearly show us that even when we consider some of the species to be cosmopolitan, specific patterns of distribution can be completely different. However, we must also stress that the number of known localities for both species is relatively low and, in the future, when the number of records of these species will be higher, a distribution pattern may look different.

Conclusions

Paramacrobiotus fairbanksi described originally from Alaska, USA, is now known from almost all zoogeographic realms. The identification of this species is possible based on morphometric characters alone because of the overlap in measurements of all measured structures. Moreover, the analysis shows low genetic variability among *Pam. fairbanksi* populations from various geographical locations, which may in general suggest that interspecies genetic variability in tardigrades is very low too or could be' the effects of *Wolbachia* infection. The species fits the 'Everything is Everywhere' hypothesis and is an example of a parthenogenetic species with wide distribution. Despite very low genetic variation, some indiscrete morphological variations were observed. Since all the studied populations were cultured and bred in the same laboratory conditions, such variation may have been caused by epigenetic effects, and were not the result of different temperatures, food sources and seasonality.

Data Availability

The datasets generated and/or analysed during the current study are available in the GenBank repository (all accession numbers listed in Table 2: ON911917-18, ON872386, ON872380-81, ON911919, ON872387, ON872382, ON911920-21, ON872388, ON872383,

ON911922-23, ON872389 and ON872384-85). The data of all sequences will be available for public access within a few days.

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Acknowledgements

Studies have been partially conducted in the framework of activities of BARg (Biodiversity and Astrobiology Research group). The PK is scholarship holder of Passport to the future – Interdisciplinary doctoral studies at the Faculty of Biology, Adam Mickiewicz University, Poznań POWR.03.02.00-00-I006/17 and her study was also supported by Minigrant Edition II, contract number POWER6/2021/2ed, Adam Mickiewicz University, POWR.03.02.00-00- I006/17. The work of MM was supported by National Science Centre, Poland, grant no. 2021/43/D/NZ8/00344 and grant no. 1220/146/2021 from the Small Grants Programme of the University of Gdansk (i.e., Ugrants-first competition).

The authors also wish to thank Cambridge Proofreading LLC (http://proofreading.org) for their linguistic assistance.

Authors Contributions

Conceptualization, P.K. and Ł.K.; data curation, P.K.; formal analysis, P.K.; investigation, P.K., M.M., and Ł.K.; methodology, P.K., M.M. and Ł.K.; supervision, Ł.K.; validation, P.K., W.S., M.M. and Ł.K.; visualization, P.K.; writing—original draft, P.K., M.M. and Ł.K.; writing—review and editing, P.K., W.S., M.M. and Ł.K. All authors have read and agreed to the published version of the manuscript.

Ethics declarations

All procedures were conducted in accordance with the guidelines. Also, none of the moss samples were collected from the region which requires permission.

Competing interests

The authors declare no competing interests.

Table 7. Measurements [in µm] and pt values of selected morphological structures of individuals of *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf 2010 Albanian population mounted in Hoyer's medium (N – number of specimens/structures measured; RANGE refers to the smallest and the largest structure among all measured specimens; SD – standard deviation, *pt* – ratio of the length of a given structure to the length of the buccal tube expressed as a percentage).

CHARACTER	N	RANGE						MEAN		SD	
		µm			*pt*			µm	*pt*	µm	*pt*
Body length	16	426	–	654		–		549		73	
Buccopharyngeal tube											
Buccal tube length	16	31.7	–	57.8		–		52.1	–	6.4	–
Stylet support insertion point	15	24.6	–	44.8	*76.1*	–	*80.8*	40.7	*78.7*	5.0	*1.4*
Buccal tube external width	15	7.5	–	14.2	*19.2*	–	*24.6*	11.1	*21.4*	1.8	*1.8*
Buccal tube internal width	15	5.5	–	10.9	*16.1*	–	*19.8*	9.0	*17.2*	1.3	*1.0*
Ventral lamina length	14	18.2	–	34.1	*57.4*	–	*61.5*	30.3	*58.7*	4.1	*1.3*
Placoid lengths											
Macroplacoid 1	15	6.1	–	9.6	*13.5*	–	*16.8*	8.2	*15.3*	1.0	*1.1*
Macroplacoid 2	16	3.8	–	8.3	*11.0*	–	*14.5*	6.6	*12.7*	1.1	*0.9*
Macroplacoid 3	16	5.8	–	11.1	*16.6*	–	*19.4*	9.4	*18.0*	1.3	*0.9*
Microplacoid	16	3.5	–	6.3	*8.3*	–	*10.9*	4.8	*9.2*	0.6	*0.9*
Macroplacoid row	15	16.8	–	32.0	*50.6*	–	*56.1*	27.2	*52.5*	3.6	*1.5*
Placoid row	15	21.2	–	40.4	*66.0*	–	*71.0*	35.3	*68.2*	4.8	*1.4*
Claw 1 heights											
External primary branch	16	10.1	–	16.0	*23.4*	–	*31.9*	13.9	*26.9*	1.6	*2.2*
External secondary branch	16	7.2	–	13.2	*18.7*	–	*24.3*	10.7	*20.6*	1.4	*1.5*
Internal primary branch	16	9.5	–	17.3	*24.1*	–	*30.0*	13.4	*25.8*	1.7	*2.0*
Internal secondary branch	16	6.6	–	13.7	*18.5*	–	*23.6*	10.6	*20.4*	1.6	*1.4*
Claw 2 heights											
External primary branch	16	10.3	–	18.3	*24.6*	–	*32.6*	14.8	*28.5*	1.8	*2.1*
External secondary branch	16	8.2	–	13.3	*19.8*	–	*25.8*	11.5	*22.2*	1.4	*1.4*
Internal primary branch	16	9.8	–	16.6	*23.9*	–	*31.1*	13.5	*26.1*	1.6	*1.8*
Internal secondary branch	15	8.2	–	13.8	*16.7*	–	*25.9*	10.7	*20.7*	1.6	*2.3*
Claw 3 heights											
External primary branch	16	11.5	–	19.0	*27.9*	–	*36.5*	15.4	*29.7*	1.6	*2.2*
External secondary branch	16	8.9	–	14.3	*20.1*	–	*28.1*	11.8	*22.7*	1.4	*2.3*
Internal primary branch	16	9.7	–	15.9	*23.7*	–	*30.7*	13.5	*26.0*	1.5	*1.7*
Internal secondary branch	15	7.7	–	13.1	*18.2*	–	*24.2*	10.9	*21.0*	1.3	*1.6*
Claw 4 heights											
Anterior primary branch	16	11.5	–	20.0	*26.3*	–	*36.3*	15.8	*30.4*	1.9	*2.5*
Anterior secondary branch	16	8.3	–	14.4	*20.0*	–	*26.1*	11.9	*22.9*	1.7	*1.8*
Posterior primary branch	16	11.9	–	20.5	*28.4*	–	*37.6*	16.0	*30.9*	1.9	*2.5*
Posterior secondary branch	16	7.4	–	14.2	*20.7*	–	*25.7*	12.2	*23.4*	1.6	*1.3*

Table 8. Measurements [in µm] of selected morphological structures of eggs of *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf 2010 Albanian population mounted in Hoyer's medium (N – number of specimens/structures measured, RANGE refers to the smallest and the largest structure among all measured eggs; SD – standard deviation).

CHARACTER	N	RANGE			MEAN	SD
Egg bare diameter	15	63.8	–	95.4	77.9	9.3

Egg full diameter	15	86.2	–	116.9	98.5	8.3
Process height	45	7.6	–	17.3	12.0	2.0
Process base width	45	9.7	–	20.3	15.5	2.2
Process base/height ratio	45	100%	–	177%	130%	17%
Inter-process distance	42	1.3	–	7.8	3.6	1.6
Number of processes on the egg circumference	15	13	–	16	14.1	1.1

Table 9. Measurements [in µm] and pt values of selected morphological structures of individuals of *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf 2010 Canadian population mounted in Hoyer's medium (N – number of specimens/structures measured; RANGE refers to the smallest and the largest structure among all measured specimens; SD – standard deviation, *pt* – ratio of the length of a given structure to the length of the buccal tube expressed as a percentage).

CHARACTER	**N**	**RANGE**						**MEAN**		**SD**	
		µm			***pt***			**µm**	***pt***	**µm**	***pt***
Body length	17	303	–	772		–		564		133	
Buccopharyngeal tube											
Buccal tube length	17	33.8	–	63.7		–		52.7	–	8.6	–
Stylet support insertion point	17	26.3	–	51.0	*75.3*	–	*80.0*	41.2	*78.3*	6.8	*1.2*
Buccal tube external width	17	6.2	–	13.7	*15.4*	–	*23.8*	11.3	*21.3*	2.3	*1.9*
Buccal tube internal width	17	4.5	–	11.4	*11.0*	–	*18.1*	8.2	*15.4*	1.9	*1.8*
Ventral lamina length	15	24.5	–	38.3	*56.4*	–	*64.7*	32.6	*60.4*	4.9	*2.6*
Placoid lengths											
Macroplacoid 1	17	4.7	–	11.7	*12.5*	–	*19.9*	8.9	*16.7*	2.1	*1.8*
Macroplacoid 2	17	3.9	–	10.4	*11.2*	–	*16.8*	7.8	*14.6*	1.9	*1.6*
Macroplacoid 3	17	5.0	–	12.1	*14.9*	–	*20.9*	9.7	*18.2*	2.0	*1.6*
Microplacoid	17	3.0	–	5.2	*6.2*	–	*9.7*	4.1	*7.8*	0.6	*0.9*
Macroplacoid row	16	21.5	–	36.0	*51.8*	–	*59.5*	30.2	*55.9*	4.8	*2.3*
Placoid row	17	23.6	–	46.8	*68.5*	–	*75.8*	37.8	*71.6*	6.9	*2.4*
Claw 1 heights											
External primary branch	16	8.2	–	17.9	*21.9*	–	*30.7*	14.4	*27.4*	2.9	*2.4*
External secondary branch	16	5.5	–	14.8	*16.2*	–	*25.2*	10.9	*20.6*	2.3	*1.9*
Internal primary branch	16	8.4	–	16.1	*20.7*	–	*29.0*	13.2	*25.2*	2.5	*2.2*
Internal secondary branch	16	6.9	–	13.9	*17.1*	–	*24.1*	10.9	*20.8*	2.0	*2.1*
Claw 2 heights											
External primary branch	17	9.2	–	18.1	*22.7*	–	*30.3*	14.6	*27.6*	3.0	*2.7*
External secondary branch	16	7.2	–	15.5	*17.7*	–	*26.4*	11.9	*22.4*	2.6	*2.2*
Internal primary branch	17	8.0	–	16.2	*20.9*	–	*27.7*	13.3	*25.3*	2.3	*1.9*
Internal secondary branch	17	6.9	–	13.8	*19.0*	–	*23.6*	11.1	*21.1*	2.1	*1.3*
Claw 3 heights											
External primary branch	17	9.0	–	18.6	*20.4*	–	*31.9*	14.9	*28.3*	3.1	*3.2*
External secondary branch	17	7.1	–	14.4	*17.0*	–	*25.9*	11.7	*22.0*	2.5	*2.4*
Internal primary branch	17	8.5	–	18.3	*23.0*	–	*31.5*	14.2	*26.9*	2.7	*2.0*
Internal secondary branch	17	7.3	–	14.6	*17.9*	–	*24.9*	11.8	*22.4*	2.1	*1.7*
Claw 4 heights											
Anterior primary branch	17	10.2	–	19.2	*25.1*	–	*34.6*	15.9	*30.3*	2.5	*2.5*
Anterior secondary branch	17	6.6	–	14.6	*16.3*	–	*25.6*	11.9	*22.5*	2.3	*2.1*
Posterior primary branch	17	10.9	–	21.2	*27.1*	–	*36.2*	16.3	*31.1*	2.8	*2.6*
Posterior secondary branch	17	7.8	–	15.0	*20.4*	–	*27.8*	12.4	*23.5*	2.0	*1.9*

Table 10. Measurements [in µm] of selected morphological structures of eggs of *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf 2010 Canadian population mounted in Hoyer's medium (N – number of specimens/structures measured, RANGE refers to the smallest and the largest structure among all measured eggs; SD – standard deviation).

CHARACTER	N	RANGE			MEAN	SD
Egg bare diameter	15	62.9	–	89.7	75.0	9.5
Egg full diameter	15	88.7	–	117.5	101.5	9.3
Process height	42	11.3	–	17.0	13.9	1.5
Process base width	42	13.6	–	18.9	15.9	1.2
Process base/height ratio	42	100%	–	136%	115%	10%
Inter-process distance	41	1.0	–	5.5	2.7	1.0
Number of processes on the egg circumference	14	10	–	12	10.8	0.8

Table 11. Measurements [in µm] and pt values of selected morphological structures of individuals of *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf 2010 Madeira population mounted in Hoyer's medium (N – number of specimens/structures measured; RANGE refers to the smallest and the largest structure among all measured specimens; SD – standard deviation, *pt* – ratio of the length of a given structure to the length of the buccal tube expressed as a percentage).

CHARACTER	N	RANGE						MEAN		SD	
		µm			*pt*			µm	*pt*	µm	*pt*
Body length	15	476	–	1036		–		714		174	
Buccopharyngeal tube											
Buccal tube length	15	49.4	–	69.3		–		59.3	–	7.2	–
Stylet support insertion point	15	37.9	–	53.8	*76.0*	–	*81.4*	46.4	*78.2*	5.6	*1.5*
Buccal tube external width	15	10.4	–	16.5	*21.0*	–	*25.9*	13.8	*23.2*	2.2	*1.6*
Buccal tube internal width	15	7.0	–	12.2	*14.1*	–	*19.1*	9.8	*16.4*	1.9	*1.5*
Ventral lamina length	13	29.5	–	39.6	*55.4*	–	*60.8*	34.7	*58.7*	3.7	*1.6*
Placoid lengths											
Macroplacoid 1	15	8.3	–	14.1	*16.7*	–	*21.8*	11.5	*19.3*	2.2	*1.6*
Macroplacoid 2	15	7.6	–	13.2	*14.9*	–	*19.1*	10.1	*16.9*	1.8	*1.3*
Macroplacoid 3	15	8.6	–	16.5	*16.7*	–	*24.0*	13.1	*21.8*	2.6	*2.1*
Microplacoid	15	3.2	–	5.9	*6.0*	–	*8.6*	4.1	*7.0*	0.8	*0.8*
Macroplacoid row	15	31.0	–	47.5	*60.1*	–	*71.7*	38.8	*65.2*	6.1	*2.9*
Placoid row	15	40.4	–	60.1	*78.8*	–	*86.8*	49.6	*83.4*	7.4	*2.8*
Claw 1 heights											
External primary branch	15	13.8	–	21.8	*27.9*	–	*31.9*	17.8	*30.0*	2.5	*1.5*
External secondary branch	15	9.5	–	15.7	*18.5*	–	*25.3*	12.9	*21.8*	1.7	*1.8*
Internal primary branch	15	13.4	–	19.6	*24.6*	–	*31.2*	16.3	*27.5*	2.2	*1.6*
Internal secondary branch	15	10.0	–	16.0	*18.0*	–	*24.7*	13.1	*22.1*	2.1	*1.8*
Claw 2 heights											
External primary branch	15	14.2	–	22.3	*28.4*	–	*32.7*	18.3	*30.8*	2.5	*1.3*
External secondary branch	15	11.8	–	17.4	*22.4*	–	*26.9*	14.6	*24.6*	1.9	*1.5*
Internal primary branch	15	13.9	–	20.4	*26.5*	–	*31.8*	16.9	*28.5*	2.3	*1.5*
Internal secondary branch	15	10.2	–	17.6	*20.6*	–	*25.8*	13.6	*22.9*	2.2	*1.7*
Claw 3 heights											

External primary branch	15	14.9	–	21.4	*24.4*	–	*32.9*	17.8	*30.1*	2.1	*2.2*
External secondary branch	15	11.6	–	16.5	*20.1*	–	*27.7*	14.3	*24.3*	1.7	*2.0*
Internal primary branch	15	14.0	–	21.2	*25.5*	–	*33.1*	17.3	*29.2*	2.5	*1.9*
Internal secondary branch	15	11.0	–	21.0	*21.0*	–	*30.7*	14.2	*23.8*	2.7	*2.3*
Claw 4 heights											
Anterior primary branch	15	16.7	–	23.2	*31.7*	–	*37.6*	20.1	*34.0*	2.4	*1.7*
Anterior secondary branch	15	12.2	–	18.6	*22.9*	–	*29.5*	15.5	*26.2*	1.8	*1.9*
Posterior primary branch	15	14.6	–	22.2	*28.8*	–	*35.5*	19.0	*32.1*	2.7	*1.6*
Posterior secondary branch	15	10.3	–	18.1	*20.4*	–	*26.2*	14.6	*24.5*	2.4	*1.5*

Table 12. Measurements [in µm] of selected morphological structures of eggs of *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf 2010 Madeira population mounted in Hoyer's medium (N – number of specimens/structures measured, RANGE refers to the smallest and the largest structure among all measured eggs; SD – standard deviation).

CHARACTER	N	RANGE			MEAN	SD
Egg bare diameter	15	74.9	–	97.6	82.9	6.3
Egg full diameter	15	107.7	–	141.7	117.5	8.3
Process height	43	13.7	–	23.0	17.7	2.2
Process base width	43	15.1	–	23.8	18.7	2.0
Process base/height ratio	43	93%	–	125%	106%	9%
Inter-process distance	44	3.0	–	6.2	4.6	0.7
Number of processes on the egg circumference	15	11	–	14	12.4	0.9

Table 13. Measurements [in µm] and pt values of selected morphological structures of individuals of *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf 2010 Mongolian population mounted in Hoyer's medium (N – number of specimens/structures measured; RANGE refers to the smallest and the largest structure among all measured specimens; SD – standard deviation, *pt* – ratio of the length of a given structure to the length of the buccal tube expressed as a percentage).

CHARACTER	N	RANGE						MEAN		SD	
		µm			*pt*			µm	*pt*	µm	*pt*
Body length	14	553	–	864		–		717		104	
Buccopharyngeal tube											
Buccal tube length	14	53.7	–	67.6		–		61.5	–	3.6	–
Stylet support insertion point	13	40.7	–	51.2	*73.4*	–	*78.8*	46.9	*76.9*	2.8	*1.4*
Buccal tube external width	14	12.3	–	18.1	*20.1*	–	*26.8*	13.7	*22.2*	1.6	*1.9*
Buccal tube internal width	14	9.0	–	14.8	*14.3*	–	*21.9*	10.5	*17.0*	1.4	*1.7*
Ventral lamina length	11	36.0	–	43.1	*60.0*	–	*65.8*	39.0	*62.9*	2.2	*1.8*
Placoid lengths											
Macroplacoid 1	14	10.4	–	13.1	*16.2*	–	*21.8*	11.4	*18.6*	0.9	*1.5*
Macroplacoid 2	14	8.7	–	11.2	*14.1*	–	*18.8*	10.0	*16.3*	0.8	*1.4*
Macroplacoid 3	14	11.0	–	15.0	*17.5*	–	*25.0*	12.7	*20.8*	1.1	*1.8*
Microplacoid	14	4.6	–	7.1	*6.8*	–	*10.9*	5.6	*9.1*	0.7	*1.0*
Macroplacoid row	14	33.2	–	41.4	*56.6*	–	*64.9*	37.0	*60.2*	2.0	*2.5*
Placoid row	14	43.9	–	54.8	*74.1*	–	*84.6*	49.4	*80.5*	2.6	*3.1*
Claw 1 heights											
External primary branch	14	15.1	–	18.1	*23.7*	–	*30.1*	16.8	*27.3*	1.0	*1.9*

External secondary branch	14	11.7	–	15.2	*18.7*	–	*24.3*	13.6	*22.2*	1.1	*1.9*
Internal primary branch	14	14.8	–	18.2	*23.6*	–	*29.9*	16.5	*27.0*	1.1	*2.2*
Internal secondary branch	14	11.6	–	15.1	*18.7*	–	*24.4*	13.1	*21.3*	1.0	*1.9*
Claw 2 heights											
External primary branch	14	16.2	–	21.0	*26.2*	–	*35.1*	18.0	*29.4*	1.3	*2.5*
External secondary branch	14	12.6	–	16.9	*19.7*	–	*28.2*	14.6	*23.8*	1.3	*2.2*
Internal primary branch	14	13.0	–	19.6	*20.9*	–	*32.0*	15.9	*25.9*	2.1	*3.0*
Internal secondary branch	14	9.6	–	15.0	*15.5*	–	*24.6*	13.0	*21.2*	1.7	*2.3*
Claw 3 heights											
External primary branch	14	13.6	–	19.6	*21.8*	–	*31.5*	17.2	*28.0*	1.6	*2.8*
External secondary branch	14	10.7	–	16.4	*18.5*	–	*25.2*	13.4	*21.7*	1.7	*2.3*
Internal primary branch	14	13.6	–	18.0	*21.8*	–	*30.8*	16.5	*26.8*	1.4	*2.2*
Internal secondary branch	14	11.1	–	16.4	*17.9*	–	*25.1*	13.4	*21.8*	1.6	*2.3*
Claw 4 heights											
Anterior primary branch	14	15.6	–	21.9	*26.4*	–	*35.1*	18.6	*30.3*	1.7	*2.6*
Anterior secondary branch	14	10.7	–	17.1	*16.7*	–	*26.4*	14.3	*23.3*	1.7	*2.8*
Posterior primary branch	14	14.5	–	22.8	*23.6*	–	*34.5*	18.5	*30.1*	2.0	*2.9*
Posterior secondary branch	14	11.0	–	17.5	*18.5*	–	*28.5*	14.2	*23.1*	2.2	*3.3*

Table 14. Measurements [in µm] of selected morphological structures of eggs of *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf 2010 Mongolian population mounted in Hoyer's medium (N – number of specimens/structures measured, RANGE refers to the smallest and the largest structure among all measured eggs; SD – standard deviation).

CHARACTER	**N**	**RANGE**			**MEAN**	**SD**
Egg bare diameter	6	64.0	–	83.5	73.0	8.0
Egg full diameter	6	87.4	–	112.4	96.8	9.5
Process height	16	11.0	–	16.9	14.2	1.9
Process base width	15	15.0	–	21.7	17.7	2.3
Process base/height ratio	15	114%	–	137%	124%	7%
Inter-process distance	12	2.2	–	3.8	3.1	0.5
Number of processes on the egg circumference	5	11	–	15	12.3	1.9

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SM.03RawmeasurementfileofParamacrobiotusfairbanksifromAlbania.xlsx
- SM.04RawmeasurementfileofParamacrobiotusfairbanksifromCanada.xlsx
- SM.05RawmeasurementfileofParamacrobiotusfairbanksifromMadeira.xlsx
- SM.06RawmeasurementfileofParamacrobiotusfairbanksifromMongolia.xlsx
- SM.07Rscriptforstatisticalanalysis.r
- SM.08AdultmeasurementandptvaluesfileforanalysisinR.xlsx
- SM.09EggmeasurementfileforanalysisinR.xlsx
- SM.10analysisofvarianceanovatestwithposthoccomparingpairsofmeasurementsapplyingbonferronicorrectionresults
- SM.11PCArandomizationtests.pdf

Supplementary Information 1

Species	Population	Longitude	Latitude	Cor. Longitude	Cor. Latitude
Paramacrobiotus fairbanksi Schill, Förster, Dandekar	1	41°19'36"N	19°49'08"E	41.326667	19.818889
Paramacrobiotus fairbanksi Schill, Förster, Dandekar	2	67°39’S	46°09’E	-67.650000	46.150000
Paramacrobiotus fairbanksi Schill, Förster, Dandekar	3	51°24'21"N	116°14'27"W	51.405833	-116.240833
Paramacrobiotus fairbanksi Schill, Förster, Dandekar	4	44°26'N	10°51'E	44.433333	10.850000
Paramacrobiotus fairbanksi Schill, Förster, Dandekar	5	32°44'37.3"N	16°54'14.4"W	32.743694	-16.904000
Paramacrobiotus fairbanksi Schill, Förster, Dandekar	6	47°49'57.0"N	107°31'26.8"E	47.832500	107.524111
Paramacrobiotus fairbanksi Schill, Förster, Dandekar	7	50°03'44"N	19°57'26"E	50.062222	19.957222
Paramacrobiotus fairbanksi Schill, Förster, Dandekar	8	40°52’42”N	03°50’45”W	40.878333	-3.845833
Paramacrobiotus fairbanksi Schill, Förster, Dandekar	9	64°50'N	147°43'E	64.833333	147.716667
Paramacrobiotus gadabouti Kayastha, Stec, Mioduch	1	32°44'36.7"N	16°54'28.0"W	32.743528	-16.907778
Paramacrobiotus gadabouti Kayastha, Stec, Mioduch	2	32°49'06"N	16°59'19"W	32.818333	-16.988611
Paramacrobiotus gadabouti Kayastha, Stec, Mioduch	3	31°57'16''S	115°50'40''E	-31.954444	115.844444
Paramacrobiotus gadabouti Kayastha, Stec, Mioduch	4	48°51'35.5"N	2°23'40''E	48.859861	2.394444
Paramacrobiotus gadabouti Kayastha, Stec, Mioduch	5	36°73'92''N	8°72'99''E	36.739200	8.729900

Supplementary Information 2

```
#enmevaluate script
install.packages("devtools", dependencies = TRUE)
library(devtools)
install_github("jamiemkass/ENMeval")
install.packages("MASS", dependencies = TRUE)

library(ENMeval)
library(raster)
library(MASS)

bio1 <- raster("bio1.asc")
bio2 <- raster("bio2.asc")
bio3 <- raster("bio3.asc")
bio4 <- raster("bio4.asc")
bio5 <- raster("bio5.asc")
bio6 <- raster("bio6.asc")
bio7 <- raster("bio7.asc")
bio8 <- raster("bio8.asc")
bio9 <- raster("bio9.asc")
bio10 <- raster("bio10.asc")
bio11 <- raster("bio11.asc")
bio12 <- raster("bio12.asc")
bio13 <- raster("bio13.asc")
```

```
bio14 <- raster("bio14.asc")
bio15 <- raster("bio15.asc")
bio16 <- raster("bio16.asc")
bio17 <- raster("bio17.asc")
bio18 <- raster("bio18.asc")
bio19 <- raster("bio19.asc")


env <- stack(bio1, bio2, bio3, bio4, bio5, bio6, bio7, bio8, bio9, bio10, bio11, bio12, bio13,
bio14, bio15, bio16, bio17, bio18, bio19)


env


#load in your occurrence points

pf <- read.csv("pf_pts.csv")[,-1]

#make a bias file

occur.raspf <- rasterize(pf, env, 1)
plot(occur.raspf)

presences <- which(values(occur.raspf) == 1)
pres.locs <- coordinates(occur.raspf)[presences, ]

dens <- kde2d(pres.locs[,1], pres.locs[,2], n = c(nrow(occur.raspf), ncol(occur.raspf)), lims =
c(extent(env)[1], extent(env)[2], extent(env)[3], extent(env)[4]))
dens.ras <- raster(dens, env)
dens.ras2 <- resample(dens.ras, env)
#plot(dens.ras2)

writeRaster(dens.ras2, "biasfile.asc", overwrite = TRUE)
```

```
length(which(!is.na(values(subset(env, 1)))))

#If this number is far in excess of 10,000, then use 10,000 background points.
#If this number is comprable to, or smaller than 10,000, then use 5,000, 1,000, 500,


bg <- xyFromCell(dens.ras2, sample(which(!is.na(values(subset(env, 1)))), 10000,
prob=values(dens.ras2)[!is.na(values(subset(env, 1)))]))
colnames(bg) <- colnames(pf)

enmeval_results <- ENMevaluate(pf, env, bg, tune.args = list(fc = c("L","LQ","H", "LQH",
"LQHP", "LQHPT"), rm = 1:5), partitions = "jackknife", algorithm = "maxnet")

enmeval_results@results

write.csv(enmeval_results@results, "enmeval_results.csv")


#extract raster values script
##set the working directory!

#install.packages("raster")

library(raster)

pts <- read.csv("at_pts.csv")

coordinates(pts) <- ~longitude+latitude

x <- raster("at_avg.asc")

plot(x, col = terrain.colors(255))
```

```
points(pts)

rasvalue <- extract(x, pts) ##Note: This would also work with a raster stack to do multiple at the same time

combined <- cbind(pts@data, pts@coords, rasvalue)

write.csv(combined, "rasvalues.csv", row.names = FALSE, na = "")
```

Supplementary Information 3

assets.researchsquare.com

Supplementary Information 4

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Supplementary Information 5

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Supplementary Information 6

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Supplementary Information 7

```
#ANOVA with post-hoc comparing pairs of measurements applying Bonferroni correction for animal and egg morphometrics
#R script for eggs

attach(jaja)
jaja1=lm(EBD~POPUL)
summary(jaja1)
pairwise.t.test(EBD, POPUL, p.adj = "bonf")
shapiro.test(residuals(jaja1))

jaja2=lm(EFD~POPUL)
```

```
summary(jaja2)
pairwise.t.test(EFD, POPUL, p.adj = "bonf")
shapiro.test(residuals(jaja2))

jaja2=lm(EFD~POPUL)
summary(jaja2)
pairwise.t.test(EFD, POPUL, p.adj = "bonf")
shapiro.test(residuals(jaja2))

wypustka=lm(PH1~POPUL)
summary(wypustka)
pairwise.t.test(PH1, POPUL, p.adj = "bonf")
shapiro.test(residuals(wypustka))

wypustka=lm(PH2~POPUL)
summary(wypustka)
pairwise.t.test(PH2, POPUL, p.adj = "bonf")
shapiro.test(residuals(wypustka))

wypustka=lm(PH3~POPUL)
summary(wypustka)
pairwise.t.test(PH3, POPUL, p.adj = "bonf")
shapiro.test(residuals(wypustka))


boxplot(EBD ~ POPUL, col = c("orange", "lightgreen", "orange", "orange", "orange",
"orange", "orange"))
boxplot(EFD ~ POPUL, col = c("orange", "lightgreen", "orange", "orange", "orange",
"orange", "orange"))
boxplot(PH1 ~ POPUL, col = c("orange", "lightgreen", "orange", "orange", "orange",
"orange", "orange"))

#R script for adults
```

```
m1=lm(BLm~pop)
summary(m1)
pairwise.t.test(BLm, pop, p.adj = "bonf")
shapiro.test(residuals(m1))

m2=lm(BTLm~pop)
summary(m2)
pairwise.t.test(BTLm, pop, p.adj = "bonf")
shapiro.test(residuals(m2))

m3=lm(SSIPm~pop)
summary(m3)
pairwise.t.test(SSIPm, pop, p.adj = "bonf")
shapiro.test(residuals(m3))

m4=lm(BTEWm~pop)
summary(m4)
pairwise.t.test(BTEWm, pop, p.adj="bonf")
shapiro.test(residuals(m4))

m5=lm(M1m~pop)
summary(m5)
pairwise.t.test(M1m, pop, p.adj="bonf")
shapiro.test(residuals(m5))

m6=lm(M2m~pop)
summary(m6)
pairwise.t.test(M2m, pop, p.adj="bonf")
shapiro.test(residuals(m6))

m7=lm(M3m~pop)
summary(m7)
pairwise.t.test(M3m, pop, p.adj="bonf")
shapiro.test(residuals(m7))
```

```
m8=lm(Mim~pop)
summary(m8)
pairwise.t.test(Mim, pop, p.adj="bonf")
shapiro.test(residuals(m8))

m9=lm(MRm~pop)
summary(m9)
pairwise.t.test(MRm, pop, p.adj="bonf")
shapiro.test(residuals(m9))

m10=lm(PRm~pop)
summary(m10)
pairwise.t.test(PRm, pop, p.adj="bonf")
shapiro.test(residuals(m10))

m11=lm(BLpt~pop)
summary(m11)
pairwise.t.test(BLpt, pop, p.adj = "bonf")
shapiro.test(residuals(m11))

m12=lm(SSIPpt~pop)
summary(m12)
pairwise.t.test(SSIPpt, pop, p.adj = "bonf")
shapiro.test(residuals(m12))

m13=lm(BTEWpt~pop)
summary(m13)
pairwise.t.test(BTEWpt, pop, p.adj="bonf")
shapiro.test(residuals(m13))

m14=lm(M1pt~pop)
summary(m14)
pairwise.t.test(M1pt, pop, p.adj="bonf")
```

```
shapiro.test(residuals(m14))

m15=lm(M2pt~pop)
summary(m15)
pairwise.t.test(M2pt, pop, p.adj="bonf")
shapiro.test(residuals(m15))

m16=lm(M3pt~pop)
summary(m16)
pairwise.t.test(M3pt, pop, p.adj="bonf")
shapiro.test(residuals(m16))

m17=lm(Mipt~pop)
summary(m17)
pairwise.t.test(Mipt, pop, p.adj="bonf")
shapiro.test(residuals(m17))

m18=lm(MRpt~pop)
summary(m18)
pairwise.t.test(MRpt, pop, p.adj="bonf")
shapiro.test(residuals(m18))

m19=lm(PRpt~pop)
summary(m19)
pairwise.t.test(PRpt, pop, p.adj="bonf")
shapiro.test(residuals(m19))

boxplot(BLm ~ pop, col = c("orange", "lightgreen", "orange","lightgreen",  "orange",
"orange", "orange", "orange"))
boxplot(BTLm ~ pop, col = c("orange", "lightgreen", "orange","lightgreen",  "orange",
"orange", "orange", "orange"))
boxplot(M2m~pop, col = c("orange", "lightgreen", "orange","lightgreen",  "orange",
"orange", "orange", "orange"))
boxplot(SSIPm~pop, col = c("orange", "lightgreen", "orange","lightgreen",  "orange",
```

```
"orange", "orange", "orange"))
boxplot(BLpt~pop, col = c("orange", "lightgreen", "orange","lightgreen",  "orange",
"orange", "orange", "orange"))
boxplot(BTEWpt~pop, col = c("orange", "lightgreen", "orange","lightgreen",  "orange",
"orange", "orange", "orange"))
boxplot(M1pt~pop, col = c("orange", "lightgreen", "orange","lightgreen",  "orange",
"orange", "orange", "orange"))
boxplot(M3pt~pop, col = c("orange", "lightgreen", "orange","lightgreen",  "orange",
"orange", "orange", "orange"))

#For PCA from Stec et al.2021

library(vegan)
library(FactoMineR)
library(missMDA)
library(ggplot2)
library(plyr)
library(ggExtra)
library(gridExtra)
library(ggpubr)
library(gtools)
library(ggpubr)



if (!"pairwiseAdonis" %in% rownames(installed.packages()))
{devtools::install_github("pmartinezarbizu/pairwiseAdonis/pairwiseAdonis")}
library(pairwiseAdonis)

set.seed(123456)

data_permute = function(x){
  for (i in 1:ncol(x)){
    x[,i]=base::sample(x[,i],size=length(x[,i]),replace=F)}
  return(x)
```

```
}

calculate_psi = function(x) {
  psi = sum((1-x$eig[,1])^2)
  return(psi)}

  calculate_phi = function(x){
  phi = sqrt((sum(x$eig[,1]^2)-ncol(x$call$X))/(ncol(x$call$X)*(ncol(x$call$X)-1)))
  return(phi)
}

f = function(x) {
  r = quantile(x, probs = c(0.0, 0.25, 0.5, 0.75, 0.95))
  names(r) = c("ymin", "lower", "middle", "upper", "ymax")
  r
}

adj.alpha.BH = function(pvals, alpha){
  signifs = sort(pvals) < (1:length(pvals))/length(pvals)*alpha
  max(((1:length(pvals))/length(pvals)*alpha)[signifs])
}

color_scheme =
c("AL"="#d73027","AQ"="#fc8d59","CA"="#fee090","MD"="#91bfdb","MN"="#4575b4",
"PL"="#45b0b4", "US"="#75b445")
label_scheme = c("AL","AQ","CA","MD","MN", "PL", "US")

#ANIMALS
data_animals=animals[,3:12] #for measurements and [,3:11] for pt values

res.ncp<-estim_ncpPCA(data_animals)
animals_imputed=data.frame(imputePCA(data_animals,ncp=res.ncp[[1]])$completeObs)
animals_pca=FactoMineR::PCA(animals_imputed,scale.unit=T)
```

```
nulls = list()
for (i in 1:1000){
  nulls[[i]] =
FactoMineR::PCA(data_permute(animals_imputed),scale.unit=T,graph=FALSE)
}

true_psi = calculate_psi(animals_pca)
nulls_psi = as.vector(unlist(lapply(nulls,calculate_psi)))
psi_p_animals = mean(nulls_psi>true_psi)

p_psi_animals = ggplot(data.frame(values=nulls_psi))+
  theme_bw()+
  geom_density(aes(x=values),fill="black",alpha=0.5)+
  geom_vline(xintercept=true_psi,col="red")+
  ggtitle("Figure SM11.1 - Animals - psi")+
  ylab("Density")+
  xlab("psi")+
  annotate("text", x = 20, y = 1.2, label = paste("Red - true psi
value:",round(true_psi,2)),col="red",hjust = 0)+
  annotate("text", x = 20, y = 1.1, label = paste("Black - null model values:
mean",round(mean(nulls_psi),2)),col="black",hjust = 0)+
  annotate("text", x = 20, y = 1.0, label = "One tailed p-value <0.001",col="black",hjust = 0)

true_phi = calculate_phi(animals_pca)
nulls_phi = as.vector(unlist(lapply(nulls,calculate_phi)))
phi_p_animals = mean(nulls_phi>true_phi)

p_phi_animals = ggplot(data.frame(values=nulls_phi))+
  theme_bw()+
  geom_density(aes(x=values),fill="black",alpha=0.5)+
  geom_vline(xintercept=true_phi,col="red")+
  ggtitle("Figure SM11.2 - Animals - phi")+
  ylab("Density")+
  xlab("phi")+
```

```
  annotate("text", x = 0.2, y = 90, label = paste("Red - true phi
value:",round(true_phi,2)),col="red",hjust = 0)+
  annotate("text", x = 0.2, y = 85, label = paste("Black - null model values:
mean",round(mean(nulls_phi),2)),col="black",hjust = 0)+
  annotate("text", x = 0.2, y = 80, label = "One tailed p-value <0.001",col="black",hjust = 0)

exp_variances = lapply(nulls, function(x){x$eig[,2]})
exp_variances = do.call(rbind,exp_variances)
exp_variances  = tidyr::gather(data.frame(exp_variances),"Comp","value")
x_order =
stringr::str_remove_all(as.character(mixedsort(unique(exp_variances$Comp))),"comp.")

x_order =
as.character(mixedsort(unique(exp_variances$Comp)))[order(as.numeric(x_order))]

true_variances = data.frame(animals_pca$eig[,2])
colnames(true_variances)=c("value")
true_variances$Component = x_order

p_box_animals = ggplot(exp_variances)+
  stat_summary(fun.data = f, geom="boxplot",aes(y=value,x=Comp))+
  theme_bw()+
  geom_point(data=true_variances,aes(x=Component,y=value),col="red")+
  scale_x_discrete(limits=x_order, labels=1:26)+
  ggtitle("Figure SM11.3 - Animals - explained variance by component")+
  ylab("Variance explained %")+
  xlab("Principal Components")+
  annotate("text", x = 11, y = 39, label = "Red: true value",col="red",hjust = 0)+
  annotate("text", x = 11, y = 37, label = "Black: null model values",col="black",hjust = 0)+
  annotate("text", x = 11, y = 35, label = "Whiskers represent 0-95% range of null model
values",col="black",hjust = 0)

animals_ind_plot=data.frame(animals_pca$ind$coord)
animals_ind_plot$population=animals$pop
```

```
animals_var_plot=data.frame(animals_pca$var$coord)
animals_var_plot$variable=rownames(animals_var_plot)

find_hull <- function(df) df[chull(df$Dim.1, df$Dim.2), ]
hulls_animals <- ddply(animals_ind_plot, "population", find_hull)

animals_ind_plot$population =
factor(animals_ind_plot$population,levels=c("AL","AQ","CA","IT","MD","MN", "PL",
"US"))

p.scores.animals=ggplot(animals_ind_plot)+
  theme_bw()+
  geom_point(aes(x=Dim.1,y=Dim.2,col=population))+
  geom_polygon(data = hulls_animals, aes(x=Dim.1,y=Dim.2,col=population),alpha=0)+

coord_fixed(ratio=diff(range(animals_ind_plot$Dim.1))/diff(range(animals_ind_plot$Dim.2)
))+

theme(panel.grid=element_blank(),legend.position="none",axis.title.x=element_text(size=10)
,axis.title.y=element_text(size=10))+
  xlab(paste("PC1",round(animals_pca$eig[1,2],2),"%",sep=" "))+
  ylab(paste("PC2",round(animals_pca$eig[2,2],2),"%",sep=" "))+
  scale_color_manual(values=color_scheme)+
  scale_fill_manual(values=color_scheme)



p.PC1=ggplot(animals_ind_plot)+
  theme_bw()+

theme(panel.grid=element_blank(),legend.position="none",axis.title.x=element_text(size=10)
,axis.title.y=element_text(size=10),aspect.ratio = 1)+
  geom_boxplot(aes(x=population,y=Dim.1,fill=population))+
  coord_flip()+
```

```
  scale_color_manual(values=color_scheme)+
  scale_fill_manual(values=color_scheme)+
  scale_y_discrete(labels = label_scheme)

p.PC2=ggplot(animals_ind_plot)+
  theme_bw()+

theme(panel.grid=element_blank(),legend.position="none",axis.title.x=element_text(size=10)
,axis.title.y=element_text(size=10),aspect.ratio = 1)+
  geom_boxplot(aes(x=population,y=Dim.2,fill=population))+
  scale_color_manual(values=color_scheme)+
  scale_fill_manual(values=color_scheme)+
  scale_x_discrete(labels = label_scheme)

p.loadings.animals=ggplot(animals_var_plot)+
  theme_bw()+

theme(panel.grid=element_blank(),axis.title.x=element_text(size=10),axis.title.y=element_te
xt(size=10))+
  geom_hline(aes(yintercept=0),col="grey")+
  geom_vline(aes(xintercept=0),col="grey")+

annotate("path",x=cos(seq(0,2*pi,length.out=100)),y=sin(seq(0,2*pi,length.out=100)),col="g
rey")+
  coord_fixed(ratio=1)+
  geom_segment(aes(x=0,y=0,xend=Dim.1,yend=Dim.2), arrow = arrow(length = unit(0.02,
"npc")))+
  geom_text(aes(x=Dim.1,y=Dim.2,label=variable),size=2.5)+
  xlab(paste("PC1",round(animals_pca$eig[1,2],2),"%",sep=" "))+
  ylab(paste("PC2",round(animals_pca$eig[2,2],2),"%",sep=" "))

pdf("animals_PCA.pdf")
ggarrange(p.scores.animals,p.PC2,p.PC1,p.loadings.animals,ncol=2,nrow=2,align="hv")
dev.off()
```

```
# EGGS
data_eggs=eggs[,3:11]

res.ncp<-estim_ncpPCA(data_eggs)
eggs_imputed=data.frame(imputePCA(data_eggs,ncp=res.ncp[[1]])$completeObs)
eggs_pca=FactoMineR::PCA(eggs_imputed,scale.unit=T)

eggs_ind_plot=data.frame(eggs_pca$ind$coord)
eggs_ind_plot$population=eggs$POPUL

eggs_var_plot=data.frame(eggs_pca$var$coord)
eggs_var_plot$variable=rownames(eggs_var_plot)

nulls = list()
for (i in 1:1000){
  nulls[[i]] = FactoMineR::PCA(data_permute(eggs_imputed),scale.unit=T,graph=FALSE)
}

true_psi = calculate_psi(eggs_pca)
nulls_psi = as.vector(unlist(lapply(nulls,calculate_psi)))
psi_p_eggs = mean(nulls_psi>true_psi)

p_psi_eggs = ggplot(data.frame(values=nulls_psi))+
  theme_bw()+
  geom_density(aes(x=values),fill="black",alpha=0.5)+
  geom_vline(xintercept=true_psi,col="red")+
  ggtitle("Figure SM11.4 - Eggs - psi")+
  ylab("Density")+
  xlab("psi")+
  annotate("text", x = 3, y = 4, label = paste("Red - true psi
value:",round(true_psi,2)),col="red",hjust = 0)+
  annotate("text", x = 3, y = 3.75, label = paste("Black - null model values:
```

```
mean",round(mean(nulls_psi),2)),col="black",hjust = 0)+
  annotate("text", x = 3, y = 3.5, label = "One tailed p-value <0.001",col="black",hjust = 0)

true_phi = calculate_phi(eggs_pca)
nulls_phi = as.vector(unlist(lapply(nulls,calculate_phi)))
phi_p_eggs = mean(nulls_phi>true_phi)

p_phi_eggs = ggplot(data.frame(values=nulls_phi))+
  theme_bw()+
  geom_density(aes(x=values),fill="black",alpha=0.5)+
  geom_vline(xintercept=true_phi,col="red")+
  ggtitle("Figure SM11.5 - Eggs - phi")+
  ylab("Density")+
  xlab("phi")+
  annotate("text", x = 0.2, y = 24, label = paste("Red - true phi
value:",round(true_phi,2)),col="red",hjust = 0)+
  annotate("text", x = 0.2, y = 22, label = paste("Black - null model values:
mean",round(mean(nulls_phi),2)),col="black",hjust = 0)+
  annotate("text", x = 0.2, y = 20, label = "One tailed p-value <0.001",col="black",hjust = 0)

exp_variances = lapply(nulls, function(x){x$eig[,2]})
exp_variances = do.call(rbind,exp_variances)
exp_variances  = tidyr::gather(data.frame(exp_variances),"Comp","value")

true_variances = data.frame(eggs_pca$eig[,2])
colnames(true_variances)=c("value")
true_variances$Component = levels(as.factor(exp_variances$Comp))

p_box_eggs = ggplot(exp_variances)+
  stat_summary(fun.data = f, geom="boxplot",aes(y=value,x=Comp))+
  theme_bw()+
  geom_point(data=true_variances,aes(x=Component,y=value),col="red")+
  scale_x_discrete(labels=1:7)+
  ggtitle("Figure SM11.6 - Eggs - explained variance by component")+
```

```
  ylab("Variance explained %")+
  xlab("Principal Components")+
  annotate("text", x = 3, y = 39, label = "Red: true value",col="red",hjust = 0)+
  annotate("text", x = 3, y = 37, label = "Black: null model values",col="black",hjust = 0)+
  annotate("text", x = 3, y = 35, label = "Whiskers represent 0-95% range of null model
values",col="black",hjust = 0)

find_hull <- function(df) df[chull(df$Dim.1, df$Dim.2), ]
hulls_eggs <- ddply(eggs_ind_plot, "population", find_hull)

eggs_ind_plot$population =
factor(eggs_ind_plot$population,levels=c("AL","AQ","CA","MD","MN", "PL", "US"))

p.scores.eggs=ggplot(eggs_ind_plot)+
  theme_bw()+
  geom_point(aes(x=Dim.1,y=Dim.2,col=population))+
  geom_polygon(data = hulls_eggs, aes(x=Dim.1,y=Dim.2,col=population),alpha=0)+
  coord_fixed(ratio=diff(range(eggs_ind_plot$Dim.1))/diff(range(eggs_ind_plot$Dim.2)))+

theme(panel.grid=element_blank(),legend.position="none",axis.title.x=element_text(size=10)
,axis.title.y=element_text(size=10))+
  xlab(paste("PC1",round(eggs_pca$eig[1,2],2),"%",sep=" "))+
  ylab(paste("PC2",round(eggs_pca$eig[2,2],2),"%",sep=" "))+
  scale_color_manual(values=color_scheme)+
  scale_fill_manual(values=color_scheme)

p.PC1=ggplot(eggs_ind_plot)+
  theme_bw()+

theme(panel.grid=element_blank(),legend.position="none",axis.title.x=element_text(size=10)
,axis.title.y=element_text(size=10),aspect.ratio = 1)+
  geom_boxplot(aes(x=population,y=Dim.1,fill=population))+
  coord_flip()+
  scale_color_manual(values=color_scheme)+
```

```
  scale_fill_manual(values=color_scheme)+
  scale_y_discrete(labels = label_scheme)

p.PC2=ggplot(eggs_ind_plot)+
  theme_bw()+

theme(panel.grid=element_blank(),legend.position="none",axis.title.x=element_text(size=10)
,axis.title.y=element_text(size=10),aspect.ratio = 1)+
  geom_boxplot(aes(x=population,y=Dim.2,fill=population))+
  scale_color_manual(values=color_scheme)+
  scale_fill_manual(values=color_scheme)+
  scale_x_discrete(labels = label_scheme)

p.loadings.eggs=ggplot(eggs_var_plot)+
  theme_bw()+

theme(panel.grid=element_blank(),axis.title.x=element_text(size=10),axis.title.y=element_te
xt(size=10))+
  geom_hline(aes(yintercept=0),col="grey")+
  geom_vline(aes(xintercept=0),col="grey")+

annotate("path",x=cos(seq(0,2*pi,length.out=100)),y=sin(seq(0,2*pi,length.out=100)),col="g
rey")+
  coord_fixed(ratio=1)+
  geom_segment(aes(x=0,y=0,xend=Dim.1,yend=Dim.2), arrow = arrow(length = unit(0.02,
"npc")))+
  geom_text(aes(x=Dim.1,y=Dim.2,label=variable),size=2.5)+
  xlab(paste("PC1",round(eggs_pca$eig[1,2],2),"%",sep=" "))+
  ylab(paste("PC2",round(eggs_pca$eig[2,2],2),"%",sep=" "))

pdf("eggs_PCA.pdf")
ggarrange(p.scores.eggs,p.PC2,p.PC1,p.loadings.eggs,ncol=2,nrow=2,align="hv")
dev.off()
```

```
pdf("SM.11_PCArandomization_tests.pdf", onefile = TRUE)
p_psi_animals
p_phi_animals
p_box_animals
p_psi_eggs
p_phi_eggs
p_box_eggs
dev.off()

adonis.eggs =
adonis(eggs_pca$ind$coord[,1:2]~Species,data.frame(Species=eggs$POPUL),method =
"euclidean",perm=10000)
adonis.animals =
adonis(animals_pca$ind$coord[,1:2]~Species,data.frame(Species=animals$pop),method =
"euclidean",perm=10000)

adonis.eggs.p = pairwise.adonis(eggs_pca$ind$coord[,1:2],eggs$POPUL,sim.method =
"euclidean",perm=10000,p.adjust.m = "BH")
adonis.animals.p = pairwise.adonis(animals_pca$ind$coord[,1:2],animals$pop,sim.method =
"euclidean", perm=10000,p.adjust.m = "BH")

animals.critic.p.BH = adj.alpha.BH(adonis.animals.p$p.value,0.05)
eggs.critic.p.BH = adj.alpha.BH(adonis.eggs.p$p.value,0.05)


write.table(adonis.eggs.p, file="adonis.eggs.csv",sep="\t")
write.table(adonis.animals.p, file="adonis.animals.csv",sep="\t")
```

Supplementary Information 8

SPECIMEN		pop	BLpt	SSIPpt	BTEWpt		M1pt	M2pt	M3pt	Mipt	MRpt
	PRpt	BLm	BTLm	SSIPm	BTEWm		M1m	M2m	M3m	Mim	MRm
	PRm										
PL 1	PL	1037	80.4	22.1	18.8	15.2	20.3	10.1	10.8	75.0	614

	59.2	47.6	13.1	11.1	9	12	6	34.4	44.4			
PL 2	PL	1111	81.1	18.4	17.2	14.9	20.9	8.8	57.2	77.4	633	57
	46.2	10.5	9.8	8.5	11.9	5	32.6	44.1				
PL 3	PL	968	80.2	21.3	19.9	16.2	22.0	9.5	62.3	81.1	549	
	56.7	45.5	12.1	11.3	9.2	12.5	5.4	35.3	46			
PL 4	PL	961	79.7	20.6	18.1	15.3	19.4	8.8	56.9	77.6	535	
	55.7	44.4	11.5	10.1	8.5	10.8	4.9	31.7	43.2			
PL 5	PL	890	80.9	19.8	16.0	12.9	18.9	8.8	53.2	71.1	373	
	41.9	33.9	8.3	6.7	5.4	7.9	3.7	22.3	29.8			
PL 6	PL	1066	79.6	20.8	16.0	15.4	21.6	8.0	55.7	73.3	534	
	50.1	39.9	10.44	8	7.7	10.8	4	27.9	36.7			
PL 7	PL	942	80.7	20.9	17.5	16.5	20.3	9.9	59.0	79.1	468	
	49.7	40.1	10.4	8.7	8.2	10.1	4.9	29.3	39.3			
PL 8	PL	836	79.3	20.7	16.7	13.5	19.8	8.8	55.2	72.7	371	
	44.4	35.2	9.2	7.4	6	8.8	3.9	24.5	32.3			
PL 9	PL	1005	80.7	21.0	18.3	16.3	21.5	9.8	58.6	81.4	556	
	55.3	44.6	11.6	10.1	9	11.9	5.4	32.4	45			
PL 10	PL	887	81.3	20.4	15.1	14.2	19.2	8.9	51.4	69.5	369	
	41.6	33.8	8.5	6.3	5.9	8	3.7	21.4	28.9			
PL 11	PL	920	80.9	20.5	16.0	13.4	20.0	9.4	56.1	74.5	390	
	42.4	34.3	8.7	6.8	5.7	8.5	4	23.8	31.6			
PL 12	PL	928	80.8	21.1	16.7	14.4	19.2	9.0	54.9	74.1	401	
	43.2	34.9	9.1	7.2	6.2	8.3	3.9	23.7	32			
PL 13	PL	883	81.7	20.3	15.4	14.1	19.4	8.5	53.1	71.4	414	
	46.9	38.3	9.5	7.2	6.6	9.1	4	24.9	33.5			
PL 14	PL	1002	79.6	21.0	18.4	15.6	20.5	9.4	58.1	79.5	605	
	60.4	48.1	12.7	11.1	9.4	12.4	5.7	35.1	48			
PL 15	PL	913	81.1	20.1	16.4	14.1	19.3	9.1	53.5	71.4	440	
	48.2	39.1	9.7	7.9	6.8	9.3	4.4	25.8	34.4			
IT 1	IT	966	81.2	22.5	19.1	15.7	20.6	9.2	59.3	76.8	526.9	
	54.5	44.3	12.3	10.4	8.6	11.2	5.0	32.4	41.9			
IT 2	IT	969	80.6	27.9	21.8	19.4	20.5	8.9	63.1	82.4	633.0	
	65.3	52.7	18.2	14.2	12.7	13.4	5.8	41.2	53.8			
IT 3	IT	989	80.3	23.5	17.3	17.8	21.5	8.3	57.4	72.9	522.5	

	52.8	42.4	12.4	9.1	9.4	11.4	4.4	30.3	38.5		
IT 4	IT	1161	80.9	24.4	18.4	14.8	20.0	7.9	57.3	72.6	674.8
	58.1	47.0	14.2	10.7	8.6	11.6	4.6	33.3	42.2		
USA 1	USA	840	80.3	22.7	17.4	14.5	18.9	30.2	48.2	74.1	473.5
	56.4	46.4	12.8	9.8	8.2	10.9	4.4	27.2	42.9		
USA 2	USA	858	80.3	32.1	19.3	17.5	18.9	30.2	58.0	74.1	470.0
	54.8	46.4	17.6	10.6	9.6	10.9	4.4	31.8	42.9		
USA 3	USA	988	79.1	24.0	18.5	14.5	18.7	7.4	59.7	71.5	557.8
	56.5	44.7	13.6	10.5	8.2	10.6	4.2	33.7	40.4		
USA 4	USA	896	80.7	22.1	18.1	14.2	18.4	8.1	58.3	72.2	522.5
	58.3	47.0	12.9	10.6	8.3	10.7	4.7	34.0	42.1		
USA 5	USA	988	81.1	24.9	19.2	16.3	19.5	7.3	57.7	75.9	572.1
	57.9	46.9	14.4	11.1	9.4	11.3	4.2	33.4	44.0		
USA 6	USA	1008	80.4	23.3	20.4	17.7	18.8	7.4	60.1	76.7	590.8
	58.6	47.1	13.7	12.0	10.4	11.0	4.3	35.2	45.0		
USA 7	USA	772	80.3	29.1	11.2	16.0	18.9	30.2	48.6	74.1	491.0
	63.6	46.4	18.5	7.1	10.2	10.9	4.4	30.9	42.9		
USA 8	USA	763	80.3	27.6	14.3	13.1	18.9	30.2	48.7	74.1	442.0
	57.9	46.4	16.0	8.3	7.6	10.9	4.4	28.2	42.9		
USA 9	USA	871	80.5	27.1	22.0	16.4	19.5	8.1	57.1	74.4	495.4
	56.9	45.8	15.4	12.5	9.3	11.1	4.6	32.5	42.3		
Antar 1		ANTAR		1129	81.1	22.1	18.4	15.9	21.1	10.9	59.0
	81.0	719.0	63.7	51.7	14.1	11.7	10.1	13.4	6.9	37.6	51.6
Antar 2		ANTAR		946	80.8	22.5	16.4	14.1	19.8	8.8	54.1
	71.7	488.8	51.7	41.8	11.6	8.5	7.3	10.2	4.6	28.0	37.1
Antar 3		ANTAR		1121	79.4	22.4	18.3	15.7	20.9	9.1	58.1
	79.2	686.6	61.2	48.6	13.7	11.2	9.6	12.8	5.6	35.6	48.5
Antar 4		ANTAR		1030	82.6	22.5	16.8	15.3	20.7	8.4	58.2
	78.2	562.5	54.6	45.1	12.3	9.2	8.4	11.3	4.6	31.8	42.7
Antar 5		ANTAR		1222	79.6	22.3	17.2	15.4	20.3	8.5	56.2
	77.0	702.7	57.5	45.8	12.8	9.9	8.8	11.7	4.9	32.3	44.3
Antar 6		ANTAR		1044	80.5	22.5	18.3	15.5	20.6	8.4	58.2
	78.5	588.7	56.4	45.4	12.7	10.3	8.8	11.6	4.8	32.8	44.3
Antar 7		ANTAR		885	80.1	22.1	16.4	13.9	19.4	8.6	56.4

	76.0	420.5	47.5	38.1	10.5	7.8	6.6	9.2	4.1	26.8	36.1
Antar 8		ANTAR		1030	81.1	22.2	17.9	14.1	20.6	9.0	58.1
	79.4	604.9	58.7	47.6	13.0	10.5	8.3	12.1	5.3	34.1	46.6
Antar 9		ANTAR		885	82.0	22.1	17.1	14.9	20.3	8.6	57.7
	78.0	444.0	50.2	41.1	11.1	8.6	7.5	10.2	4.3	29.0	39.1
Antar 10		ANTAR		920	80.4	22.0	16.9	14.8	19.8	8.6	56.6
	77.9	470.0	51.1	41.1	11.3	8.6	7.6	10.1	4.4	28.9	39.8
Antar 11		ANTAR		980	81.5	22.3	16.5	15.0	20.7	8.9	57.7
	78.4	530.0	54.1	44.1	12.0	8.9	8.1	11.2	4.8	31.2	42.4
Antar 12		ANTAR		989	81.0	22.1	17.4	14.9	20.2	9.0	57.7
	77.9	563.5	57.0	46.1	12.6	9.9	8.5	11.5	5.1	32.9	44.4
Antar 13		ANTAR		1003	80.4	21.8	17.6	15.4	20.6	9.0	57.6
	78.1	593.4	59.2	47.6	12.9	10.4	9.1	12.2	5.3	34.1	46.2
Antar 14		ANTAR		1221	81.6	22.0	18.0	14.7	20.8	8.9	57.5
	78.2	821.5	67.3	54.9	14.8	12.1	9.9	14.0	6.0	38.7	52.6
Antar 15		ANTAR		998	81.0	21.9	17.9	15.4	20.9	9.0	57.8
	78.4	596.9	59.8	48.5	13.1	10.7	9.2	12.5	5.4	34.6	46.9
Antar 16		ANTAR		1142	80.6	22.3	17.6	15.5	20.3	8.2	57.8
	78.3	720.3	63.1	50.9	14.1	11.1	9.8	12.8	5.2	36.5	49.4
Albania 1		ALBANIA		1124.05		76.14	24.57	16.03	12.02	17.37	8.28
	52.36	68.94	649.1	57.8	44.0	14.2	9.3	6.9	10.0	4.8	30.2
	39.8										
Albania 2		ALBANIA		1008.46		79.39	19.21	16.14	13.07	19.08	9.30
	54.92	68.30	518.6	51.4	40.8	9.9	8.3	6.7	9.8	4.8	28.2
	35.1										
Albania 3		ALBANIA		1346.03		77.85	23.57		11.85	18.42	10.90
	53.11	66.98	426.0	31.7	24.6	7.5		3.8	5.8	3.5	16.8
	21.2										
Albania 4		ALBANIA		1113.61		80.84		14.07	13.09	16.63	8.72
	51.70	69.30	599.9	53.9	43.6		7.6	7.1	9.0	4.7	27.9
	37.3										
Albania 5		ALBANIA		993.52	79.23	21.48	13.48	11.01	17.40	8.67	51.78
	65.95	445.9	44.9	35.6	9.6	6.1	4.9	7.8	3.9	23.2	29.6
Albania 6		ALBANIA		1051.64		78.52	23.37	14.86	12.40	16.62	9.66

53.18	67.97	575.9	54.8	43.0	12.8	8.1	6.8	9.1	5.3	29.1	37.2
Albania 7	ALBANIA	947.91	78.93	20.62	16.03	12.83	18.58	10.32	50.76	66.12	479.6
50.6	39.9	10.4	8.1	6.5	9.4	5.2	25.7	33.5			
Albania 8	ALBANIA	1102.28		21.28	16.49	13.98	19.18	10.85			
637.2	57.8		12.3	9.5	8.1	11.1	6.3				
Albania 9	ALBANIA	988.81	80.39	19.90	13.62	12.32	18.57	8.25	50.64	68.39	532.1
53.8	43.3	10.7	7.3	6.6	10.0	4.4	27.3	36.8			
Albania 10	ALBANIA	1124.05		76.14	24.57	16.03	12.02	17.37	8.28	52.36	68.94
649.1	57.8	44.0	14.2	9.3	6.9	10.0	4.8	30.2	39.8		
Albania 11	ALBANIA	1147.31		78.57	20.64	16.81	14.53	18.53	8.49	56.12	70.97
653.7	57.0	44.8	11.8	9.6	8.3	10.6	4.8	32.0	40.4		
Albania 12	ALBANIA	962.28	78.75	21.43	15.26	12.55	16.64	10.16	51.81	69.58	496.3
51.6	40.6	11.1	7.9	6.5	8.6	5.2	26.7	35.9			
Albania 13	ALBANIA	1013.24		77.15	19.94	15.95	13.40	19.44	9.17	53.27	69.75
550.3	54.3	41.9	10.8	8.7	7.3	10.6	5.0	28.9	37.9		
Albania 14	ALBANIA	1020.04		80.46	19.54	14.94	11.94	18.25	8.52	51.97	66.83
512.7	50.3	40.4	9.8	7.5	6.0	9.2	4.3	26.1	33.6		
Albania 15	ALBANIA	982.01	79.36	19.17	15.87	12.82	18.22	8.99	52.42	67.23	514.8
52.4	41.6	10.1	8.3	6.7	9.6	4.7	27.5	35.2			
Albania 16	ALBANIA	1008.72		79.06	21.68	13.81	12.69	18.47	9.35	51.69	67.72
543.5	53.9	42.6	11.7	7.4	6.8	10.0	5.0	27.9	36.5		
CN1	CN	982.97	76.95	21.01	16.47	15.53	17.33	8.04	53.03	68.48	432.80
44.03	33.88	9.25	7.25	6.84	7.63	3.54	23.35	30.15			
CN2	CN	1208.88		79.96	21.44	16.16	15.96	16.51	6.80	56.56	70.78
770.30	63.72	50.95	13.66	10.30	10.17	10.52	4.33	36.04	45.10		
CN3	CN	1226.09		77.78	20.61	17.64	14.51	18.01	6.18	55.28	74.37
772.07	62.97	48.98	12.98	11.11	9.14	11.34	3.89	34.81	46.83		
CN4	CN	1190.13		77.52	21.22	17.04	16.79	19.08	8.35	57.38	74.32

	734.19	61.69	47.82	13.09	10.51	10.36	11.77	5.15	35.40	45.85	
CN5	CN	1106.74		79.87	23.54	18.44	16.77	19.30	7.10	59.52	73.51
	642.24	58.03	46.35	13.66	10.70	9.73	11.20	4.12	34.54	42.66	
CN6	CN	749.37	79.23	19.49	16.80	14.35	16.97	7.59	51.83	68.97	437.18
	58.34	46.22	11.37	9.80	8.37	9.90	4.43	30.24	40.24		
CN7	CN	1244.56		78.74	15.36	12.48	11.25	17.26	7.72	53.12	68.80
	504.67	40.55	31.93	6.23	5.06	4.56	7.00	3.13	21.54	27.90	
CN8	CN	1016.62		78.44	20.40	16.13	14.58	19.47	7.68	54.51	68.99
	499.26	49.11	38.52	10.02	7.92	7.16	9.56	3.77	26.77	33.88	
CN9	CN	976.13	78.64	23.69	16.98	14.46	18.60	7.22	56.75	71.12	494.90
	50.70	39.87	12.01	8.61	7.33	9.43	3.66	28.77	36.06		
CN10	CN	1103.87		79.04	21.53	16.52	14.98	20.94	7.44	59.13	70.88
	636.82	57.69	45.60	12.42	9.53	8.64	12.08	4.29	34.11	40.89	
CN11	CN	1141.33		75.34	21.51	19.92	16.06	18.90	7.13	58.29	71.31
	667.45	58.48	44.06	12.58	11.65	9.39	11.05	4.17	34.09	41.70	
CN12	CN	949.67	79.10	21.99	17.86	13.12	19.42	9.70	55.53	73.54	506.08
	53.29	42.15	11.72	9.52	6.99	10.35	5.17	29.59	39.19		
CN13	CN	898.10	77.97	21.17	13.80	11.43	14.87	8.77		69.77	303.29
	33.77	26.33	7.15	4.66	3.86	5.02	2.96		23.56		
CN14	CN	1057.23		78.42	22.22	14.99	13.67	15.71	7.60	54.24	70.45
	513.39	48.56	38.08	10.79	7.28	6.64	7.63	3.69	26.34	34.21	
CN15	CN	1108.69		76.50	22.73	19.32	15.44	20.19	7.84	57.49	75.82
	649.14	58.55	44.79	13.31	11.31	9.04	11.82	4.59	33.66	44.39	
CN16	CN	1099.08		79.05	23.78	17.21	16.11	19.63	8.83	57.89	75.10
	587.46	53.45	42.25	12.71	9.20	8.61	10.49	4.72	30.94	40.14	
CN17	CN	1008.50		78.74	21.07	16.48	13.37	17.58	8.57	54.03	70.24
	428.41	42.48	33.45	8.95	7.00	5.68	7.47	3.64	22.95	29.84	
MD1	MD	1396.30		78.78	23.36	20.18	15.99	23.97	7.91	65.79	85.62
	953.53	68.29	53.80	15.95	13.78	10.92	16.37	5.40	44.93	58.47	
MD2	MD	1495.84		77.64	23.61	20.35	19.04	23.83	8.57	66.18	86.81
	1036.32		69.28	53.79	16.36	14.10	13.19	16.51	5.94	45.85	60.14
MD3	MD	987.61	79.33	22.64	17.89	14.96	20.37	7.49	63.65	82.82	501.21
	50.75	40.26	11.49	9.08	7.59	10.34	3.80	32.30	42.03		
MD4	MD	1199.75		76.95	24.97	20.13	18.11	22.44	6.68	64.34	84.40

	731.37	60.96	46.91	15.22	12.27	11.04	13.68	4.07	39.22	51.45	
MD5	MD	1118.64		79.76	22.71	17.02	14.94	19.10	6.03	63.39	80.12
	601.38	53.76	42.88	12.21	9.15	8.03	10.27	3.24	34.08	43.07	
MD6	MD	1216.63		77.62	22.66	20.88	19.08	23.50	7.23	68.44	86.40
	791.42	65.05	50.49	14.74	13.58	12.41	15.29	4.70	44.52	56.20	
MD7	MD	1261.94		81.38	24.07	19.41	16.73	22.61	7.06	63.70	81.76
	752.24	59.61	48.51	14.35	11.57	9.97	13.48	4.21	37.97	48.74	
MD8	MD	963.69	77.53	20.99	16.74	15.66	20.95	6.67	63.55	82.01	475.58
	49.35	38.26	10.36	8.26	7.73	10.34	3.29	31.36	40.47		
MD9	MD	979.43	76.53	21.43	18.32	16.75	20.87	6.91	63.23	81.60	484.82
	49.50	37.88	10.61	9.07	8.29	10.33	3.42	31.30	40.39		
MD10	MD	1093.00		76.04	21.36	16.94	15.35	16.74	8.52	60.05	
	78.83	563.33	51.54	39.19	11.01	8.73	7.91	8.63	4.39	30.95	40.63
MD11	MD	1394.86		77.81	25.57	21.81	17.07	23.85	6.38	67.65	
	86.67	854.91	61.29	47.69	15.67	13.37	10.46	14.62	3.91	41.46	53.12
MD12	MD	1187.92		79.19	25.86	20.63	17.62	23.24	6.28	66.78	
	84.32	745.66	62.77	49.71	16.23	12.95	11.06	14.59	3.94	41.92	52.93
MD13	MD	1144.18		76.09	21.03	19.33	16.63	21.30	6.24	67.14	
	86.06	769.92	67.29	51.20	14.15	13.01	11.19	14.33	4.20	45.18	57.91
MD14	MD	1067.73		79.86	23.30	18.77	16.99	20.98	6.28	62.18	
	78.80	580.10	54.33	43.39	12.66	10.20	9.23	11.40	3.41	33.78	42.81
MD15	MD	1313.28		77.94	24.86	20.42	17.96	23.56	6.49	71.72	
	84.27	870.18	66.26	51.64	16.47	13.53	11.90	15.61	4.30	47.52	55.84
MON1	MON	1220.72		78.81	20.63	17.03	15.86	21.33	9.36	57.95	
	81.29	762.83	62.49	49.25	12.89	10.64	9.91	13.33	5.85	36.21	50.80
MON2	MON	1157.98		78.55	20.72	18.26	15.62	20.22	8.32	56.59	
	75.43	715.40	61.78	48.53	12.80	11.28	9.65	12.49	5.14	34.96	46.60
MON3	MON	1254.89		76.29	21.44	17.97	15.65	21.93	10.25	60.23	
	80.85	767.99	61.20	46.69	13.12	11.00	9.58	13.42	6.27	36.86	49.48
MON4	MON	1386.48		76.63	22.47	18.29	16.33	21.56	9.41	60.91	
	80.88	848.39	61.19	46.89	13.75	11.19	9.99	13.19	5.76	37.27	49.49
MON5	MON	1299.29		76.92	23.99	19.56	16.34	21.21	10.60	62.18	
	82.37	864.29	66.52	51.17	15.96	13.01	10.87	14.11	7.05	41.36	54.79
MON6	MON	1336.84		78.08	25.07	21.82	18.30	25.02	10.90	64.89	

	83.50	801.97	59.99	46.84	15.04	13.09	10.98	15.01	6.54	38.93	50.09
MON7		MON	1399.07		76.27	21.17	19.98	18.28	20.63	9.12	61.95
	84.57	857.91	61.32	46.77	12.98	12.25	11.21	12.65	5.59	37.99	51.86
MON8		MON	861.55	78.16	20.22	18.66	14.89	18.07	8.89	56.66	77.74
	552.60	64.14	50.13	12.97	11.97	9.55	11.59	5.70	36.34	49.86	
MON9		MON	998.67		26.84	16.16	14.51	20.20	6.82	57.12	74.13
	674.70	67.56		18.13	10.92	9.80	13.65	4.61	38.59	50.08	
MON10		MON	1265.24		77.22	22.68	20.07	17.35	21.15	9.01	60.39
	80.34	728.65	57.59	44.47	13.06	11.56	9.99	12.18	5.19	34.78	46.27
MON11		MON	974.60	75.88	20.06	16.75	14.13	17.45	8.06	59.21	79.24
	621.50	63.77	48.39	12.79	10.68	9.01	11.13	5.14	37.76	50.53	
MON12		MON	1079.91		77.15	21.68	18.00	18.83	21.97	9.50	62.88
	84.26	625.16	57.89	44.66	12.55	10.42	10.90	12.72	5.50	36.40	48.78
MON13		MON	1090.80		75.71	22.90	19.55	16.20	20.44	8.67	61.80
	81.70	585.98	53.72	40.67	12.30	10.50	8.70	10.98	4.66	33.20	43.89
MON14		MON	1030.99		73.41	21.53	17.81	15.78	19.50	9.04	60.52
	80.12	631.69	61.27	44.98	13.19	10.91	9.67	11.95	5.54	37.08	49.09

Supplementary Information 9

CHARACTER		POPUL		EBD	EFD	PH1	PH2	PH3	PBW1	PBW2
	PBW3	NPEC								
	PL 1	PL	65.2	92.2	15.1	13.4	14	18.7	15.3	15.6	11
	PL 2	PL	64.2	87.9	13.2	14	13.7	16.2	15.8	16.6	11
	PL 3	PL	62.4	91.2	16	15.3	16.2	16.5	15.3	16.5	12
	PL 4	PL	59.4	84.6	14.5	16.4	13.4	17.6	19.2	15.5	11
	PL 5	PL	59.4	84.9	13.5	14.1	13.1	15.7	16.1	14.9	12
	PL 6	PL	68	93.1	15	14.4	16.5	16.6	15.4	16.8	11
	PL 7	PL	64.6	91.5	14.7	14.1	15.2	16.6	14.5	15.6	12
	PL 8	PL	61.5	89.56	15.4	14.8	15.1	16.8	18.4	15.1	12
	PL 9	PL	68.5	87.5	12.1	11.5	12.2	15.9	15.4	14.3	12
	PL 10	PL	64.4	93.7	14.6	15.7	14.8	16.4	17.7	15.6	12
	PL 11	PL	64.9	91	13.6	13.3	13.8	17.6	16.3	16.4	11
	PL 12	PL	68.3	90.2	12	11.7	13.4	16	15.9	17.5	11
	PL 13	PL	72.1	96.6	13.9	13.8	13.4	17.9	18.3	17.5	11

PL 14	PL	74.5	98.3	12.7	13	11.7	15.7	17.4	15.7	12	
PL 15	PL	75	98.7	12.9	13.2	13.3	17.2	16	16	12	
USA 1	USA	62.1	84.1	13.4	12.76	13.76	17.9	15.65	16.5	12	
USA 2	USA	81.86	104.84	9.31	6.25	8.3	18.58	20.29	16.68	11	
USA 3	USA	82.57	105.83	11.52	11.91	12.11	22.16	22.56	18.07	10	
USA 4	USA	69.32	90.15	9.8			14.1			14	
USA 5	USA	67.4	93.2	11.2			17			11	
USA 6	USA	67.8	97.9	10.4			21.3			14	
USA 7	USA	67.8	84.8	10.3			14.7			16	
USA 8	USA	72	92.1	11.7			20.2			17	
USA 9	USA	77	95.9	14.3			21.3			15	
USA 10	USA	73.6	86.3	9.9				17.7			13
USA 11	USA	67.8	90.9	11.2				18.2			17
USA 12	USA	62.8	86.7	11.9				16.5			12
USA 13	USA	69.3	89.4	11.6				14.5			14
USA 14	USA	70.9	96.3	12.5				19.7			14
USA 15	USA	71.3	92.9	13.1				16.1			12
USA 16	USA	70.9	89.4	11.4				17.2			20
USA 17	USA	67.8	94	11.2				18.6			13
USA 18	USA	73.2	89.4	12.1				17.4			15
Antar 1	ANTAR		76.03	98.27	12.39	12.13	12.18	17.2	16.7		
16.7	12										
Antar 2	ANTAR		77.3	100.7	13.5	13.6	13.5	19.6	19.3		
19.6	12										
Antar 3	ANTAR		79.45	99.65	10.55	10.24	10.1	15.2	14.9		
14.6	12										
Antar 4	ANTAR		68.5	90.2	12.86	12.7	12.2	17.3	17.4		
16.8	12										
Antar 5	ANTAR		82	107	13.6	13.4	13.1	19.8	19.2		
19.1	12										
Antar 6	ANTAR		77.3	101.4	11.1	11.6	11.4	14.8	15.1		
14.9	12										
Antar 7	ANTAR		71.5	95.5	12.6	12.8	12.5	16.4	16.9		
16.2	12										

Antar 8	ANTAR	72.56	95.46	12.88	12.81	12.81	17.8	17.2	17.1 12
Antar 9	ANTAR	75.42	98.2	12.4	12.6	12.3	16.4	16.8	16.2 12
Antar 10	ANTAR	77.36	98.32	11.5	11.7	11.8	15.4	15.7	15.9 12
Antar 11	ANTAR	81.66	103.2	11.71	11.33	11.8	15.6	15.8	15.9 12
Antar 12	ANTAR	77.2	98.2	12.4	12.1	11.9	17.1	17.2	17.1 12
Antar 13	ANTAR	75.81	99.29	12.4	12.6	12.7	17.2	17.1	16.9 12
Antar 14	ANTAR	78.1	100.7	13.56	14.45	12.09	18.4	19.1	16.8 12
Antar 15	ANTAR	78.7	100.1	12.1	12.02	13.01	16.7	16.2	17.5 12
Antar 16	ANTAR	75.3	98.98	11.57	12.2	11.5	15.71	16.56	15.3 12
Albania 1	ALBANIA	76.48	89.48	10.19	11.07	11.26	13.48	15.8	16.12 15
Albania 2	ALBANIA	68.11	89.47	15	11.44	12.76	18.75	13.54	17.34 13
Albania 3	ALBANIA	85.89	106.58	14.44	17.33	10.05	16.91	20.27	16 14
Albania 4	ALBANIA	89.34	104.39	10.51	14.58	13.1	15.03	16.97	16.54 14
Albania 5	ALBANIA	95.39	116.86	13.31	14.69	13.1	19.88	17.03	16.28 16
Albania 6	ALBANIA	63.84	89.56	8.79	7.63	10.95	9.71	11.23	10.97 16
Albania 7	ALBANIA	69.55	94.07	15.31	13.2	14.16	17.64	15.73	19.65 14
Albania 8	ALBANIA	63.75	86.17	13.25	12.54	12.96	14.56	14.53	14.42 13

Albania 9		ALBANIA		78.14	95.28	12.1	9.6	11.36	14.67	14.59
14.81	13									
Albania 10		ALBANIA		85.96	100.61	8.01	10.53	12.55	14.2	14.17
13.23	13									
Albania 11		ALBANIA		72.72	100.46	13.64	12.56	12.7	15.52	16.97
16.82	15									
Albania 12		ALBANIA		84.6	103.2	11.41	10.41	10.97	16.73	16.69
16.84	15									
Albania 13		ALBANIA		80.06	106.12	10.83	11.83	13.9	14.69	13.67
16.82	14									
Albania 14		ALBANIA		77.4	96.5	9.53	9.65	10.54	13.73	14.26
15.18	14									
Albania 15		ALBANIA		77.24	98.72	12.85	12.43	12.47	15.53	13.97
13.78	13									
CN1	CN	68.47	93.8	14.18	14.86	15.95	15.56	15.48	16.5	11
CN2	CN	80.48	104.76	12.43	12.99	14.53	15.1	14.79	15.31	10
CN3	CN	69.75	93.64	11.29	13.67	14.46	14.64	14.92	15.93	12
CN4	CN	68.44	96.12	15.59	12.78	14.56	15.8	15.27	17.65	10
CN5	CN	68.65	92.5	16.63	12.69	14.52	17.87	16.38	16.18	12
CN6	CN	62.86	98.24	16.95	15.03	16.61	16.94	17.72	16.63	11
CN7	CN	63.91	94.76	12.98	14.86	13.09	15.14	18.06	17.84	11
CN8	CN	81.14	108.12	13.27	12.32	13.54	13.73	13.59	13.97	12
CN9	CN	79.18	99.99	12.76	12.51	12.92	15.4	15.6	15.92	10
CN10	CN	67.01	95.6	15.17	14.77	14.34	16.66	15.84	18.9	10
CN11	CN	89.73	115.73	15.01	13.79	12.94	16.14	17.03	16.18	10
CN12	CN	89.06	117.48	11.28	11.26	12.2	14.97	13.93	14.45	10
CN13	CN	88.89	112.32							
CN14	CN	79.32	110.58	15	15.65	15.25	15.01	17.21	15.91	11
CN15	CN	67.46	88.71	11.27	12.98	13.28	14.57	15.9	15.38	11
MD1	MD	80.89	108.54	16.37	17.43	17.72	19.45	17.38	16.58	13
MD2	MD	83.41	116.14	14.54	15.91	17.21	15.1	16.53	16.22	12
MD3	MD	79.67	117.7	19.55	17.35	18.44	20.36	19.25	18.01	12
MD4	MD	97.61	141.69	22.98	22.78	21.86	23.83	22.38	20.41	14
MD5	MD	84.37	118.66	16.58	15.55	17.58	17.6	17.41	19.03	12

MD6	MD	78.3	112.72	16.49	17.92	17.99	18.91	21.07	19.3	13
MD7	MD	75.19	117.14	17.32	18.79	20.96	18.25	18.22	20.5	12
MD8	MD	90.34	124.62	19.79	19.46	20.71	22.74	20.53	21.67	11
MD9	MD	81.87	113.18	14.93	17.6	19.88	16.92	19.643	19.56	13
MD10	MD	74.88	110.9	13.65	15.6	14.31	17.12	19.07	17.86	12
MD11	MD	85.37	112.71	15	14.66	14.95	17.03	16.23	17.12	13
MD12	MD	86.73	122.24	17.79	18.65	17.07	18.08	17.28	16.89	11
MD13	MD	75.17	107.65	16.39			16.41			14
MD14	MD	80.77	118.74	16.56	20.34	17.16	17.72	23.5	19.2	12
MD15	MD	88.76	120.15	19.13	18.94	17.26	18.26	18.46	17.76	12
MON1	MON	64.04	87.44	13.23	13.82	16.5	16.58	18.36	18.82	15
MON2	MON	64.08	91.08	14.17	13.86	13.44	16.35	17.18	17.15	
MON3	MON	80.2	112.35	16.71	16.68	16.41	21.49	21.67	20.38	11
MON4	MON	83.47	103.51	12.39						
MON5	MON	74.04	96.36	14.69	16.86	12.9	16.69	19.83	16.06	11
MON6	MON	72.25	90.14	11.04	12.37	12.04	15.15	15.01	15.18	12

Supplementary Information 10

```
#Results from R for egg measurements

Call:
lm(formula = EBD ~ POPUL)

Residuals:
    Min      1Q  Median      3Q     Max
-14.148  -4.606  -0.578   3.937  17.492

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   77.898      1.769  44.031  < 2e-16 ***
POPULANTAR    -1.386      2.463  -0.563  0.57488
POPULCN       -2.941      2.502  -1.176  0.24276
POPULMD        4.991      2.502   1.995  0.04901 *
POPULMON      -4.885      3.310  -1.476  0.14337
POPULPL      -11.738      2.502  -4.691  9.3e-06 ***
POPULUSA      -7.040      2.395  -2.939  0.00416 **
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˙â€™ 0.1
â€˜â€™ 1

Residual standard error: 6.852 on 93 degrees of freedom
Multiple R-squared:  0.3723,     Adjusted R-squared:  0.3318
```

```
F-statistic: 9.192 on 6 and 93 DF,  p-value: 6.771e-08
Pairwise comparisons using t tests with pooled SD

data:  EBD and POPUL

      ALBANIA ANTAR  CN     MD      MON    PL
ANTAR 1.0000  -      -      -       -      -
CN    1.0000  1.0000 -      -       -      -
MD    1.0000  0.2343 0.0433 -       -      -
MON   1.0000  1.0000 1.0000 0.0764  -      -
PL    0.0002  0.0013 0.0143 3.5e-08 0.8645 -
USA   0.0873  0.3848 1.0000 5.2e-05 1.0000 1.0000

P value adjustment method: bonferroni
Shapiro-Wilk normality test

data:  residuals(jaja1)
W = 0.98358, p-value = 0.2497

Call:
lm(formula = EFD ~ POPUL)

Residuals:
    Min      1Q  Median      3Q     Max
-12.780  -4.801  -0.388   3.315  24.171

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  98.4980     1.8248  53.977  < 2e-16 ***
POPULANTAR    0.5751     2.5400   0.226  0.82137
POPULCN       2.9920     2.5807   1.159  0.24927
POPULMD      19.0207     2.5807   7.370 6.85e-11 ***
POPULMON     -1.6847     3.4139  -0.493  0.62284
POPULPL      -7.1007     2.5807  -2.751  0.00713 **
POPULUSA     -6.0469     2.4708  -2.447  0.01627 *
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˙â€™ 0.1
â€˜â€™ 1

Residual standard error: 7.067 on 93 degrees of freedom
Multiple R-squared:  0.5965,    Adjusted R-squared:  0.5705
F-statistic: 22.92 on 6 and 93 DF,  p-value: < 2.2e-16

> pairwise.t.test(EFD, POPUL, p.adj = "bonf")

     Pairwise comparisons using t tests with pooled SD

data:  EFD and POPUL

      ALBANIA ANTAR   CN      MD      MON    PL
ANTAR 1.0000  -       -       -       -      -
CN    1.0000  1.0000  -       -       -      -
MD    1.4e-09 2.4e-09 3.1e-07 -       -      -
MON   1.0000  1.0000  1.0000  5.9e-07 -      -
PL    0.1498  0.0681  0.0037  2.4e-15 1.0000 -
USA   0.3417  0.1604  0.0088  2.1e-15 1.0000 1.0000
```

```
P value adjustment method: bonferroni
> shapiro.test(residuals(jaja2))

     Shapiro-Wilk normality test

data:  residuals(jaja2)
W = 0.95743, p-value = 0.002649

Call:
lm(formula = PH1 ~ POPUL)

Residuals:
    Min      1Q  Median      3Q     Max
-3.9347 -1.1026  0.0294  1.1549  5.8420

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  11.9447     0.4532  26.355  < 2e-16 ***
POPULANTAR    0.3753     0.6309   0.595  0.55333
POPULCN       1.8989     0.6523   2.911  0.00452 **
POPULMD       5.1933     0.6409   8.103  2.2e-12 ***
POPULMON      1.7603     0.8479   2.076  0.04067 *
POPULPL       2.0020     0.6409   3.123  0.00239 **
POPULUSA     -0.4541     0.6137  -0.740  0.46118
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˙â€™ 0.1
â€˜â€™ 1

Residual standard error: 1.755 on 92 degrees of freedom
  (1 observation deleted due to missingness)
Multiple R-squared:  0.5405,     Adjusted R-squared:  0.5105
F-statistic: 18.04 on 6 and 92 DF,  p-value: 9.97e-14

> pairwise.t.test(PH1, POPUL, p.adj = "bonf")

     Pairwise comparisons using t tests with pooled SD

data:  PH1 and POPUL

      ALBANIA ANTAR   CN      MD      MON    PL
ANTAR 1.0000  -       -       -       -      -
CN    0.0949  0.4155  -       -       -      -
MD    4.6e-11 4.3e-10 4.7e-05 -       -      -
MON   0.8541  1.0000  1.0000  0.0023  -      -
PL    0.0502  0.2417  1.0000  6.2e-05 1.0000 -
USA   1.0000  1.0000  0.0062  2.3e-13 0.1851 0.0027

P value adjustment method: bonferroni
> shapiro.test(residuals(wypustka))

     Shapiro-Wilk normality test

data:  residuals(wypustka)
W = 0.98904, p-value = 0.5948
Call:
```

```
lm(formula = PH2 ~ POPUL)

Residuals:
    Min      1Q  Median      3Q     Max
-4.3360 -0.8952 -0.0602  1.1436  5.3640

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  11.9660     0.4645  25.759  < 2e-16 ***
POPULANTAR    0.4265     0.6466   0.660  0.51154
POPULCN       1.6169     0.6686   2.418  0.01802 *
POPULMD       5.9611     0.6686   8.916 2.15e-13 ***
POPULMON      2.7520     0.9291   2.962  0.00409 **
POPULPL       1.9473     0.6570   2.964  0.00407 **
POPULUSA     -1.6593     1.1379  -1.458  0.14894
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˙â€™ 0.1
â€˜â€™ 1

Residual standard error: 1.799 on 75 degrees of freedom
  (18 observations deleted due to missingness)
Multiple R-squared:  0.5988,     Adjusted R-squared:  0.5667
F-statistic: 18.65 on 6 and 75 DF,  p-value: 3.786e-13

> pairwise.t.test(PH2, POPUL, p.adj = "bonf")

     Pairwise comparisons using t tests with pooled SD

data:  PH2 and POPUL

      ALBANIA ANTAR   CN      MD      MON   PL
ANTAR 1.000   -       -       -       -     -
CN    0.378   1.000   -       -       -     -
MD    4.5e-12 4.2e-11 2.7e-07 -       -     -
MON   0.086   0.289   1.000   0.021   -     -
PL    0.085   0.447   1.000   1.3e-06 1.000 -
USA   1.000   1.000   0.114   8.5e-08 0.026 0.046

P value adjustment method: bonferroni
> shapiro.test(residuals(wypustka))

     Shapiro-Wilk normality test

data:  residuals(wypustka)
W = 0.97981, p-value = 0.2235
Call:
lm(formula = PH3 ~ POPUL)

Residuals:
    Min      1Q  Median      3Q     Max
-3.7686 -0.8745 -0.0446  0.8254  3.7814

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept) 12.188667   0.389224  31.315  < 2e-16 ***
POPULANTAR  -0.008042   0.541777  -0.015 0.988197
```

```
POPULCN      1.967762   0.560189   3.513 0.000755 ***
POPULMD      5.889905   0.560189  10.514  < 2e-16 ***
POPULMON     2.069333   0.778448   2.658 0.009595 **
POPULPL      1.798000   0.550446   3.266 0.001644 **
POPULUSA    -0.798667   0.953400  -0.838 0.404859
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˙â€™ 0.1
â€˜â€™ 1

Residual standard error: 1.507 on 75 degrees of freedom
  (18 observations deleted due to missingness)
Multiple R-squared:  0.6763,     Adjusted R-squared:  0.6504
F-statistic: 26.11 on 6 and 75 DF,  p-value: < 2.2e-16

> pairwise.t.test(PH3, POPUL, p.adj = "bonf")

     Pairwise comparisons using t tests with pooled SD

data:  PH3 and POPUL

      ALBANIA ANTAR   CN      MD      MON     PL
ANTAR 1.00000 -       -       -       -       -
CN    0.01586 0.01269 -       -       -       -
MD    4.4e-15 2.1e-15 3.2e-08 -       -       -
MON   0.20150 0.18500 1.00000 0.00013 -       -
PL    0.03452 0.02802 1.00000 5.2e-09 1.00000 -
USA   1.00000 1.00000 0.10740 2.2e-08 0.23244 0.16861

P value adjustment method: bonferroni
> shapiro.test(residuals(wypustka))

     Shapiro-Wilk normality test

data:  residuals(wypustka)
W = 0.98984, p-value = 0.7725

#Results from R for adult single characters
Call:
lm(formula = BLm ~ pop)

Residuals:
    Min      1Q  Median      3Q     Max
-260.22  -70.46   -3.61   78.55  322.19

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   549.04      28.64  19.173  < 2e-16 ***
popANTAR       45.54      40.50   1.124 0.263583
popCN          14.47      39.90   0.363 0.717605
popIT          40.24      64.03   0.628 0.531163
popMD         165.09      41.17   4.010 0.000118 ***
popMON        168.04      41.92   4.009 0.000119 ***
popPL         -65.57      41.17  -1.593 0.114431
popUSA        -36.26      47.73  -0.760 0.449258
---
```

```
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˜â€™ 0.1
â€˜â€™ 1

Residual standard error: 114.5 on 98 degrees of freedom
Multiple R-squared:  0.3587,     Adjusted R-squared:  0.3129
F-statistic: 7.832 on 7 and 98 DF,  p-value: 1.633e-07

> pairwise.t.test(BLm, pop, p.adj = "bonf")

     Pairwise comparisons using t tests with pooled SD

data:  BLm and pop

      ALBANIA ANTAR  CN     IT     MD      MON     PL
ANTAR 1.0000  -      -      -      -       -       -
CN    1.0000  1.0000 -      -      -       -       -
IT    1.0000  1.0000 1.0000 -      -       -       -
MD    0.0033  0.1274 0.0096 1.0000 -       -       -
MON   0.0033  0.1208 0.0095 1.0000 1.0000  -       -
PL    1.0000  0.2294 1.0000 1.0000 7.9e-06 8.9e-06 -
USA   1.0000  1.0000 1.0000 1.0000 0.0018  0.0018  1.0000

P value adjustment method: bonferroni
> shapiro.test(residuals(m1))

     Shapiro-Wilk normality test

data:  residuals(m1)
W = 0.99355, p-value = 0.9024

Call:
lm(formula = BTLm ~ pop)

Residuals:
     Min       1Q   Median       3Q      Max
-20.4569  -3.4909   0.2939   5.1052  11.0488

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  52.1069     1.5773  33.036  < 2e-16 ***
popANTAR      4.9563     2.2306   2.222 0.028589 *
popCN         0.5643     2.1976   0.257 0.797883
popIT         5.5806     3.5269   1.582 0.116803
popMD         7.2285     2.2675   3.188 0.001924 **
popMON        9.3524     2.3089   4.051 0.000102 ***
popPL        -1.9269     2.2675  -0.850 0.397515
popUSA        5.7653     2.6288   2.193 0.030662 *
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˜â€™ 0.1
â€˜â€™ 1

Residual standard error: 6.309 on 98 degrees of freedom
Multiple R-squared:  0.2869,     Adjusted R-squared:  0.236
F-statistic: 5.633 on 7 and 98 DF,  p-value: 1.755e-05

> pairwise.t.test(BTLm, pop, p.adj = "bonf")
```

```
     Pairwise comparisons using t tests with pooled SD

data:  BTLm and pop

      ALBANIA ANTAR   CN      IT      MD      MON     PL
ANTAR 0.80048 -       -       -       -       -       -
CN    1.00000 1.00000 -       -       -       -       -
IT    1.00000 1.00000 1.00000 -       -       -       -
MD    0.05387 1.00000 0.10124 1.00000 -       -       -
MON   0.00286 1.00000 0.00570 1.00000 1.00000 -       -
PL    1.00000 0.08610 1.00000 1.00000 0.00378 0.00015 -
USA   0.85853 1.00000 1.00000 1.00000 1.00000 1.00000 0.13218

P value adjustment method: bonferroni
> shapiro.test(residuals(m2))

     Shapiro-Wilk normality test

data:  residuals(m2)
W = 0.96767, p-value = 0.0109

Call:
lm(formula = SSIPm ~ pop)

Residuals:
     Min       1Q   Median       3Q      Max
-16.0680  -2.3406   0.0921   3.2620   9.7012

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  40.7080     1.2802  31.798  < 2e-16 ***
popANTAR      5.4357     1.7820   3.050  0.00295 **
popCN         0.5408     1.7564   0.308  0.75881
popIT         5.8695     2.7901   2.104  0.03802 *
popMD         5.6653     1.8105   3.129  0.00232 **
popMON        6.1720     1.8788   3.285  0.00142 **
popPL        -0.3147     1.8105  -0.174  0.86239
popUSA        5.6598     2.0905   2.707  0.00803 **
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˙â€™ 0.1
â€˜â€™ 1

Residual standard error: 4.958 on 96 degrees of freedom
  (2 observations deleted due to missingness)
Multiple R-squared:  0.2597,     Adjusted R-squared:  0.2057
F-statistic: 4.812 on 7 and 96 DF,  p-value: 0.0001125

> pairwise.t.test(SSIPm, pop, p.adj = "bonf")

     Pairwise comparisons using t tests with pooled SD

data:  SSIPm and pop

      ALBANIA ANTAR CN    IT    MD    MON   PL
ANTAR 0.083   -     -     -     -     -     -
```

```
CN    1.000   0.157 -     -     -     -     -
IT    1.000   1.000 1.000 -     -     -     -
MD    0.065   1.000 0.123 1.000 -     -     -
MON   0.040   1.000 0.075 1.000 1.000 -     -
PL    1.000   0.048 1.000 0.813 0.038 0.023 -
USA   0.225   1.000 0.391 1.000 1.000 1.000 0.146

P value adjustment method: bonferroni
> shapiro.test(residuals(m3))

     Shapiro-Wilk normality test

data:  residuals(m3)
W = 0.96927, p-value = 0.01606

Call:
lm(formula = BTEWm ~ pop)

Residuals:
    Min      1Q  Median      3Q     Max
-5.0582 -1.2560 -0.0663  1.3593  4.4493

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  11.1193     0.4842  22.964  < 2e-16 ***
popANTAR      1.5469     0.6740   2.295 0.023878 *
popCN         0.1689     0.6643   0.254 0.799844
popIT         3.1532     1.0553   2.988 0.003558 **
popMD         2.7127     0.6848   3.961 0.000142 ***
popMON        2.5614     0.6969   3.675 0.000389 ***
popPL        -0.7633     0.6848  -1.115 0.267722
popUSA        3.8618     0.7907   4.884  4.1e-06 ***
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˙â€™ 0.1
â€˜â€™ 1

Residual standard error: 1.875 on 97 degrees of freedom
  (1 observation deleted due to missingness)
Multiple R-squared:  0.4098,    Adjusted R-squared:  0.3672
F-statistic:  9.62 on 7 and 97 DF,  p-value: 4.889e-09

> pairwise.t.test(BTEWm, pop, p.adj="bonf")

     Pairwise comparisons using t tests with pooled SD

data:  BTEWm and pop

      ALBANIA ANTAR   CN      IT      MD      MON     PL
ANTAR 0.66858 -       -       -       -       -       -
CN    1.00000 1.00000 -       -       -       -       -
IT    0.09961 1.00000 0.14376 -       -       -       -
MD    0.00398 1.00000 0.00638 1.00000 -       -       -
MON   0.01090 1.00000 0.01756 1.00000 1.00000 -       -
PL    1.00000 0.02505 1.00000 0.00963 5.2e-05 0.00018 -
USA   0.00011 0.10746 0.00018 1.00000 1.00000 1.00000 1.9e-06
```

```
P value adjustment method: bonferroni
> shapiro.test(residuals(m4))

     Shapiro-Wilk normality test

data:  residuals(m4)
W = 0.99325, p-value = 0.887

Call:
lm(formula = M1m ~ pop)

Residuals:
    Min      1Q  Median      3Q     Max
-4.2465 -0.9865  0.0533  1.3340  3.1100

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   8.1960     0.4265  19.217  < 2e-16 ***
popANTAR      1.7665     0.5937   2.976  0.00369 **
popCN         0.7105     0.5851   1.214  0.22763
popIT         2.9240     0.9295   3.146  0.00220 **
popMD         3.3140     0.6032   5.494 3.16e-07 ***
popMON        3.1911     0.6138   5.199 1.11e-06 ***
popPL         0.4507     0.6032   0.747  0.45676
popUSA        2.0707     0.6965   2.973  0.00372 **
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˙â€™ 0.1
â€˜â€™ 1

Residual standard error: 1.652 on 97 degrees of freedom
  (1 observation deleted due to missingness)
Multiple R-squared:  0.3775,     Adjusted R-squared:  0.3326
F-statistic: 8.403 on 7 and 97 DF,  p-value: 5.322e-08

> pairwise.t.test(M1m, pop, p.adj="bonf")

     Pairwise comparisons using t tests with pooled SD

data:  M1m and pop

      ALBANIA ANTAR   CN      IT      MD      MON     PL
ANTAR 0.10334 -       -       -       -       -       -
CN    1.00000 1.00000 -       -       -       -       -
IT    0.06161 1.00000 0.49765 -       -       -       -
MD    8.8e-06 0.29632 0.00064 1.00000 -       -       -
MON   3.1e-05 0.57251 0.00192 1.00000 1.00000 -       -
PL    1.00000 0.81185 1.00000 0.25542 0.00020 0.00061 -
USA   0.10412 1.00000 1.00000 1.00000 1.00000 1.00000 0.61876

P value adjustment method: bonferroni
> shapiro.test(residuals(m5))

     Shapiro-Wilk normality test

data:  residuals(m5)
W = 0.98552, p-value = 0.3129
```

```
Call:
lm(formula = M2m ~ pop)

Residuals:
    Min      1Q  Median      3Q     Max
-3.9347 -0.8628  0.0550  0.9974  3.1287

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   6.6200     0.3506  18.883  < 2e-16 ***
popANTAR      1.9719     0.4958   3.977 0.000134 ***
popCN         1.1747     0.4884   2.405 0.018050 *
popIT         3.1875     0.7839   4.066 9.66e-05 ***
popMD         3.4413     0.5040   6.828 7.25e-10 ***
popMON        3.3664     0.5132   6.560 2.55e-09 ***
popPL         0.8533     0.5040   1.693 0.093600 .
popUSA        2.3989     0.5843   4.106 8.36e-05 ***
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˙â€™ 0.1
â€˜â€™ 1

Residual standard error: 1.402 on 98 degrees of freedom
Multiple R-squared:   0.45,Adjusted R-squared:  0.4107
F-statistic: 11.45 on 7 and 98 DF,  p-value: 1.51e-10

> pairwise.t.test(M2m, pop, p.adj="bonf")

      Pairwise comparisons using t tests with pooled SD

data:  M2m and pop

      ALBANIA ANTAR   CN      IT      MD      MON     PL
ANTAR 0.00374 -       -       -       -       -       -
CN    0.50541 1.00000 -       -       -       -       -
IT    0.00271 1.00000 0.31574 -       -       -       -
MD    2.0e-08 0.12318 0.00041 1.00000 -       -       -
MON   7.2e-08 0.21785 0.00101 1.00000 1.00000 -       -
PL    1.00000 0.80545 1.00000 0.10869 5.6e-05 0.00015 -
USA   0.00234 1.00000 1.00000 1.00000 1.00000 1.00000 0.29010

P value adjustment method: bonferroni
> shapiro.test(residuals(m6))

      Shapiro-Wilk normality test

data:  residuals(m6)
W = 0.99388, p-value = 0.9208

Call:
lm(formula = M3m ~ pop)

Residuals:
    Min      1Q  Median      3Q     Max
-4.6424 -0.8453  0.0100  1.1212  3.4573
```

```
Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   9.4012     0.4143  22.693  < 2e-16 ***
popANTAR      2.2775     0.5859   3.887 0.000184 ***
popCN         0.2611     0.5772   0.452 0.652005
popIT         2.5038     0.9263   2.703 0.008105 **
popMD         3.6514     0.5956   6.131 1.84e-08 ***
popMON        3.3416     0.6064   5.510 2.90e-07 ***
popPL         0.7521     0.5956   1.263 0.209650
popUSA        1.5288     0.6905   2.214 0.029139 *
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˙â€™ 0.1
â€˜â€™ 1

Residual standard error: 1.657 on 98 degrees of freedom
Multiple R-squared:  0.4237,     Adjusted R-squared:  0.3826
F-statistic: 10.29 on 7 and 98 DF,  p-value: 1.288e-09

> pairwise.t.test(M3m, pop, p.adj="bonf")

     Pairwise comparisons using t tests with pooled SD

data:  M3m and pop

      ALBANIA ANTAR   CN      IT      MD      MON     PL
ANTAR 0.00516 -       -       -       -       -       -
CN    1.00000 0.02008 -       -       -       -       -
IT    0.22694 1.00000 0.46720 -       -       -       -
MD    5.1e-07 0.64856 2.5e-06 1.00000 -       -       -
MON   8.1e-06 1.00000 3.8e-05 1.00000 1.00000 -       -
PL    1.00000 0.33465 1.00000 1.00000 0.00016 0.00162 -
USA   0.81590 1.00000 1.00000 1.00000 0.08546 0.33534 1.00000

P value adjustment method: bonferroni
> shapiro.test(residuals(m7))

     Shapiro-Wilk normality test

data:  residuals(m7)
W = 0.98011, p-value = 0.1127
Call:
lm(formula = Mim ~ pop)

Residuals:
     Min       1Q   Median       3Q      Max
-1.34312 -0.46088 -0.02444  0.30667  1.85312

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   4.7931     0.1670  28.694  < 2e-16 ***
popANTAR      0.2838     0.2362   1.201  0.23260
popCN        -0.7196     0.2327  -3.092  0.00259 **
popIT         0.1469     0.3735   0.393  0.69501
popMD        -0.6451     0.2401  -2.686  0.00848 **
popMON        0.8169     0.2445   3.341  0.00118 **
popPL        -0.1998     0.2401  -0.832  0.40745
```

```
popUSA       -0.4087     0.2784  -1.468  0.14533
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˜â€™ 0.1
â€˜â€™ 1

Residual standard error: 0.6682 on 98 degrees of freedom
Multiple R-squared:  0.3772,     Adjusted R-squared:  0.3327
F-statistic:  8.48 on 7 and 98 DF,  p-value: 4.386e-08

> pairwise.t.test(Mim, pop, p.adj="bonf")

     Pairwise comparisons using t tests with pooled SD

data:  Mim and pop

      ALBANIA ANTAR  CN       IT     MD       MON    PL
ANTAR 1.0000  -      -        -      -        -      -
CN    0.0725  0.0011 -        -      -        -      -
IT    1.0000  1.0000 0.6066   -      -        -      -
MD    0.2376  0.0055 1.0000   1.0000 -        -      -
MON   0.0331  0.8858 1.7e-07  1.0000 1.5e-06  -      -
PL    1.0000  1.0000 0.8525   1.0000 1.0000   0.0024 -
USA   1.0000  0.4079 1.0000   1.0000 1.0000   0.0012 1.0000

P value adjustment method: bonferroni
> shapiro.test(residuals(m8))

     Shapiro-Wilk normality test

data:  residuals(m8)
W = 0.97107, p-value = 0.02037

Call:
lm(formula = MRm ~ pop)

Residuals:
     Min       1Q   Median       3Q      Max
-10.3733  -3.4297   0.1004   3.0668   8.6973

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   27.183      1.098  24.749  < 2e-16 ***
popANTAR       5.615      1.529   3.673 0.000394 ***
popCN          3.013      1.529   1.971 0.051636 .
popIT          7.099      2.394   2.966 0.003811 **
popMD         11.639      1.553   7.493 3.30e-11 ***
popMON         9.797      1.581   6.198 1.43e-08 ***
popPL          1.157      1.553   0.745 0.458303
popUSA         4.696      1.794   2.618 0.010279 *
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˜â€™ 0.1
â€˜â€™ 1

Residual standard error: 4.254 on 96 degrees of freedom
  (2 observations deleted due to missingness)
Multiple R-squared:  0.4893,     Adjusted R-squared:  0.4521
```

```
F-statistic: 13.14 on 7 and 96 DF,  p-value: 9.082e-12

> pairwise.t.test(MRm, pop, p.adj="bonf")

     Pairwise comparisons using t tests with pooled SD

data:  MRm and pop

      ALBANIA ANTAR   CN      IT      MD      MON     PL
ANTAR 0.01104 -       -       -       -       -       -
CN    1.00000 1.00000 -       -       -       -       -
IT    0.10670 1.00000 1.00000 -       -       -       -
MD    9.2e-10 0.00433 4.7e-06 1.00000 -       -       -
MON   4.0e-07 0.23849 0.00092 1.00000 1.00000 -       -
PL    1.00000 0.12342 1.00000 0.41396 3.2e-08 1.0e-05 -
USA   0.28781 1.00000 1.00000 1.00000 0.00553 0.16958 1.00000

P value adjustment method: bonferroni
> shapiro.test(residuals(m9))

     Shapiro-Wilk normality test

data:  residuals(m9)
W = 0.98508, p-value = 0.2959

Call:
lm(formula = PRm ~ pop)

Residuals:
     Min       1Q   Median       3Q      Max
-14.2394  -3.5467   0.0793   3.5067  10.5267

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   35.324      1.447  24.406  < 2e-16 ***
popANTAR       9.171      2.015   4.552 1.54e-05 ***
popCN          2.475      1.986   1.247  0.21555
popIT          8.764      3.154   2.778  0.00656 **
popMD         14.289      2.047   6.981 3.65e-10 ***
popMON        14.077      2.083   6.758 1.05e-09 ***
popPL          2.623      2.047   1.281  0.20314
popUSA         7.467      2.364   3.159  0.00211 **
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˙â€™ 0.1
â€˜â€™ 1

Residual standard error: 5.606 on 97 degrees of freedom
  (1 observation deleted due to missingness)
Multiple R-squared:  0.4925,     Adjusted R-squared:  0.4559
F-statistic: 13.45 on 7 and 97 DF,  p-value: 4.992e-12

> pairwise.t.test(PRm, pop, p.adj="bonf")

     Pairwise comparisons using t tests with pooled SD

data:  PRm and pop
```

```
      ALBANIA ANTAR   CN      IT      MD      MON     PL
ANTAR 0.00043 -       -       -       -       -       -
CN    1.00000 0.02492 -       -       -       -       -
IT    0.18379 1.00000 1.00000 -       -       -       -
MD    1.0e-08 0.35428 1.2e-06 1.00000 -       -       -
MON   2.9e-08 0.52400 3.1e-06 1.00000 1.00000 -       -
PL    1.00000 0.04437 1.00000 1.00000 3.6e-06 8.7e-06 -
USA   0.05905 1.00000 0.93023 1.00000 0.13446 0.19363 1.00000

P value adjustment method: bonferroni
> shapiro.test(residuals(m10))

     Shapiro-Wilk normality test

data:  residuals(m10)
W = 0.98174, p-value = 0.1577

Call:
lm(formula = BLpt ~ pop)

Residuals:
    Min      1Q  Median      3Q     Max
-313.45  -72.60  -12.49   84.99  307.79

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 1058.374     30.805  34.357  < 2e-16 ***
popANTAR     -24.311     43.565  -0.558  0.57808
popCN          4.447     42.920   0.104  0.91768
popIT        -37.124     68.882  -0.539  0.59115
popMD        129.680     44.285   2.928  0.00424 **
popMON       109.986     45.094   2.439  0.01653 *
popPL       -101.774     44.285  -2.298  0.02368 *
popUSA      -171.263     51.342  -3.336  0.00120 **
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˙â€™ 0.1
â€˜â€™ 1

Residual standard error: 123.2 on 98 degrees of freedom
Multiple R-squared:  0.3653,    Adjusted R-squared:  0.3199
F-statistic: 8.056 on 7 and 98 DF,  p-value: 1.032e-07

> pairwise.t.test(BLpt, pop, p.adj = "bonf")

     Pairwise comparisons using t tests with pooled SD

data:  BLpt and pop

      ALBANIA ANTAR   CN      IT      MD      MON     PL
ANTAR 1.00000 -       -       -       -       -       -
CN    1.00000 1.00000 -       -       -       -       -
IT    1.00000 1.00000 1.00000 -       -       -       -
MD    0.11868 0.02119 0.14122 0.50463 -       -       -
MON   0.46272 0.10233 0.54838 1.00000 1.00000 -       -
PL    0.66300 1.00000 0.46949 1.00000 3.9e-05 0.00032 -
```

```
USA   0.03367 0.14404 0.02250 1.00000 2.4e-06 1.7e-05 1.00000

P value adjustment method: bonferroni
> shapiro.test(residuals(m11))

     Shapiro-Wilk normality test

data:  residuals(m11)
W = 0.99063, p-value = 0.6784

Call:
lm(formula = SSIPpt ~ pop)

Residuals:
    Min      1Q  Median      3Q     Max
-3.4400 -0.5499  0.1169  0.6407  3.2159

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  78.7194     0.2996 262.769  < 2e-16 ***
popANTAR      2.1368     0.4170   5.124 1.54e-06 ***
popCN        -0.4090     0.4110  -0.995  0.32219
popIT         2.0306     0.6529   3.110  0.00246 **
popMD        -0.5563     0.4237  -1.313  0.19229
popMON       -1.8666     0.4397  -4.246 5.04e-05 ***
popPL         1.8139     0.4237   4.282 4.40e-05 ***
popUSA        1.6139     0.4892   3.299  0.00136 **
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˙â€™ 0.1
â€˜â€™ 1

Residual standard error: 1.16 on 96 degrees of freedom
  (2 observations deleted due to missingness)
Multiple R-squared:  0.6028,     Adjusted R-squared:  0.5738
F-statistic: 20.81 on 7 and 96 DF,  p-value: < 2.2e-16

> pairwise.t.test(SSIPpt, pop, p.adj = "bonf")

     Pairwise comparisons using t tests with pooled SD

data:  SSIPpt and pop

      ALBANIA ANTAR   CN      IT      MD      MON     PL
ANTAR 4.3e-05 -       -       -       -       -       -
CN    1.00000 2.5e-07 -       -       -       -       -
IT    0.06895 1.00000 0.00753 -       -       -       -
MD    1.00000 1.2e-07 1.00000 0.00400 -       -       -
MON   0.00141 1.8e-13 0.02667 1.7e-06 0.10215 -       -
PL    0.00123 1.00000 1.3e-05 1.00000 5.8e-06 1.3e-11 -
USA   0.03813 1.00000 0.00150 1.00000 0.00068 1.4e-08 1.00000

P value adjustment method: bonferroni
> shapiro.test(residuals(m12))

     Shapiro-Wilk normality test
```

```
data:  residuals(m12)
W = 0.97979, p-value = 0.1129

Call:
lm(formula = BTEWpt ~ pop)

Residuals:
    Min      1Q  Median      3Q     Max
-5.9756 -0.8000 -0.0937  0.5000  6.2222

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 21.39825    0.46097  46.420  < 2e-16 ***
popANTAR     0.79550    0.64164   1.240  0.21805
popCN       -0.05892    0.63245  -0.093  0.92597
popIT        3.17675    1.00466   3.162  0.00209 **
popMD        1.82992    0.65191   2.807  0.00604 **
popMON       0.84319    0.66345   1.271  0.20680
popPL       -0.79825    0.65191  -1.224  0.22374
popUSA       4.47953    0.75276   5.951 4.24e-08 ***
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˙â€™ 0.1
â€˜â€™ 1

Residual standard error: 1.785 on 97 degrees of freedom
  (1 observation deleted due to missingness)
Multiple R-squared:  0.416,Adjusted R-squared:  0.3738
F-statistic:  9.87 on 7 and 97 DF,  p-value: 3.028e-09

> pairwise.t.test(BTEWpt, pop, p.adj="bonf")

      Pairwise comparisons using t tests with pooled SD

data:  BTEWpt and pop

      ALBANIA ANTAR   CN      IT      MD      MON     PL
ANTAR 1.00000 -       -       -       -       -       -
CN    1.00000 1.00000 -       -       -       -       -
IT    0.05856 0.53134 0.04287 -       -       -       -
MD    0.16925 1.00000 0.10002 1.00000 -       -       -
MON   1.00000 1.00000 1.00000 0.65159 1.00000 -       -
PL    1.00000 0.41192 1.00000 0.00405 0.00309 0.42264 -
USA   1.2e-06 8.7e-05 4.5e-07 1.00000 0.01846 0.00018 8.9e-09

P value adjustment method: bonferroni
> shapiro.test(residuals(m13))

      Shapiro-Wilk normality test

data:  residuals(m13)
W = 0.95386, p-value = 0.001086

Call:
lm(formula = M1pt ~ pop)

Residuals:
```

```
    Min      1Q  Median      3Q     Max
-6.6222 -0.7578  0.0790  0.9812  4.1778

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  15.2938     0.4260  35.902  < 2e-16 ***
popANTAR      2.1250     0.5930   3.584 0.000532 ***
popCN         1.4262     0.5845   2.440 0.016497 *
popIT         3.8562     0.9284   4.154 7.05e-05 ***
popMD         3.9615     0.6024   6.576 2.45e-09 ***
popMON        3.2705     0.6131   5.334 6.27e-07 ***
popPL         1.8062     0.6024   2.998 0.003450 **
popUSA        2.5284     0.6956   3.635 0.000448 ***
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˙â€™ 0.1
â€˜â€™ 1

Residual standard error: 1.65 on 97 degrees of freedom
  (1 observation deleted due to missingness)
Multiple R-squared:  0.3768,     Adjusted R-squared:  0.3319
F-statistic:  8.38 on 7 and 97 DF,  p-value: 5.57e-08

> pairwise.t.test(M1pt, pop, p.adj="bonf")

     Pairwise comparisons using t tests with pooled SD

data:  M1pt and pop

      ALBANIA ANTAR   CN      IT      MD      MON     PL
ANTAR 0.01490 -       -       -       -       -       -
CN    0.46191 1.00000 -       -       -       -       -
IT    0.00197 1.00000 0.26287 -       -       -       -
MD    6.9e-08 0.07154 0.00099 1.00000 -       -       -
MON   1.8e-05 1.00000 0.07149 1.00000 1.00000 -       -
PL    0.09660 1.00000 1.00000 0.82873 0.01521 0.52815 -
USA   0.01253 1.00000 1.00000 1.00000 1.00000 1.00000 1.00000

P value adjustment method: bonferroni
> shapiro.test(residuals(m14))

     Shapiro-Wilk normality test

data:  residuals(m14)
W = 0.9651, p-value = 0.007242

Call:
lm(formula = M2pt ~ pop)

Residuals:
    Min      1Q  Median      3Q     Max
-3.3649 -0.7859 -0.0310  0.7907  2.5388

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  12.6567     0.3211  39.414  < 2e-16 ***
popANTAR      2.3746     0.4541   5.229 9.66e-07 ***
```

```
popCN         1.9536     0.4474   4.367 3.13e-05 ***
popIT         4.2683     0.7180   5.944 4.26e-08 ***
popMD         4.2005     0.4616   9.099 1.09e-14 ***
popMON        3.6334     0.4701   7.729 9.54e-12 ***
popPL         2.1433     0.4616   4.643 1.07e-05 ***
popUSA        2.9211     0.5352   5.458 3.63e-07 ***
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˙â€™ 0.1
â€˜â€™ 1

Residual standard error: 1.284 on 98 degrees of freedom
Multiple R-squared:  0.5303,     Adjusted R-squared:  0.4967
F-statistic:  15.8 on 7 and 98 DF,  p-value: 9.854e-14

> pairwise.t.test(M2pt, pop, p.adj="bonf")

     Pairwise comparisons using t tests with pooled SD

data:  M2pt and pop

      ALBANIA ANTAR   CN      IT      MD      MON     PL
ANTAR 2.7e-05 -       -       -       -       -       -
CN    0.00088 1.00000 -       -       -       -       -
IT    1.2e-06 0.27208 0.04531 -       -       -       -
MD    3.1e-13 0.00405 9.1e-05 1.00000 -       -       -
MON   2.7e-10 0.24328 0.01296 1.00000 1.00000 -       -
PL    0.00030 1.00000 1.00000 0.11467 0.00081 0.06619 -
USA   1.0e-05 1.00000 1.00000 1.00000 0.56384 1.00000 1.00000

P value adjustment method: bonferroni
> shapiro.test(residuals(m15))

     Shapiro-Wilk normality test

data:  residuals(m15)
W = 0.9892, p-value = 0.5587

Call:
lm(formula = M3pt ~ pop)

Residuals:
    Min      1Q  Median      3Q     Max
-5.0784 -0.6722  0.0511  0.6713  4.2575

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  18.0476     0.3359  53.721  < 2e-16 ***
popANTAR      2.3899     0.4751   5.030 2.22e-06 ***
popCN         0.1735     0.4681   0.371  0.71167
popIT         2.6024     0.7512   3.464  0.00079 ***
popMD         3.7751     0.4830   7.817 6.23e-12 ***
popMON        2.7158     0.4918   5.522 2.75e-07 ***
popPL         2.1058     0.4830   4.360 3.21e-05 ***
popUSA        0.8969     0.5599   1.602  0.11242
---
```

```
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˜â€™ 0.1
â€˜â€™ 1

Residual standard error: 1.344 on 98 degrees of freedom
Multiple R-squared:  0.5094,     Adjusted R-squared:  0.4743
F-statistic: 14.53 on 7 and 98 DF,  p-value: 7.54e-13

> pairwise.t.test(M3pt, pop, p.adj="bonf")

     Pairwise comparisons using t tests with pooled SD

data:  M3pt and pop

      ALBANIA ANTAR   CN      IT      MD      MON     PL
ANTAR 6.2e-05 -       -       -       -       -       -
CN    1.00000 0.00021 -       -       -       -       -
IT    0.02212 1.00000 0.04394 -       -       -       -
MD    1.7e-10 0.14161 5.9e-10 1.00000 -       -       -
MON   7.7e-06 1.00000 2.6e-05 1.00000 1.00000 -       -
PL    0.00090 1.00000 0.00278 1.00000 0.02715 1.00000 -
USA   1.00000 0.25103 1.00000 1.00000 5.1e-05 0.05730 0.99042

P value adjustment method: bonferroni
> shapiro.test(residuals(m16))

     Shapiro-Wilk normality test

data:  residuals(m16)
W = 0.95775, p-value = 0.001934

Call:
lm(formula = Mipt ~ pop)

Residuals:
     Min       1Q   Median       3Q      Max
-10.3778  -0.5772  -0.1088   0.3753  12.5222

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   9.2436     0.8704  10.620  < 2e-16 ***
popANTAR     -0.3749     1.2310  -0.305   0.7614
popCN        -1.4472     1.2127  -1.193   0.2356
popIT        -0.6686     1.9463  -0.344   0.7319
popMD        -2.2625     1.2513  -1.808   0.0737 .
popMON       -0.1039     1.2742  -0.082   0.9352
popPL        -0.1236     1.2513  -0.099   0.9215
popUSA        8.4341     1.4507   5.814 7.64e-08 ***
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˜â€™ 0.1
â€˜â€™ 1

Residual standard error: 3.482 on 98 degrees of freedom
Multiple R-squared:  0.3891,     Adjusted R-squared:  0.3455
F-statistic: 8.918 on 7 and 98 DF,  p-value: 1.833e-08

> pairwise.t.test(Mipt, pop, p.adj="bonf")
```

```
     Pairwise comparisons using t tests with pooled SD

data:  Mipt and pop

      ALBANIA ANTAR   CN      IT      MD      MON     PL
ANTAR 1.00000 -       -       -       -       -       -
CN    1.00000 1.00000 -       -       -       -       -
IT    1.00000 1.00000 1.00000 -       -       -       -
MD    1.00000 1.00000 1.00000 1.00000 -       -       -
MON   1.00000 1.00000 1.00000 1.00000 1.00000 -       -
PL    1.00000 1.00000 1.00000 1.00000 1.00000 1.00000 -
USA   2.1e-06 6.7e-07 1.6e-08 0.00093 2.3e-09 3.0e-06 2.0e-06

P value adjustment method: bonferroni
> shapiro.test(residuals(m17))

     Shapiro-Wilk normality test

data:  residuals(m17)
W = 0.59627, p-value = 1.265e-15

Call:
lm(formula = MRpt ~ pop)

Residuals:
    Min      1Q  Median      3Q     Max
-42.267  -1.422   0.283   1.961   9.233

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  52.5405     1.3378  39.273  < 2e-16 ***
popANTAR      4.8782     1.8622   2.620 0.010233 *
popCN         3.3704     1.8622   1.810 0.073438 .
popIT         6.7345     2.9158   2.310 0.023049 *
popMD        12.6447     1.8920   6.683 1.53e-09 ***
popMON        7.6931     1.9255   3.995 0.000127 ***
popPL         0.5261     1.8920   0.278 0.781551
popUSA        2.6150     2.1847   1.197 0.234265
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˜â€™ 0.1
â€˜â€™ 1

Residual standard error: 5.181 on 96 degrees of freedom
  (2 observations deleted due to missingness)
Multiple R-squared:  0.4043,     Adjusted R-squared:  0.3609
F-statistic: 9.308 on 7 and 96 DF,  p-value: 9.349e-09

> pairwise.t.test(MRpt, pop, p.adj="bonf")

     Pairwise comparisons using t tests with pooled SD

data:  MRpt and pop

      ALBANIA ANTAR   CN      IT      MD      MON     PL
ANTAR 0.28652 -       -       -       -       -       -
```

```
CN    1.00000 1.00000 -       -       -       -       -
IT    0.64537 1.00000 1.00000 -       -       -       -
MD    4.3e-08 0.00187 7.8e-05 1.00000 -       -       -
MON   0.00355 1.00000 0.69556 1.00000 0.32639 -       -
PL    1.00000 0.60238 1.00000 1.00000 1.6e-07 0.00932 -
USA   1.00000 1.00000 1.00000 1.00000 0.00037 0.67133 1.00000

P value adjustment method: bonferroni
> shapiro.test(residuals(m18))

     Shapiro-Wilk normality test

data:  residuals(m18)
W = 0.56054, p-value = 3.911e-16

Call:
lm(formula = PRpt ~ pop)

Residuals:
    Min      1Q  Median      3Q     Max
-6.3317 -1.6004  0.0125  1.5504  6.2250

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  68.1973     0.6918  98.584  < 2e-16 ***
popANTAR      9.6902     0.9629  10.064  < 2e-16 ***
popCN         3.3586     0.9491   3.539 0.000619 ***
popIT         7.9777     1.5077   5.291 7.53e-07 ***
popMD        15.1679     0.9783  15.504  < 2e-16 ***
popMON       12.2611     0.9956  12.315  < 2e-16 ***
popPL         7.0760     0.9783   7.233 1.10e-10 ***
popUSA        5.9249     1.1296   5.245 9.17e-07 ***
---
Signif. codes:  0 â€***â€™ 0.001 â€**â€™ 0.01 â€*â€™ 0.05 â€˙â€™ 0.1
â€˜â€™ 1

Residual standard error: 2.679 on 97 degrees of freedom
  (1 observation deleted due to missingness)
Multiple R-squared:  0.7776,    Adjusted R-squared:  0.7616
F-statistic: 48.46 on 7 and 97 DF,  p-value: < 2.2e-16

> pairwise.t.test(PRpt, pop, p.adj="bonf")

     Pairwise comparisons using t tests with pooled SD

data:  PRpt and pop

      ALBANIA ANTAR   CN      IT      MD      MON     PL
ANTAR 2.7e-15 -       -       -       -       -       -
CN    0.01733 2.6e-08 -       -       -       -       -
IT    2.1e-05 1.00000 0.07041 -       -       -       -
MD    < 2e-16 3.8e-06 < 2e-16 0.00018 -       -       -
MON   < 2e-16 0.28420 1.9e-13 0.16309 0.12200 -       -
PL    3.1e-09 0.21975 0.00468 1.00000 2.0e-11 3.0e-05 -
USA   2.6e-05 0.02996 0.62261 1.00000 3.1e-11 7.4e-06 1.00000
```

```
P value adjustment method: bonferroni
> shapiro.test(residuals(m19))

      Shapiro-Wilk normality test

data:  residuals(m19)
W = 0.99166, p-value = 0.77
```

Supplementary Information 11

Figure SM11.1 − Animals measurements − psi

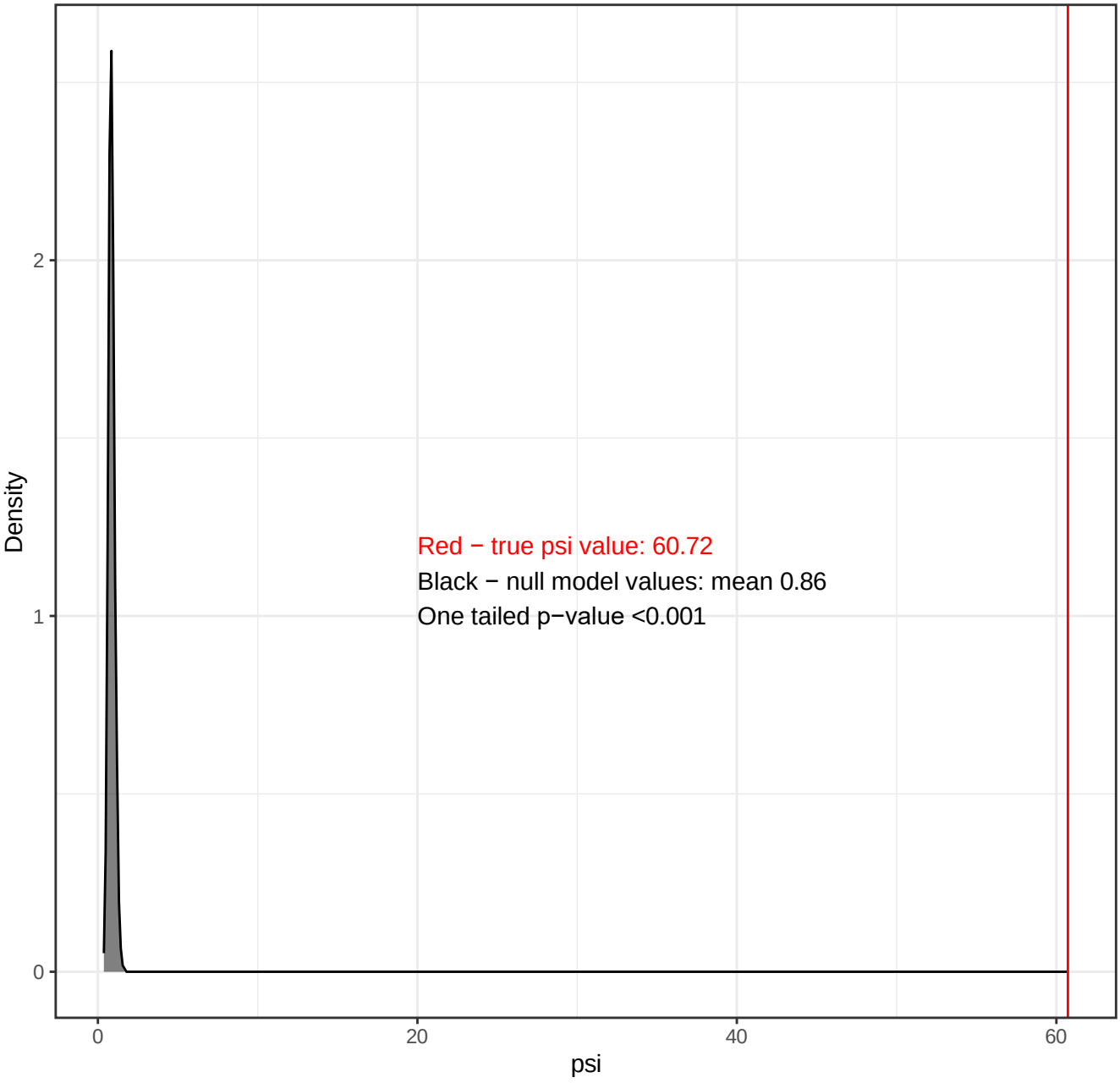


Figure SM11.2 − Animals measurements − phi

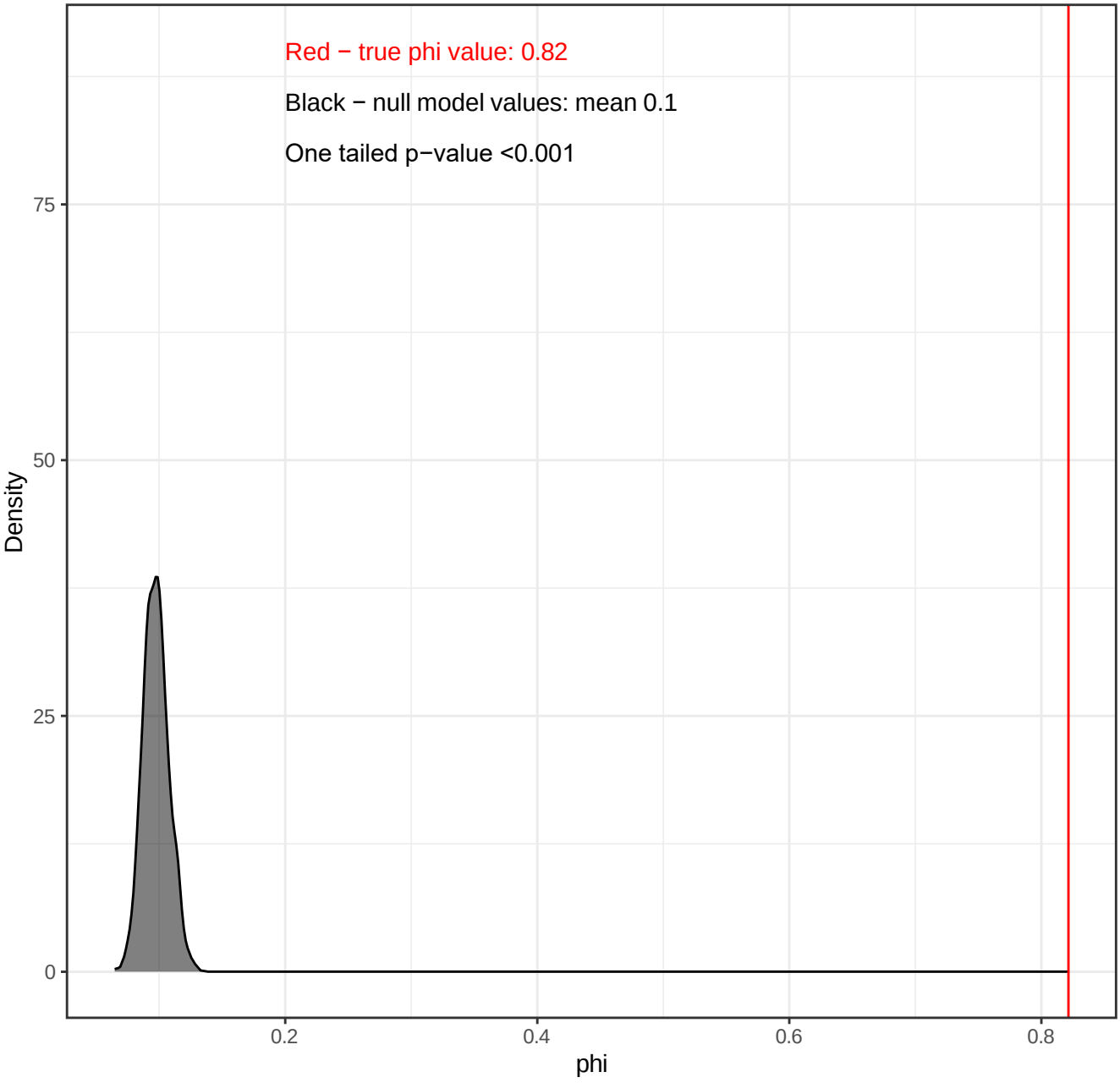


Figure SM11.3 − Animals measurements − explained variance by component

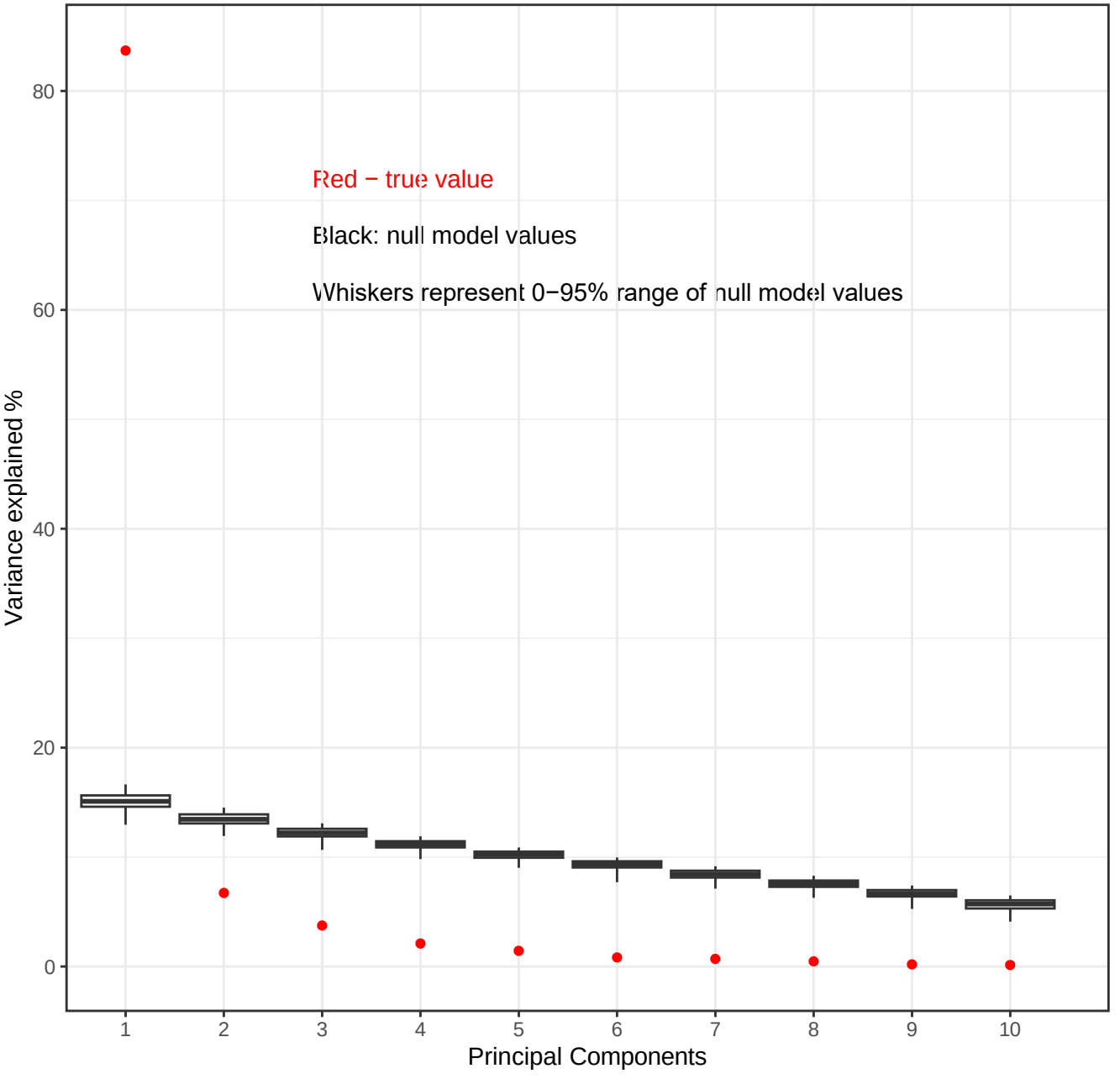


Figure SM11.4 − Eggs − psi

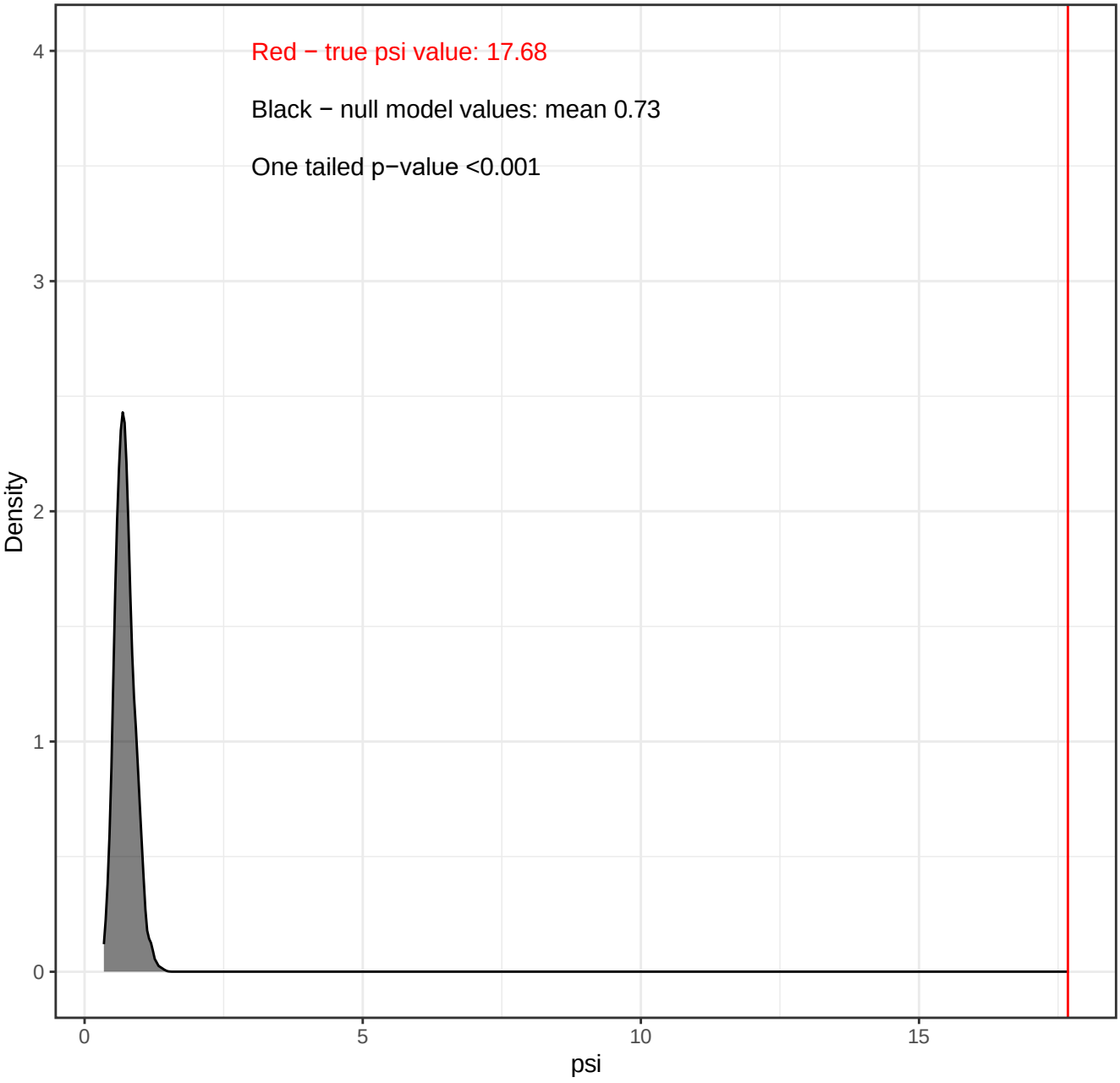


Figure SM11.5 − Eggs − phi

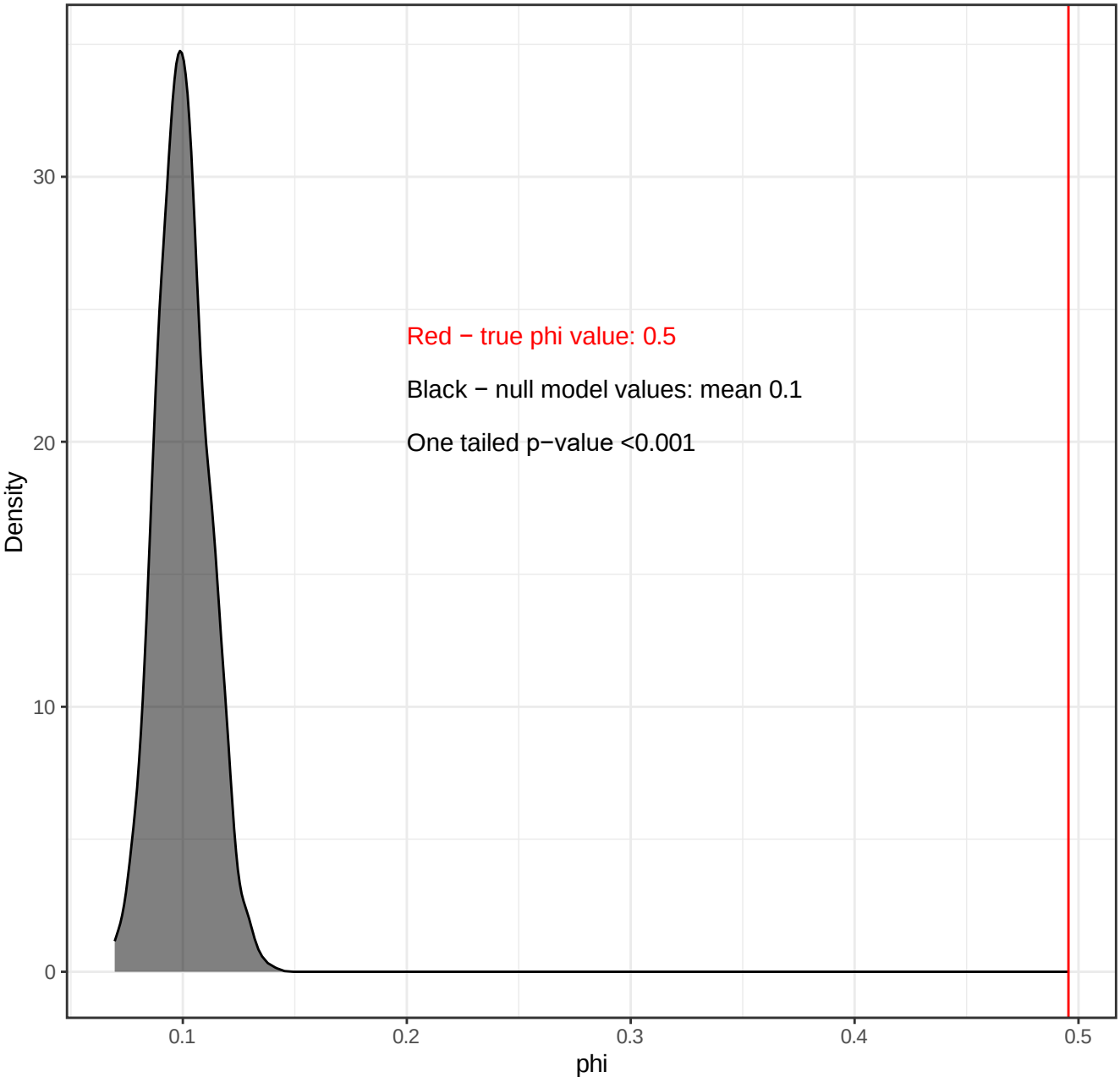


Figure SM11.6 − Eggs − explained variance by component

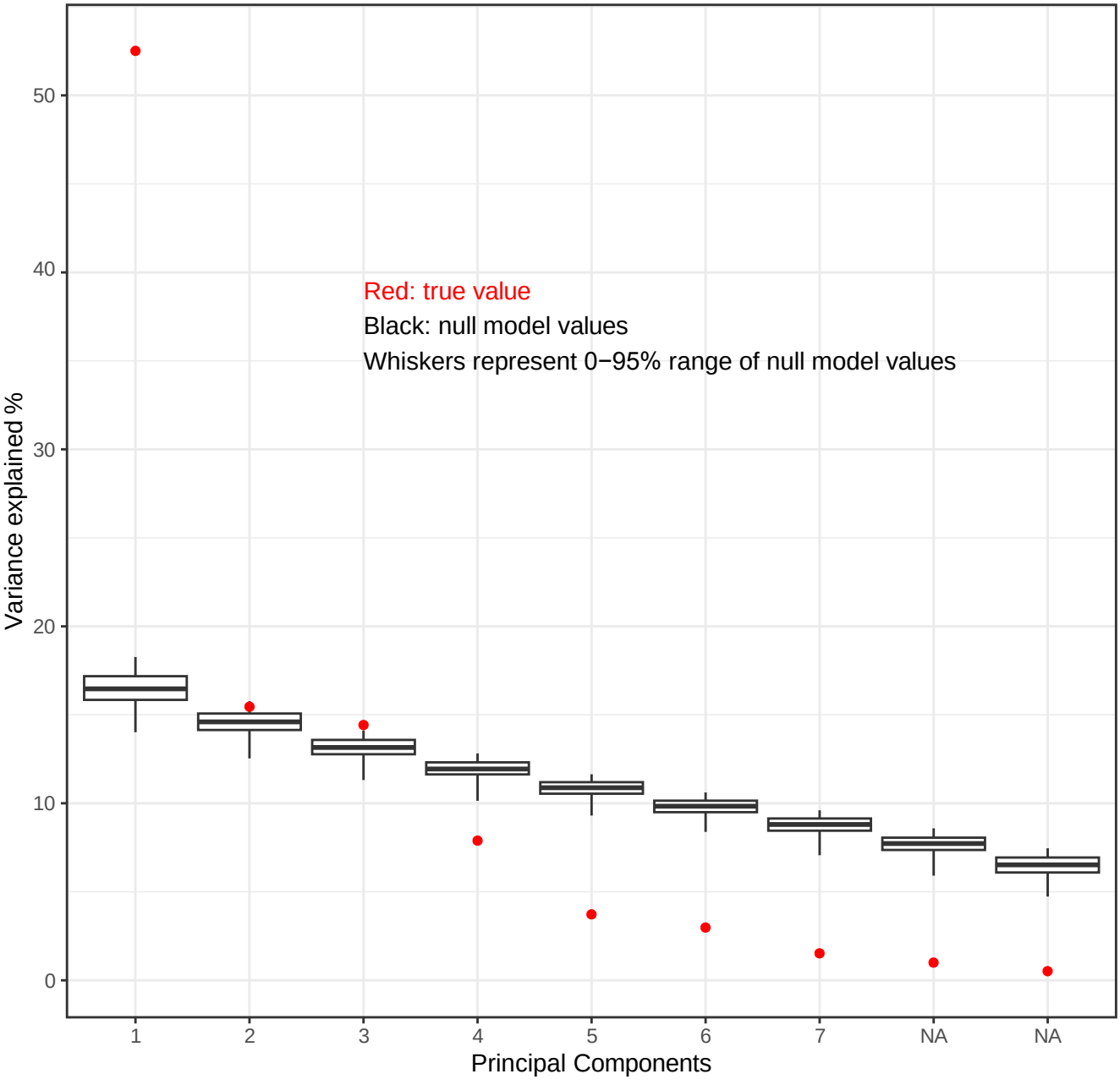


Figure SM11.7 − Animals pt − psi

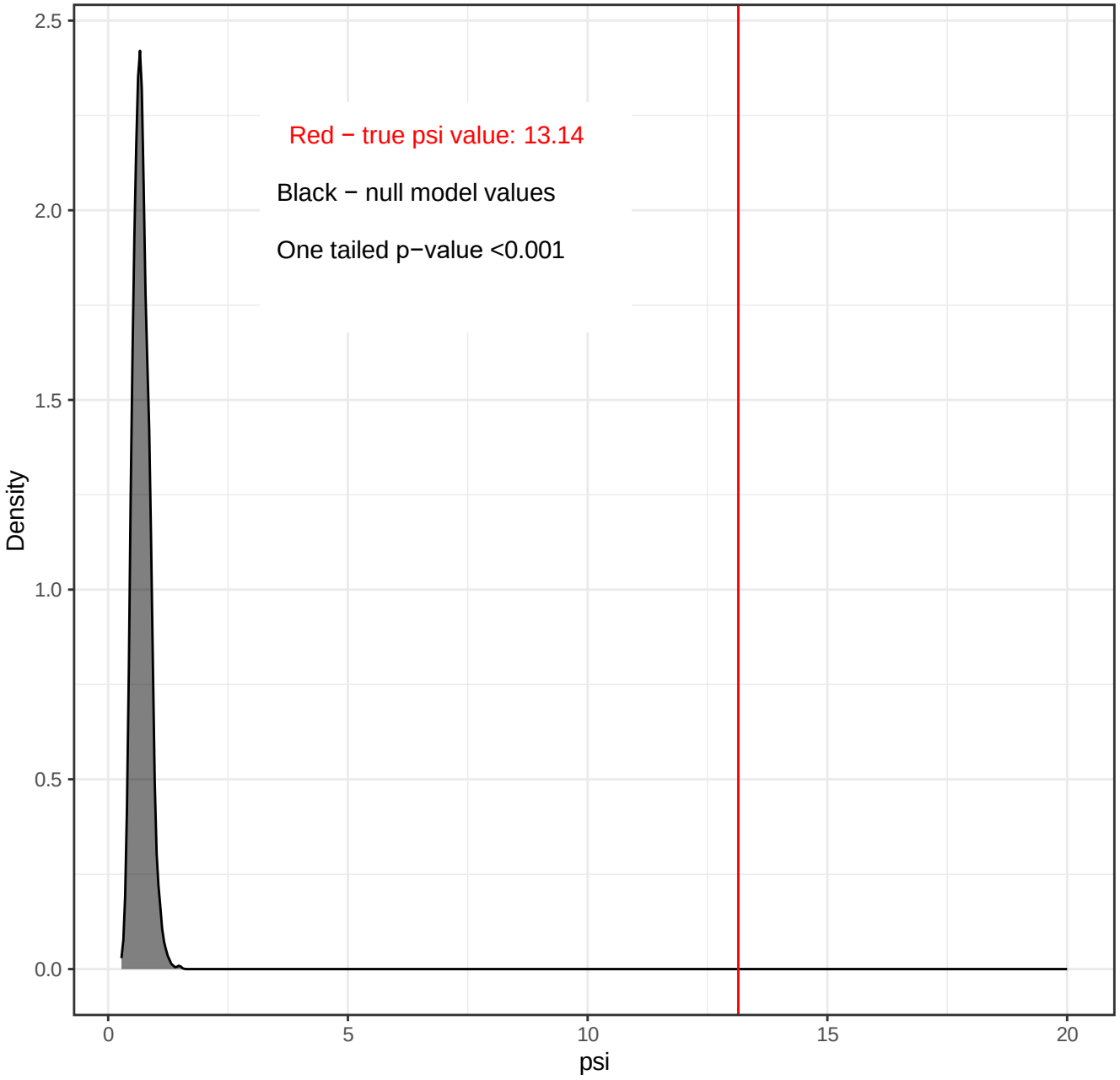


Figure SM11.8 − Animals pt − phi

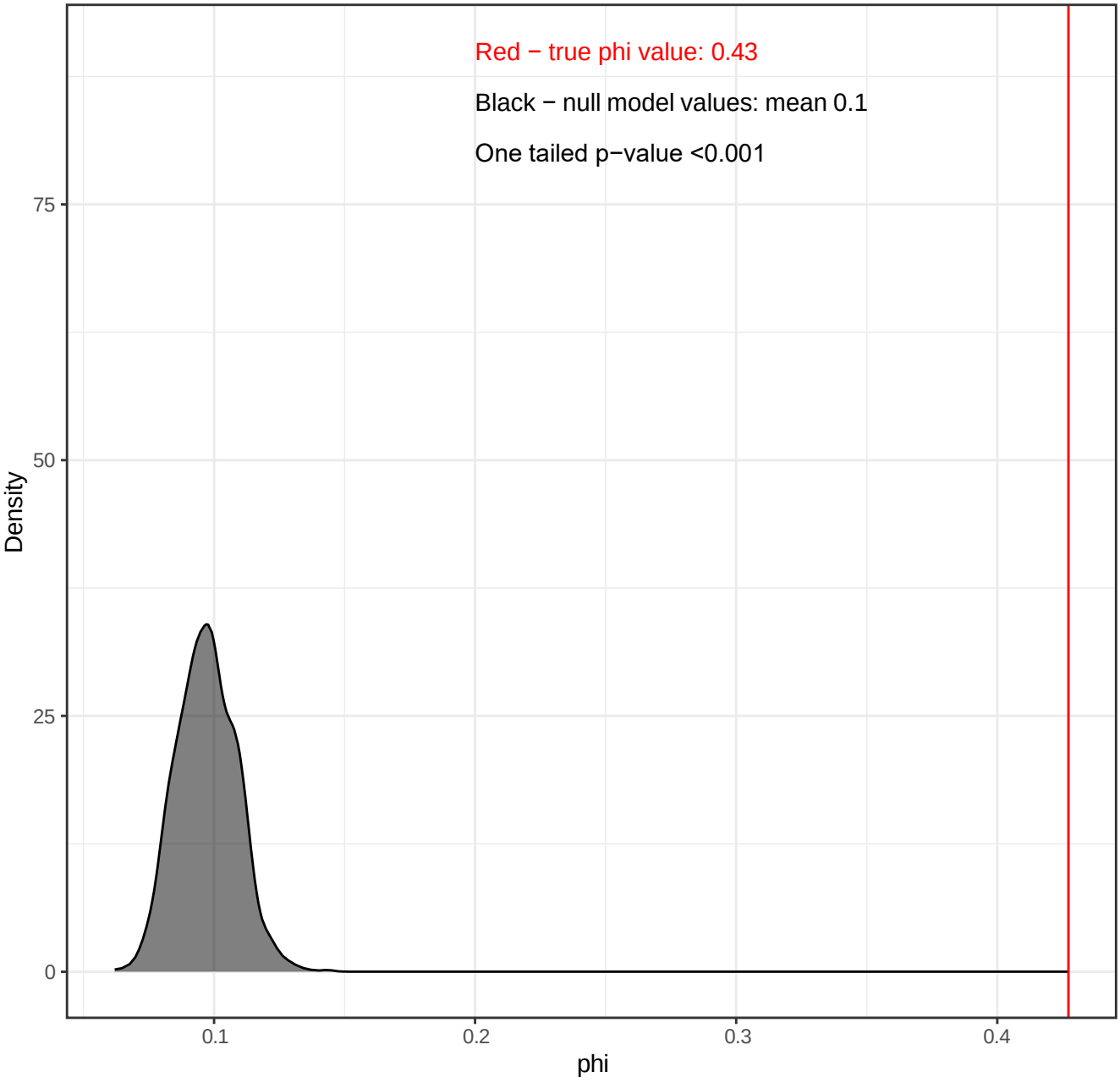
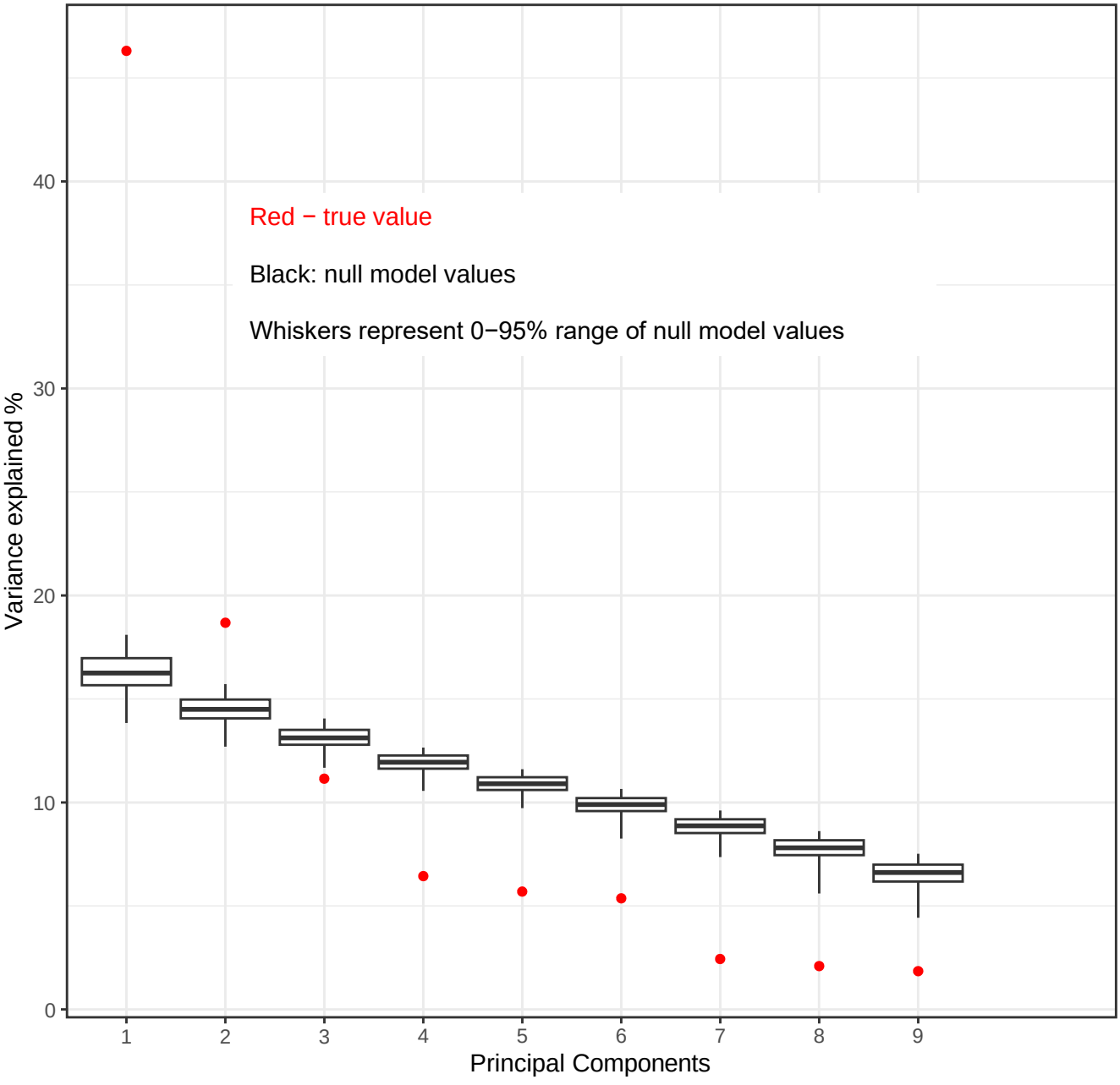


Figure SM11.9 − Animals pt − explained variance by component



Paper III

Kayastha, P., Rzymski, P., Gołdyn, B., Nagwani, A.K., Fiałkowska, E., Pajdak-Stós, A., Sobkowiak, R., Robotnikowski, G. and Kaczmarek, Ł. Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates, *Zool J Linn Soc*, zlad060 (2023) https://doi.org/10.1093/zoolinnean/zlad060

Zoological Journal of the Linnean Society, 2023, XX, 1–19
https://doi.org/10.1093/zoolinnean/zlad060
Advance access publication 22 July 2023
Original Article

OXFORD

Original Article

Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates

Pushpalata Kayastha[1,*,‡], Piotr Rzymski[2,3,‡], Bartłomiej Gołdyn[4], Amit Kumar Nagwani[5], Edyta Fiałkowska[6], Agnieszka Pajdak-Stós[6], Robert Sobkowiak[7], Gracjan Robotnikowski[4] and Łukasz Kaczmarek[1,‡]

[1]Department of Animal Taxonomy and Ecology, Faculty of Biology, Adam Mickiewicz University, Poznań, Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland
[2]Department of Environmental Medicine, Poznan University of Medical Sciences, Poznań, Poland
[3]Integrated Science Association (ISA), Universal Scientific Education and Research Network (USERN), Poznań, Poland
[4]Department of General Zoology, Faculty of Biology, Adam Mickiewicz University, Poznań, Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland
[5]Department of Bioenergetics, Faculty of Biology, Adam Mickiewicz University, Poznań, Poland,
[6]Institute of Environmental Sciences, Faculty of Biology, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland
[7]Department of Cell Biology, Faculty of Biology, Adam Mickiewicz University, Poznań, Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland

‡Equal contribution.

[*]Corresponding author. Department of Animal Taxonomy and Ecology, Faculty of Biology, Adam Mickiewicz University, Poznań, Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland. E-mail: pushpalata.kayastha@gmail.com

ABSTRACT

Perchlorates are present at high concentrations in Martian regolith and pose an additional challenge to the survival of terrestrial life on Mars. Some microinvertebrates can resist extreme conditions (e.g. low temperatures, lack of oxygen and radiation), making them suitable model species for space experiments. Clarification of whether they can tolerate high levels of perchlorates is crucial for understanding the scope of application of small invertebrates in Mars exploration. We assessed the activity of some Crustacea, Nematoda, Rotifera and Tardigrada exposed to 0.25–1.00% magnesium perchlorate. The number of active specimens decreased with exposure time and perchlorate concentration. However, exposure of selected species to 0.25% perchlorate for 24 or 72 h showed activity in some specimens. Only *Caenorhabditis elegans*, *Lecane inermis* and *Artemia salina* exhibited activity after 24 h exposure to 1.00% perchlorate. *Lecane inermis* was the only species to remain active after 72 h of incubation with 1.00% perchlorate. Transferring specimens to distilled water after perchlorate exposure generally resulted in high recovery rates. The study indicates that all the tested invertebrates resist extremely high concentrations of perchlorates, which has implications for further research on their potential use in development of biological systems with improved performance and utility on Mars.

Keywords: Crustacea; Mars exploration; Nematoda, perchlorate; regolith; Rotifera; Tardigrada

INTRODUCTION

The search for biological features with potential applications in space exploration is an active area of interest. Most of the studies are focused on microorganisms adapted to extreme environmental conditions on Earth. This is particularly important for future exploration of moons or planets, such as Mars, where sustaining potential human outposts would require *in situ* resource utilization and the development of efficient life-support systems instead of relying on life-support consumables supplied from the Earth. Microorganisms could exist in Martian history, owing to evidence indicating the presence of oceans, lacustrine and riverine environments in the distant past (although there is no proof of any biosignatures to confirm this to date) (Baker 2006, Rodriguez *et al.* 2016). At present, environmental conditions on Mars are considered too hostile for most life forms. This is attributable to low atmospheric pressure, preventing stable formation of liquid water on the surface, lack of a global magnetic field, low temperatures, increased flux of ultraviolet (UV) B and UVC, oxidative conditions and the xeric environment (Read *et al.* 2015, Erdmann *et al.* 2017, 2021a, Martínez *et al.* 2017). Nonetheless,

Received 25 November 2022; revised 28 April 2023; accepted 8 May 2023

extremophiles associated with different environments on the Earth reveal some promising features that could potentially allow them to survive in selected Martian conditions, as demonstrated experimentally (Kounaves 2007, Onofri *et al.* 2008, 2015, Berry *et al.* 2010, de Vera *et al.* 2010, de Vera 2012, Direito *et al.* 2011, Baqué *et al.* 2013, Mastascusa *et al.* 2014, Frösler *et al.* 2017, Billi *et al.* 2019, Levchenko *et al.* 2019, Merino *et al.* 2019, Panitz *et al.* 2019, Beblo-Vranesevic *et al.* 2020, Coleine and Delgado-Baquerizo 2022). Identification of such organisms and the molecular basis of their adaptations to extreme environmental conditions could open a pathway to design biological systems by genetic engineering or tools of synthetic biology that could be useful in future Martian exploration.

Apart from selected microorganisms, some invertebrates also reveal a polyextremophilic nature. Some representatives of these invertebrate groups [e.g. *Artemia franciscana* Kellogg 1906 (Crustacea), *Caenorhabditis elegans* (Maupas 1900) (Nematoda), *Milnesium tardigradum* (at present *Milnesium inceptum* Morek et al. 2019) (Tardigrada), *Richtersius coronifer* (Richters 1903) (Tardigrada), *Ramazzottius varieornatus* Bertolani and Kinchin 1993 (Tardigrada), *Mniobia russeola* (Zelinka 1891) (Rotifera) and *Macrotrachela quadricornifera* (Milne 1886) (Rotifera)] are considered to be good model species for space experiments (Planel *et al.* 1994, Bertolani *et al.* 2001, Ricci & Boschetti 2003, Ricci *et al.* 2005, Horikawa 2008, Horikawa *et al.* 2008; Jönsson *et al.* 2008, Erdmann and Kaczmarek 2017, Ishioka and Higashibata 2019, Erdmann *et al.* 2021b).

All these invertebrates are capable of cryptobiosis, a state of life characterized by extremely reduced metabolic activity, which is entered by an organism in response to adverse environmental conditions (Keilin 1959, Clegg 2001) either in all life stages (e.g. tardigrades, bdelloid rotifers) or in some of them (e.g. encysted embryos of *Artemia*, juvenile and adult stages in nematodes). This process is mediated by various cytoprotective strategies that are likely to underlie an extraordinary resistance to environmental conditions. These strategies include the synthesis and accumulation of molecules (such as trehalose, a non-reducing disaccharide) that protect cell membranes and macromolecule functions by stabilizing their structure or scavenging free radicals. Moreover, various proteins have been recognized as cytoprotectants in some invertebrates (mainly tardigrades). These molecules include intrinsically disordered proteins, such as damage suppressor protein (Dsup) and Dsup-like proteins, cytoplasmic abundant heat-soluble, secretory abundant heat-soluble and mitochondrial abundant heat-soluble proteins, heat shock proteins and hydrophilic late embryogenesis abundant (LEA) proteins (Yamaguchi *et al.* 2012, Tanaka *et al.* 2015, Hashimoto *et al.* 2016, Hesgrove and Boothby 2020, Mínguez-Toral *et al.* 2020). Other cytoprotective strategies might involve a higher tolerance to oxidative stress mediated by reactive oxygen species, the removal of damaged or dysfunctional molecules, and effective DNA repair mechanisms (Rebecchi *et al.* 2007, 2011, 2020, Guidetti *et al.* 2011, Gajardo and Beardmore 2012, Erkut *et al.* 2013, Rebecchi 2013, Kaczmarek *et al.* 2019, Møbjerg and Neves 2021, Kasianchuk *et al.* 2023). Likewise, also in rotifers, the LEA proteins ArLEA1A and ArLEA1B have been hypothesized to contribute to desiccation tolerance (Tripathi *et al.* 2012). Moreover, results show that glutathione *S*-transferase zeta recombinant protein is likely to play an important role in response to metal-induced oxidative stress (Lee *et al.* 2020). Furthermore, nematodes harbour LEA3 proteins that are important components of anhydrobiotic protection (Tyson *et al.* 2012), and the activity of ice-binding proteins shows a good correlation with the survival rate of *C. elegans* during freezing (Kuramochi *et al.* 2019). Also, LEA proteins play a pivotal role in stress tolerance in crustaceans (Zhao *et al.* 2016), in addition to protecting their cysts from desiccation and freezing (Toxopeus *et al.* 2014). Moreover, invertebrates exhibit morphological and behavioural adaptations that allow them to resist harsh conditions, including microscopic body size, different thicknesses of the cuticle that help anterior–posterior body contraction into a 'tun' in tardigrades and/or bdelloid rotifers, modifications of internal organization (in rotifers), and body folding/coiling and congregation into masses in nematodes (Watanabe 2006, Rebecchi *et al.* 2007, Marotta *et al.* 2010). In the case of monogononta rotifers, such as *L. inermis*, which are not desiccation resistant, the means of surviving in harsh environments are resting eggs (Radzikowski 2013). However, they are not produced by all strains (Pajdak-Stós *et al.* 2014). *Lecane* rotifers are often present in wastewater treatment plants, where they are subjected to different chemicals, some of which are toxic. Their persistence in activated sludge suggests high resistance to adverse conditions.

Some invertebrates from the above-mentioned taxonomic groups can survive extreme habitat conditions, such as lack of water, high and very low temperatures, high doses of radiation, high concentrations of different chemical toxicants or lack of oxygen (McSorley 2003, Wełnicz *et al.* 2011, Gajardo and Beardmore 2012, Guidetti *et al.* 2012, Rebecchi *et al.* 2020). These abilities are particularly reserved for the specimens in a cryptobiotic state, when the organisms do not reproduce. A few species have already been tested in outer space conditions, which in some respects resemble the Martian environment (extreme temperature, lack of oxygen and liquid water, and high doses of different types of radiation) (Finckenor and de Groh 2020). A few species of crustaceans, nematodes, bdelloid rotifers and tardigrades, in the anhydrobiotic state, have been tested either in outer space in low Earth orbit or in conditions simulating such extreme environments during space flight (Gaubin *et al.* 1990, Spooner *et al.* 1992, Planel *et al.* 1994, Ricci *et al.* 2005, Higashibata *et al.* 2006, 2007, Leandro *et al.* 2007, Jönsson *et al.* 2008, Selch *et al.* 2008, Rebecchi *et al.* 2009, 2011, Persson *et al.* 2011, Guidetti *et al.* 2012, Vasanthan *et al.* 2014, Rizzo *et al.* 2015, Jönsson and Wojcik 2017, Kaplan *et al.* 2020).

All these unique abilities of cryptobiotic invertebrates predispose them to experiments focused on survival beyond our planet, including Mars, the exploration of which is gaining increased attention. Importantly, however, the discovery of high concentrations of perchlorates (ClO_4^-) in the Martian regolith (a blanket of unconsolidated, loose, heterogeneous superficial deposits covering solid rock), reaching a mean of 0.6 wt %, is considered a major challenge for terrestrial life forms (Hecht *et al.* 2009; Kounaves *et al.* 2010, 2014, Glavin *et al.* 2013, Leshin *et al.* 2013, Ming *et al.* 2014, Sutter *et al.* 2017, Martin *et al.* 2020). Martian perchlorate concentrations far exceed the levels noted on Earth (Ericksen 1983, Calderón *et al.* 2014). Moreover, it is known that these highly reactive and toxic chemicals reveal bactericidal effects (Anderson *et al.* 2000, Kumarathilaka *et al.* 2016, Wadsworth and Cockell 2017, Pleus and Corey 2018). Thus, even if extremotolerant anhydrobiotic species reach Mars and encounter a microniche with favourable temperature and liquid water, the toxicity of the environment

could limit their survival in the active form, preventing reproduction and development of a population.

It is not known whether any species of Crustacea, Nematoda, Rotifera or Tardigrada (including those that can survive various extreme conditions) could withstand perchlorate at the levels expected on Mars. Judging from the experimental data obtained for other animals, it is plausible that perchlorate exposure could lead to the generation of reactive oxygen species and promote denaturation of proteins, destabilization of lipid bilayers and DNA damage, ultimately decreasing the survival of the invertebrates considered (Ingram 1981, Hallsworth *et al.* 2003, Yu *et al.* 2019, Heinz *et al.* 2020, Rzymski *et al.* 2022).

Therefore, the aim of the present study was to assess the activity of representatives of Crustacea, Nematoda, Rotifera (Monogononta) and Tardigrada in high perchlorate concentrations (0.25–1.00%), which are in the range that can be expected on Mars. To this end, we have selected species that are non-challenging in culture (to avoid the potential interference of unpredicted factors), are recognized as model organisms and/or are known for their ability to sustain various chemical stresses. Clarification in this regard is pivotal for understanding the scope of application of small invertebrates in Mars exploration and for selecting species tolerant enough to perchlorate stress to predispose them for further studies in simulated Martian conditions.

MATERIALS AND METHODS

Animal models

Crustacea

Artemia salina (Linné and Salvius 1758) cysts (size 190–260 μm) were purchased from Artemia Koral and cultured based on the protocol described by Eppley (1974). They were placed in plastic containers (500 mL) containing a solution of non-iodized salt in deionized water (concentration 3.5%). The containers were placed in control conditions (17°C and 12 h photoperiod). The cultures were constantly oxygenated with a water aerator. After 48 h, hatched nauplii of similar stage and size (instar II–III stage, 600–700 μm) were selected for experiments.

Nematoda

Caenorhabditis elegans (wild-type Bristol N2 strain) was obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota (Duluth, MN, USA) (size ~1.3 mm). Standard methods were used to culture specimens in sterile conditions (Brenner 1974). In brief, *Caenorhabditis* specimens were cultured monoxenically on solid nematode growth medium (NGM; NaCl 50 mM, peptone 0.25%, $CaCl_2$ 1 mM, cholesterol 5 μg/mL, KH_2PO_4 25 mM, $MgSO_4$ 1 mM and agar 1.7%) using *Escherichia coli* Migula 1895 (strain OP50) as food. A sterilized spatula was used to move a chunk of agar with animals onto freshly seeded NGM plates. After 4–5 days, animals were harvested by washing them off the plate. Adult, fully active and non-moulting specimens of medium body size were selected for experiments.

Rotifera

Lecane inermis Bryce 1892 (clone 1.A2.15) specimens were isolated in the winter season from a municipal Blachownia Wastewater Treatment Plant (50°47′12″ N 18°59′47″ E), near Częstochowa (Poland) (size 92–154 μm). The clone was obtained from a single parthenogenetic female transferred with a glass micropipette from a sludge sample to a separate well filled with Żywiec Zdrój spring water. Rotifers were fed with NOVO (nutrition powder used for rotifer mass culture) (Pajdak-Stós *et al.* 2017). Cultures were constantly maintained in darkness at 20°C (culture collection of Aquatic Ecosystems Group, Institute of Environmental Sciences, Jagiellonian University). Fully active specimens were extracted in 20 μL of culture medium from stock culture and used in experiments.

Tardigrada

Hypsibius exemplaris Gąsiorek *et al.* 2018 specimens (size 100–350 μm) were obtained from the commercial culture provided by Sciento (Manchester, UK) (catalogue number Z151). Specimens for this culture were extracted from a freshwater benthic sample with a type locality in a pond in Darcy Lever, Bolton, UK (53°33′32″ N, 02°23′48″ W; 75 m a.s.l.). Tardigrades were cultured according to Roszkowska *et al.* (2021). In brief, parthenogenetic females were kept in the medium (double distilled H_2O mixed with Żywiec Zdrój spring water in a 3:1 ratio) in Petri dishes, with the bottom scratched with sandpaper. Tardigrade cultures were kept in a climate chamber (photoperiod 12 h light–12 h dark, 20°C, relative humidity of 50.0%). Animals were fed *ad libitum* once per week with *Chlorella vulgaris* (Beyerinck [Beijerinck], 1890) (SAG211-11b strain). Adult, fully active and non-moulting specimens of medium body size and with full green intestines were selected for experiments.

Milnesium inceptum Morek *et al.* 2019 specimens were extracted from a moss sample in a xerothermic habitat, i.e. mosses on a concrete wall in the city centre of Poznań in Poland (Heliodor Święcicki Clinical Hospital of Poznań University of Medical Sciences at Przybyszewskiego street, 52°24′15″ N, 16°53′18″ E; 87 m a.s.l.) (size 300–850 μm). Tardigrades were obtained from samples according to a standard procedure (Dastych 1980). Fully active *Mil. inceptum* specimens were extracted directly from the sample, and laboratory culture was established. Parthenogenetic *Milnesium* females were cultured in the medium on scratched Petri dishes (for more details, see culture conditions for *Hys. exemplaris*). Rotifers (*L. inermis*) and nematodes (*C. elegans*) were added *ad libitum* as a food source once per week. Adult, fully active and non-moulting specimens of medium body size were selected for experiments.

Paramacrobiotus experimentalis Kaczmarek *et al.* 2020 specimens were extracted from a sample of mosses from soil collected near to Fort-Voyron, Antananarivo, Antananarivo Province, Madagascar (18°55′35″ S, 47°31′23″ E, 1340 m a.s.l.) (size 350–600 μm). Fully active *Pam. experimentalis* specimens were used to establish laboratory culture. In a similar manner to *Mil. inceptum* and *Hys. Exemplaris* cultures, specimens were kept in culture medium on scratched Petri dishes (for more details, see *Hys. exemplaris*). Rotifers (*L. inermis*) and nematodes (*C. elegans*) were added *ad libitum* as a food source once per week. Adult, fully active and non-moulting *Paramacrobiotus* females of medium body size, with eggs visible in the ovary, were selected for experiments.

Experimental design

All model animals were exposed to 0.25%, 0.50% or 1.00% of magnesium perchlorate [$Mg(ClO_4)_2$; Sigma-Aldrich,

Germany], corresponding to 1.5, 3.0 or 6.0 mM ClO_4^- ions, but with different protocols, adapted specifically to the organisms and explained in the following subsections. Magnesium perchlorate solutions were prepared using distilled water (pH = 7.0, electric conductivity 0.46 μS/cm). Magnesium perchlorate was selected for the experiments owing to its presence in Martian regolith, spectroscopically confirmed by the Mars Reconnaissance Orbiter (Ojha *et al.* 2015). The applied levels of perchlorate can be expected in the Martian regolith and have been used in previous chemical and biological experiments related to survival on Mars (Rzymski *et al.* 2022, 2023). All animals were exposed separately for a maximum of 24 or 72 h to each perchlorate concentration, and specimens active after these exposure times were counted. Moreover, after 24 and 72 h exposures, Nematoda, Rotifera and Tardigrada specimens were transferred to distilled water for an additional 24 h to assess the number in which activity restored. As a control, specimens of all test animals were placed for 72 h in a medium without magnesium perchlorate. All specimens survived in such conditions. The percentage of active specimens in studied groups and in all exposure models is given as a percentage of the control.

Crustacea

The experiments were set in 60 glass test tubes, and two repetitions were carried out. For each magnesium perchlorate concentration in NaCl solution and for control line, 15 test tubes with a single *A. salina* nauplius were prepared ($N = 4 \times 15 \times 2 = 120$). The test tubes were placed in the same conditions as during the breeding phase and observed for 72 h. At this time, the mobility of the individuals (indicating signs of life) was controlled. At the end of the experiments, the active and inactive nauplii were counted. Artemia is not capable of re-entering cryptobiosis after leaving the cyst, and movements of appendages are necessary for gas exchange. Thus, following standard procedures, nauplii that were not active for ≥ 10 s were considered dead (Artoxkit 1990).

Nematoda

Six repetitions for each perchlorate concentration were performed. The experiments were conducted in Petri dishes (diameter 35 mm) with scratched bottoms. Five specimens of nematodes were transferred to dishes containing different perchlorate concentrations. The Petri dishes were observed for 24 h, and the number of active or inactive specimens was counted. Thereafter, the specimens were transferred from magnesium perchlorate solution to medium (1:3, Żywiec Zdrój: distilled water) and again observed for active and inactive specimens for 24 h. Likewise, for a 72 h period, Petri dishes were observed for 72 h for active and inactive specimens, then nematodes were transferred from magnesium perchlorate solution to medium and again observed for active and inactive specimens for 24 h (PP24H). The number of active or inactive nematodes was counted.

Rotifera

Six repetitions for each perchlorate concentration were performed. Experiments were conducted in 24-well culture plates (TTP, Switzerland). Each of the two plates was prepared as follows: *L. inermis* rotifers were transferred from a stock culture (~3250 individuals/mL) with an automatic micropipette in a volume of 20 μL of culture medium in each well. The number of specimens in each well ranged from 35 to 109 specimens. The wells were divided into four experimental groups (with six replicates each), and 1 mL of the appropriate perchlorate solution was added to each well. The plates were incubated in an incubation chamber (SANYO MLR-350, Sanyo Electric) at 20°C in a 12 h light–12 h dark regime. In the first plate, active and inactive rotifers were counted for 24 h from the start. Then in all wells containing perchlorate, the medium was delicately replaced with Żywiec Zdrój spring water. In our previous research, we observed that *L. inermis* rotifers deprived of food ceased movements and withdrew into lorica, hence they were considered dead (Fiałkowska & Pajak-Stós 2018). Therefore, to avoid such interference with the results of the present study, they were fed a pinch of dry NOVO (patent Pajdak-Stós *et al.* 2017). Although the addition of food might potentially influence the effect of perchlorates (e.g. through adsorption on food particles), one should note that all animals, including those in the control groups, were fed with the same amount; thus, any significant effects on their survival had to arise from perchlorate presence in the well. The active and inactive rotifers were counted again 3 h after the feeding. The procedure was repeated for the second plate after 72 h from the start of the experiment. All active and inactive rotifers were counted, after which the medium was replaced with spring water (Żywiec Zdrój, Poland) and the rotifers were fed with a pinch of NOVO. After another 3 h, the counting was repeated.

Tardigrada

Six repetitions for each perchlorate concentration for each species were performed. Experiments were conducted in Petri dishes (diameter 35 mm) with scratched bottoms and different magnesium perchlorate concentrations. Five specimens of tardigrades were transferred to each dish and observed for a 24 h period to count the number of active or inactive tardigrades. Thereafter, the tardigrades were transferred from magnesium perchlorate solution to culture medium (1:3, Żywiec Zdrój: distilled water) and again observed for active and inactive tardigrades for 24 h. Likewise, for the 72 h period, dishes were observed for 72 h for active and inactive tardigrades, after which the tardigrades were transferred from magnesium perchlorate solution to medium and again observed for signs of activity for 24 h. The number of active or inactive tardigrades was counted.

Statistical analysis

To compare the number of active specimens in the experimental treatments, repetitions within each group were averaged, and the mean and SD number of active specimens was plotted (Figs 1–6). In the case of Rotifera, the percentage of active animals was calculated (because the original number of specimens in each well was slightly different), then the repetitions within each group were averaged and the mean value with an error bar for the percentage of active specimens was plotted. Significance bars denote differences detected by ANOVA with Tukey's post hoc test (the data did not meet the assumptions of the tests fully, but are kept here for illustration, following Glass *et al.* 1972, Harwell *et al.* 1992, Lix *et al.* 1996).

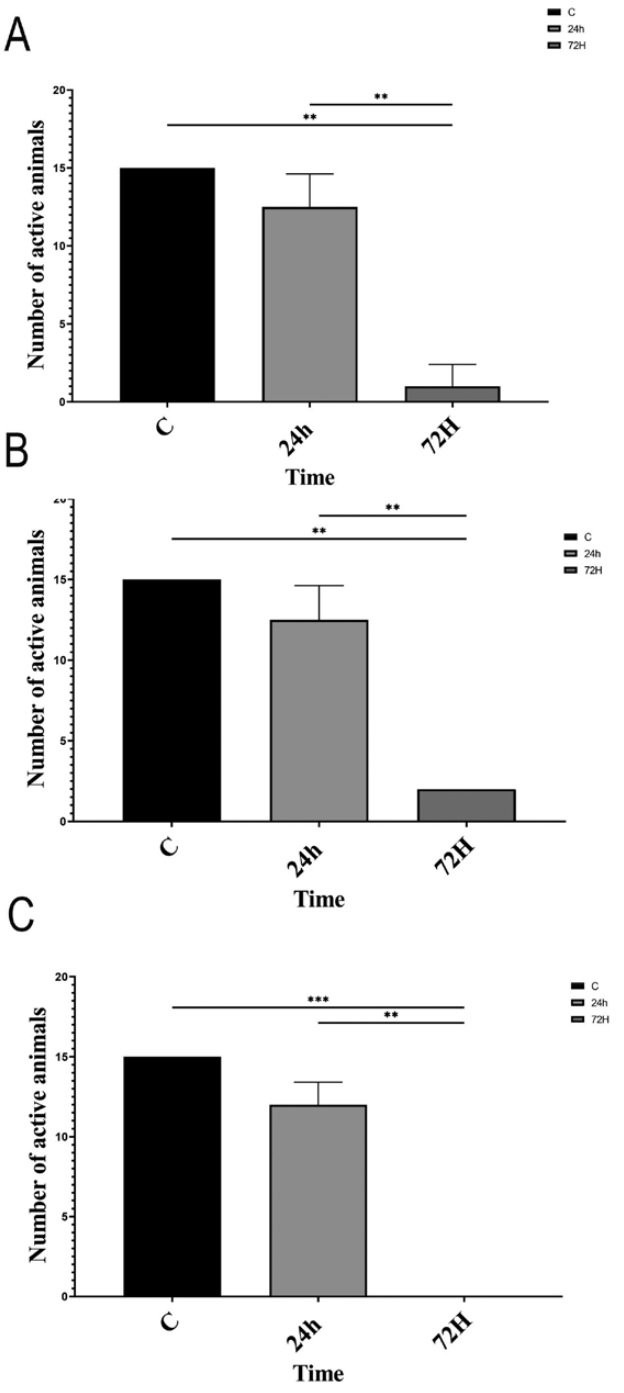


Figure 1. Activity of *Artemia salina* after 72 h exposure to 0.25% (A), 0.50% (B) and 1.00% (C) magnesium perchlorate [$Mg(ClO_4)_2$]. Abbreviation: C, 0 h. Levels of significance are represented as $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$ and $^{****}P \leq .0001$.

To test statistically for the differences between the experimental treatments, the median deactivation time (deactivation time 50%, DT50) was calculated, following the standard procedure for median lethal time calculation (Verma *et al.* 2014), as the median time after which > 50% of the individuals in a given repetition stopped showing any signs of activity. This index was not calculated in the case of *A. salina*, where we were able to collect and analyse independent data on each individual (because *A. salina* nauplii were placed individually in each test tube). The non-parametric Kruskal–Wallis test was used as a basic model to analyse the differences between the treatments. To make comparisions between the groups, the Wilcoxon pairwise test with Holm's correction was performed. A value of $P < .05$ was deemed statistically significant.

Remarks

The abbreviations of tardigrade genera follow Perry *et al.* (2019). For other taxonomic groups, one-letter abbreviations of the genus names were used.

RESULTS

Response of Crustacea to perchlorate

The activity of *A. salina* nauplii after exposure to perchlorate solutions was very high for 24 h exposure (Table 1; Fig. 7). Overall, 80%–83% of specimens showed activity even after being exposed to perchlorate for 24 h. However, the activity declined significantly when observations after 72 h were compared with 0 or 24 h records (in the case of all the perchlorate concentrations, $P < .01$). In the case of the highest level of perchlorate (1.00%), nauplii were active after 72 h of incubation (Table 1; Fig. 1). There was a significant difference in the median deactivation time of *Artemia* nauplii between the experimental treatments (Kruskal–Wallis $\chi^2 = 58.768$, d.f. = 3, $P < .0001$; Table 3; Fig. 7). All the experimental treatments showed statistical significance when compared with the control group ($P < .0001$ in all cases), but there were no significant differences between the treatments themselves.

Response of Nematoda to perchlorate

The activity of *C. elegans* after perchlorate exposure was low. Even in the lowest perchlorate concentration (0.25%), only 40% of specimens were active after 24 h and 23% after 72 h exposure. In higher concentrations, almost no active specimens were observed. However, after transferring to a culture medium, even for the highest perchlorate concentration (1.00%), 10%–13% of specimens returned to activity. In the case of 0.50% perchlorate for both 24 and 72 h, nearly one-third of animals returned to activity after transferring to the culture medium (Tables 1 and 2; Fig. 2). There was a significant difference in the median deactivation time of *C. elegans* between the experimental treatments (Kruskal–Wallis $\chi^2 = 22.824$, d.f. = 3, $P < .0001$; Table 3; Fig. 7). The control group differed significantly from all the treatments ($P = .0063$), and the 0.25% group differed from the remaining experimental groups ($P = .0063$).

Response of Rotifera to perchlorate

The activity of *L. inermis* after perchlorate exposure was very low (Tables 1 and 2; Fig. 3). Only 22.2% of rotifers were active after 24 h and only 1% after 72 h exposure to 0.25% perchlorate. However, 31%–68% resumed activity after transfer to fresh culture medium (Tables 1 and 2; Fig. 3). There was a significant difference in the median deactivation time of *L. inermis* between the experimental treatments (Kruskal–Wallis $\chi^2 = 19.68$, d.f. = 3, $P = .0002$; Table 3; Fig. 7). The individual statistical significance among groups was as follows: control vs. 0.25%, $P = .0075$; control vs. 0.50%, $P = .0063$; and control vs. 1.00%, $P = .0063$.

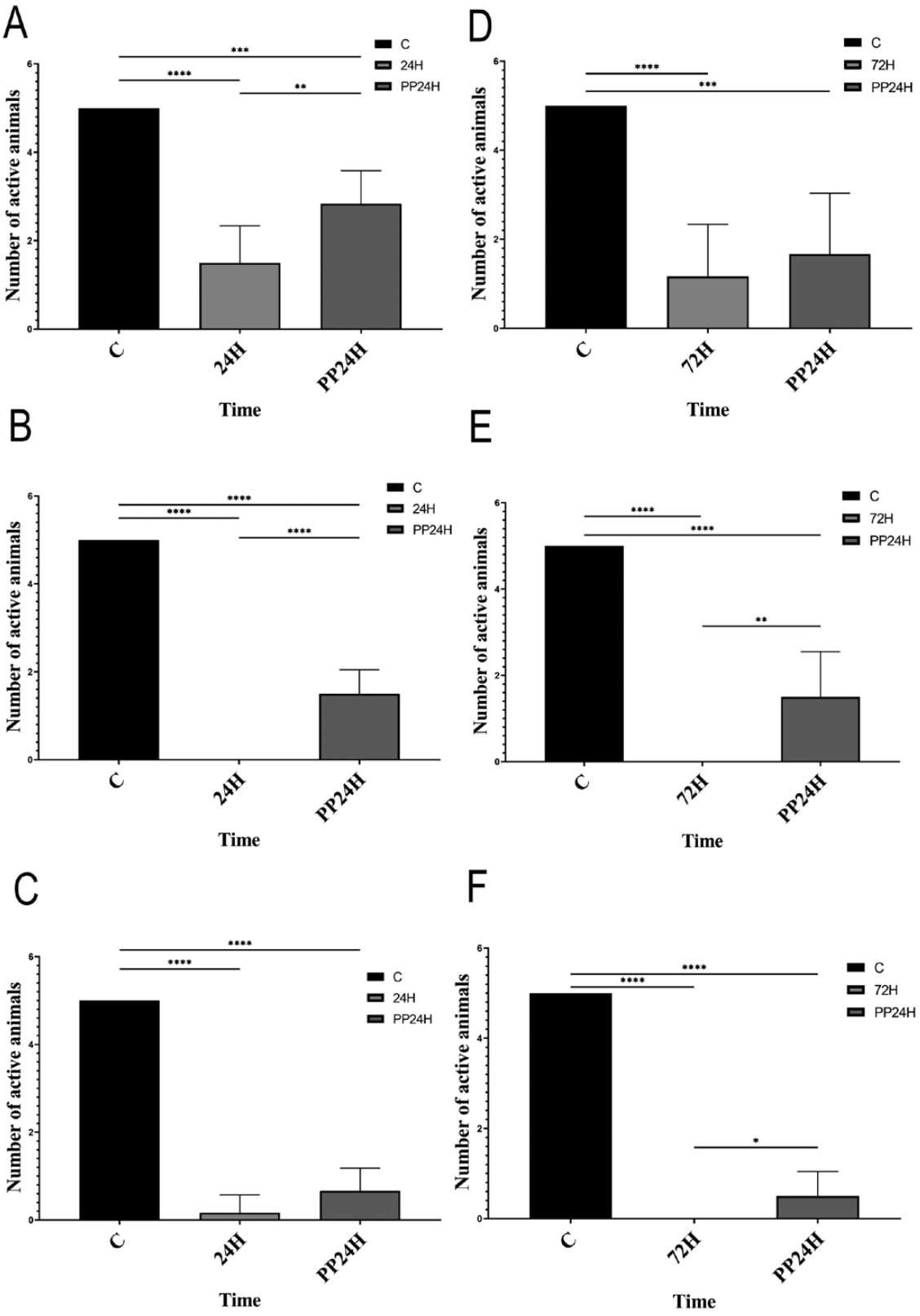


Figure 2. A–C, activity of *Caenorhabditis elegans* after 24 h exposure to 0.25% (A), 0.50% (B) and 1.00% (C) magnesium perchlorate [$Mg(ClO_4)_2$] and transfer to the culture medium for 24 h. D–F, activity of *C. elegans* after 72 h exposure to 0.25% (D), 0.50% (E) and 1.00% (F) $Mg(ClO_4)_2$ and transfer to the culture medium for 24 h. Abbreviations: C, control; PP24H, 24 h post perchlorate in culture medium. Levels of significance are represented as $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$ and $^{****}P \leq .0001$.

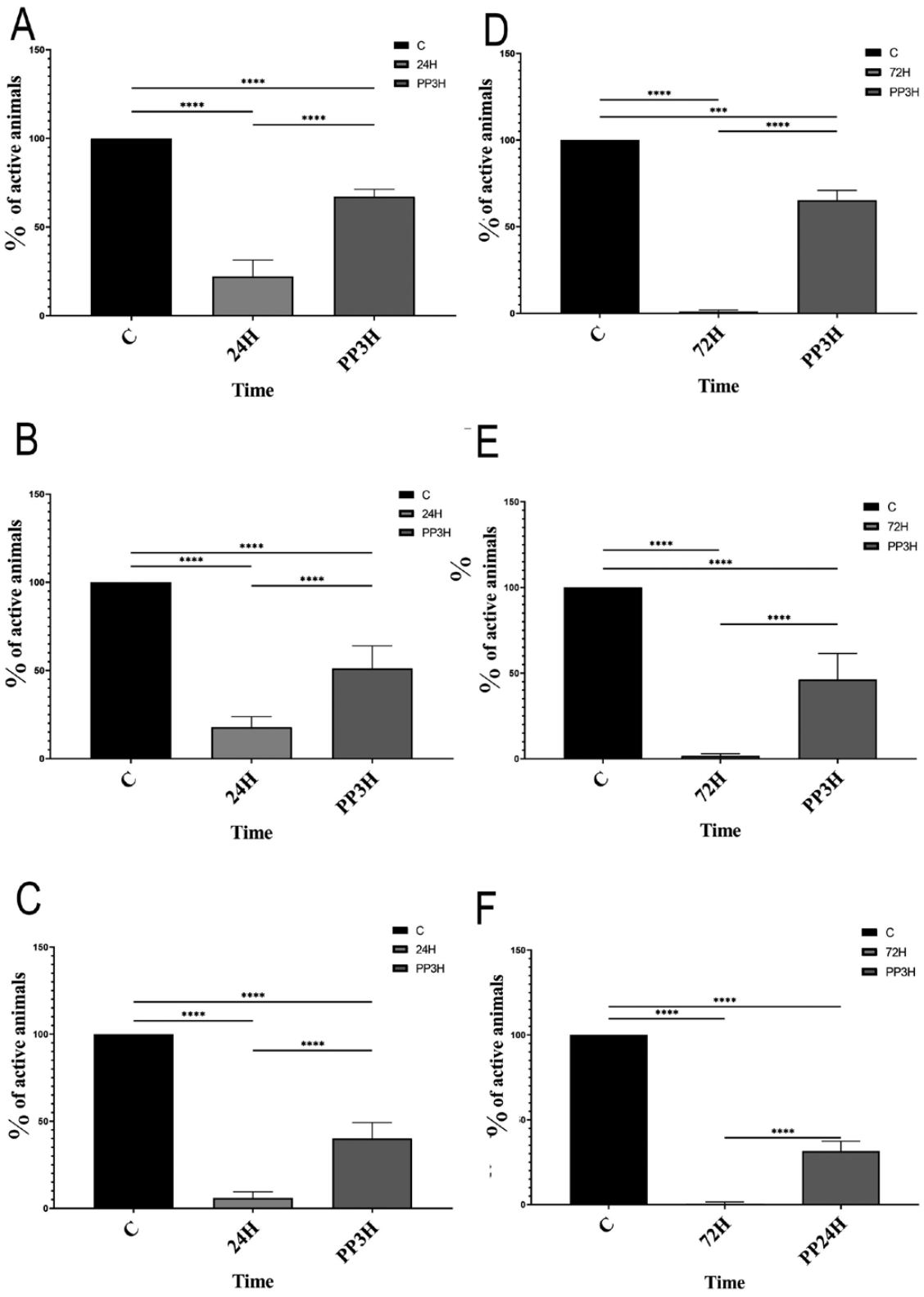


Figure 3. A–C, activity of *Lecane inermis* after 24 h exposure to 0.25% (A), 0.50% (B) and 1.00% (C) magnesium perchlorate [$Mg(ClO_4)_2$] and transfer to the culture medium for 3 h. D–F, activity of *L. inermis* after 72 h exposure to 0.25% (D), 0.50% (E) and 1.00% (F) $Mg(ClO_4)_2$ and transfer to the culture medium for 3 h. Abbreviations: C, control; PP3H, 3 h post perchlorate in culture medium. Levels of significance are represented as $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$ and $^{****}P \leq .0001$.

Response of Tardigrada to perchlorate

The response of tardigrades to perchlorate exposure revealed interspecies differences (Tables 1 and 2; Figs 4–6). In the case of *Hys. exemplaris*, all specimens were active after 24 h exposure to 0.25% perchlorate, but no activity was observed at higher concentrations. Although the frequency of active specimens of *Mil. inceptum* and *Pam. experimentalis* exposed to 0.25% perchlorate was lower (approximately one-third), active specimens

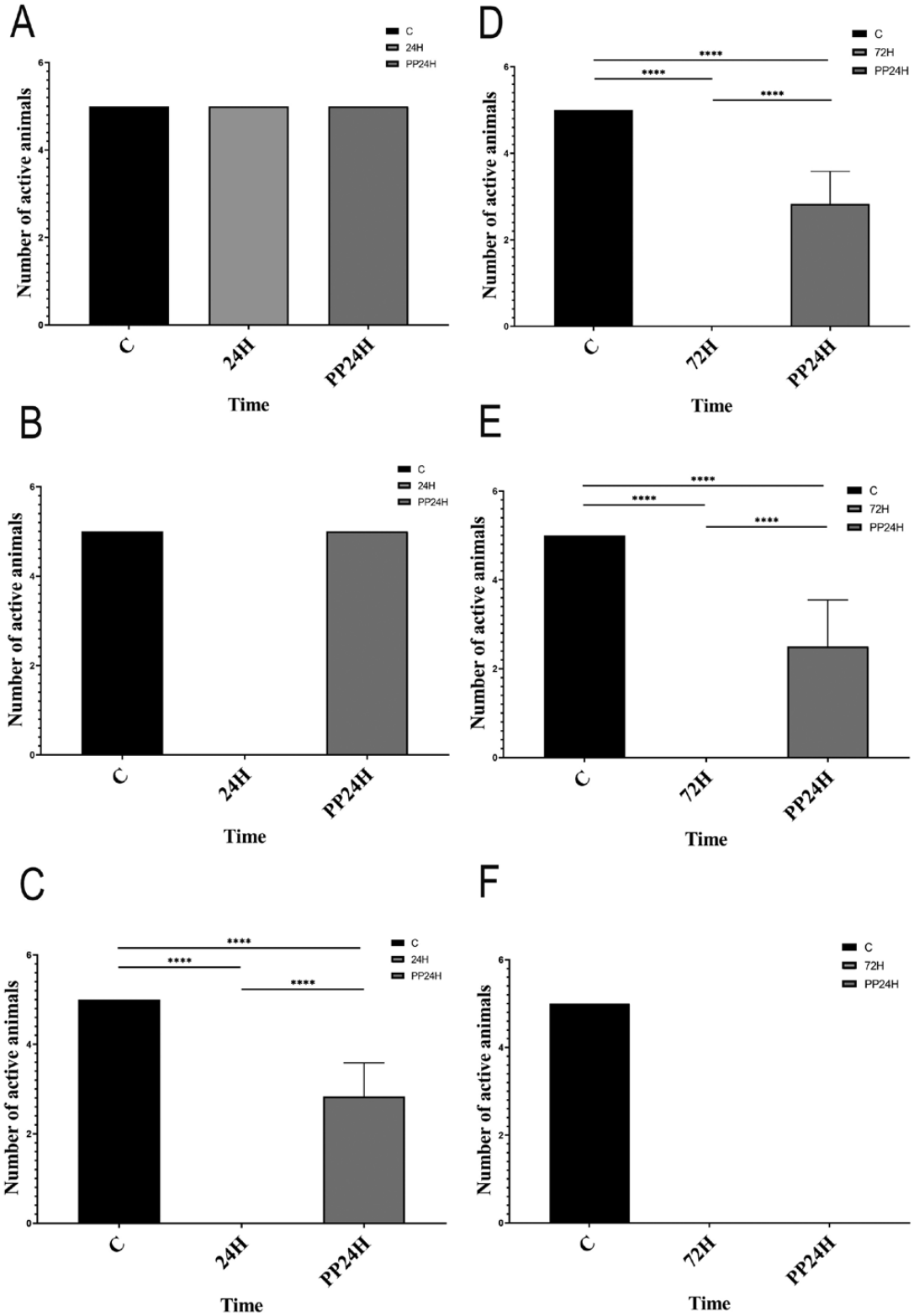


Figure 4. A–C, activity of *Hypsibius exemplaris* after 24 h exposure to 0.25% (A), 0.50% (B) and 1.00% (C) magnesium perchlorate $[Mg(ClO_4)_2]$ and transfer to the culture medium for 24 h. D–F, activity of *Hys. exemplaris* after 72 h exposure to 0.25% (D), 0.50% (E) and 1.00% (F) $Mg(ClO_4)_2$ and transfer to the culture medium for 24 h. Abbreviations: C, control; PP24H, 24 h post perchlorate in culture medium. Levels of significance are represented as: ns, not significant; $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$ and $^{****}P \leq .0001$.

were also observed after 24 h exposure to 0.50% perchlorate. Moreover, 72 h exposure of *Hys. exemplaris* to the lowest perchlorate concentration resulted in a 100% decrease in activity. *Milnesium inceptum* and *Pam. experimentalis* were able to withstand it, and about one-quarter of the specimens from the latter species were also active after 72 h incubation in 0.50%

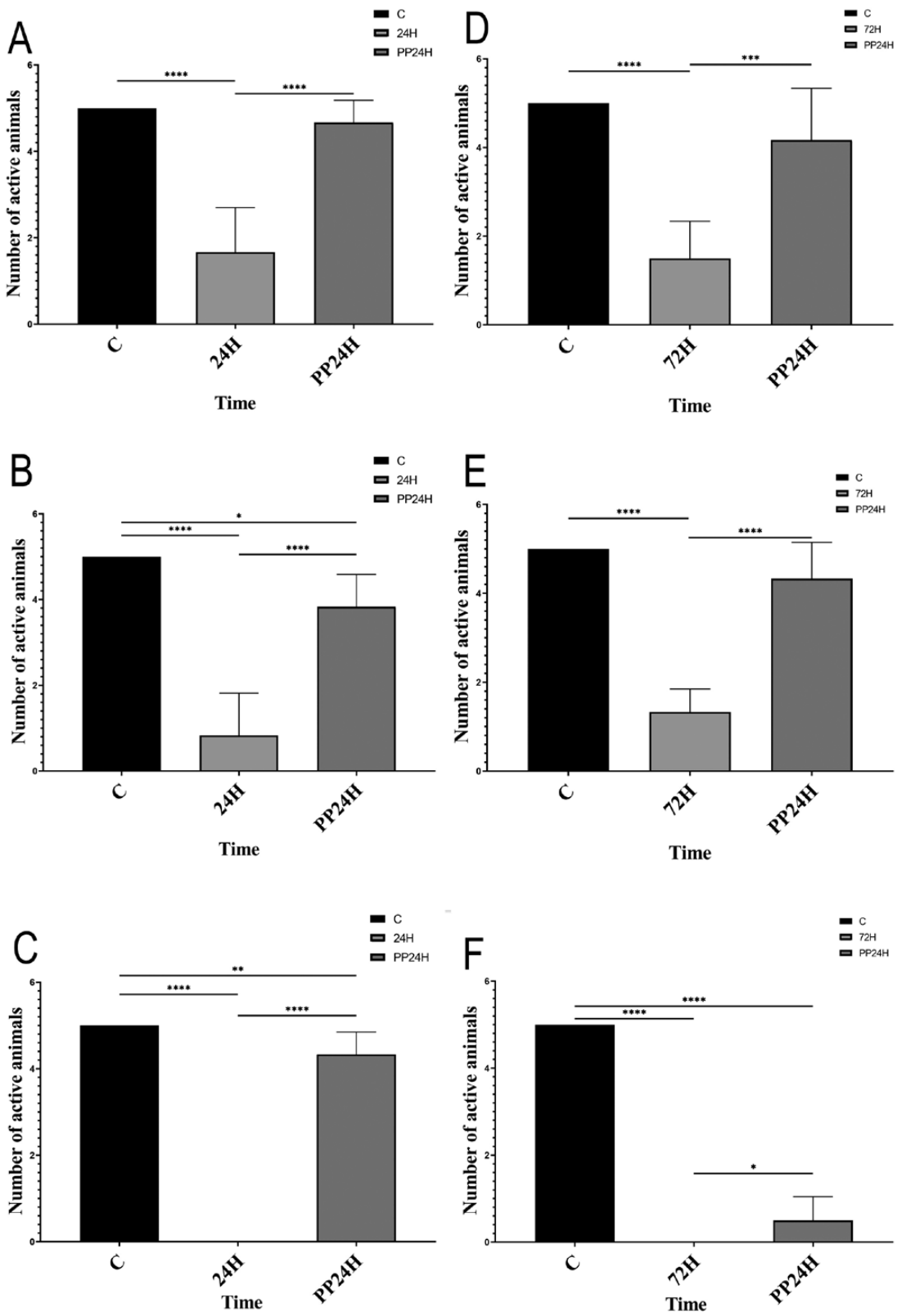


Figure 5. A–C, activity of *Paramacrobiotus experimentalis* after 24 h exposure to 0.25% (A), 0.50% (B) and 1.00% (C) magnesium perchlorate [$Mg(ClO_4)_2$] and transfer to the culture medium for 24 h. D–F, activity of *Pam. experimentalis* after 72 h exposure to 0.25% (D), 0.50% (E) and 1.00% (F) $Mg(ClO_4)_2$ and transfer to the culture medium for 24 h. Abbreviations: C, control; PP24H, 24 h post perchlorate in culture medium. Levels of significance are represented as $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$ and $^{****}P \leq .0001$.

perchlorate. No active specimen was observed after 24 or 72 h exposures to the highest perchlorate concentration. However, the frequencies of active specimens increased after the animals were transferred to culture medium. In that case, all specimens of *Hys. exemplaris* and *Mil. inceptum* and the majority of *Pam. experimentalis* returned to activity after exposure for 24 h to

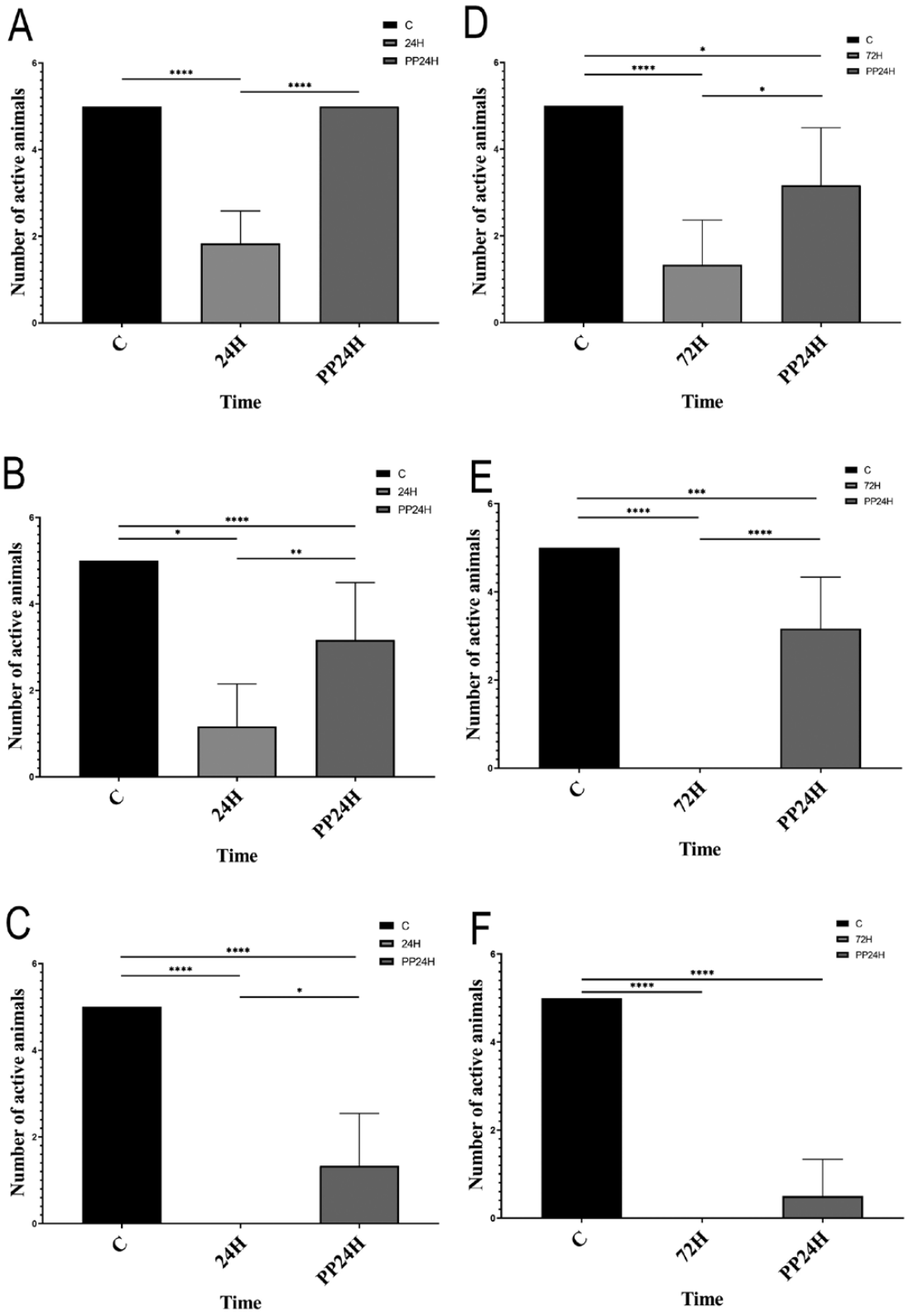


Figure 6. A–C, activity of *Milnesium inceptum* after 24 h exposure to 0.25% (A), 0.50% (B) and 1.00% (C) magnesium perchlorate $[Mg(ClO_4)_2]$ and transfer to the culture medium for 24 h. D–F, activity of *Mil. inceptum* after 72 h exposure to 0.25% (D), 0.50% (E) and 1.00% (F) $Mg(ClO_4)_2$ and transfer to the culture medium for 24 h. Abbreviations: C, control; PP24H, 24 h post perchlorate in culture medium. Levels of significance are represented as $^*P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$ and $^{****}P \leq .0001$.

0.25% perchlorate. The highest recovery of activity after 24 h exposure to 1.00% perchlorate was found for *Pam. experimentalis*, the lowest for *Mil. inceptum*. Notably, most specimens of the three species returned to activity when transferred to culture medium after 72 h exposure to 0.25 or 0.50% perchlorate. In the case of 72 h incubation at the highest perchlorate concentration,

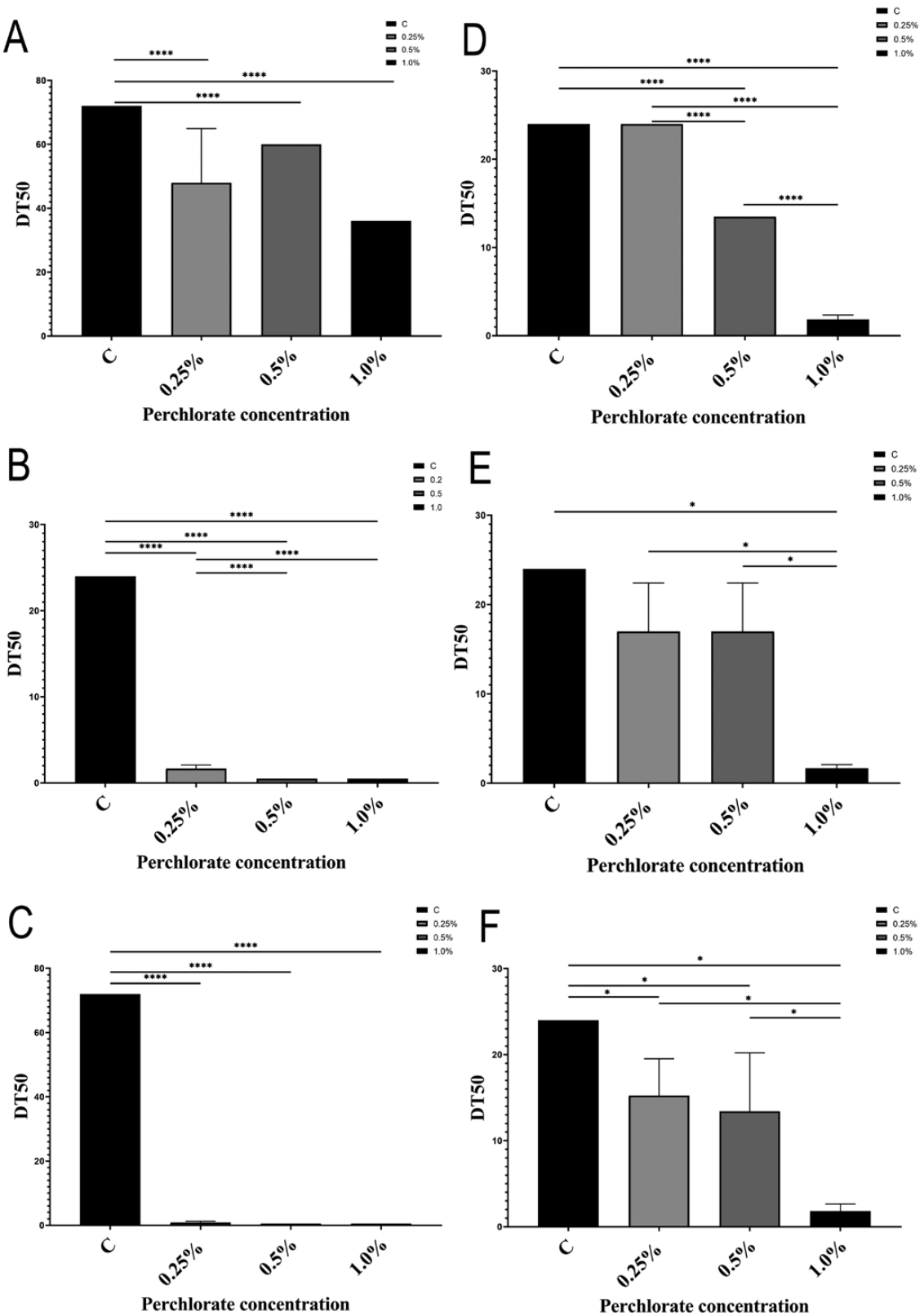


Figure 7. Median deactivation time (DT50) for: A, *Artemia salina*; B, *Caenorhabditis elegans*; C, *Lecane inermis*; D, *Hypsibius exemplaris*; E, *Paramacrobiotus experimentalis*; and F, *Milnesium inceptum*. Levels of significance are represented as $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$ and $^{****}P \leq .0001$.

only 10% of *Mil. inceptum* and *Pam. experimentalis* resumed activity, and none of *Hys. exemplaris*.

There was a significant difference in the median time of inactivation of all three tardigrades species between the experimental treatments (Table 3; Fig. 7). The values of DT50 for *Hys. exemplaris* between the experimental treatments were statistically significant (Kruskal–Wallis $\chi^2 = 22.72$, d.f. = 3, $P < .0001$; Table 3; Fig. 7). The individual statistical significance among

Table 1. The activity of the specimens of six invertebrate species exposed for 24 or 72 h to one of three concentrations of magnesium perchlorate (lack of active specimens is highlighted in grey).

Species	*N*	Time (h)	Activity (% of control)		
			0.25% perchlorate solution	0.5% perchlorate solution	1% perchlorate solution
Artemia salina	30	24	83.3	83.3	80.0
Caenorhabditis elegans	30	24	40.0	0.0	3.3
Lecane inermis	35–109	24	22.2	17.9	6.0
Hypsibius exemplaris	30	24	100.0	0.0	0.0
Paramacrobiotus experimentalis	30	24	33.3	16.7	0.0
Milnesium inceptum	30	24	36.7	23.3	0.0
A. salina	30	72	6.7	6.7	0.0
C. elegans	30	72	23.3	0.0	0.0
L. inermis	35–109	72	1.0	1.7	0.6
Hys. exemplaris	30	72	0.0	0.0	0.0
Pam. experimentalis	30	72	30.0	26.7	0.0
Mil. inceptum	30	72	26.7	0.0	0.0

Table 2. The activity of the specimens of five invertebrate species exposed for 24 or 72 h to one of three concentrations of magnesium perchlorate, then transferred to distilled water (for 24 h) (lack of active specimens is marked in grey).

Species	*N*	Time (h)	Activity (% of control)		
			0.25% perchlorate solution	0.50% perchlorate solution	1.00% perchlorate solution
Caenorhabditis elegans	30	24	56.7	30.0	13.3
Lecane inermis	35–109	24	67.2	51.2	40.2
Hypsibius exemplaris	30	24	100.0	100.0	56.7
Paramacrobiotus experimentalis	30	24	93.3	76.7	86.7
Milnesium inceptum	30	24	100.0	63.3	26.7
C. elegans	30	72	33.3	30.0	10.0
L. inermis	35–109	72	65.4	46.4	31.6
Hys. exemplaris	30	72	56.7	50.0	0.0
Pam. experimentalis	30	72	83.3	86.7	10.0
Mil. inceptum	30	72	63.3	63.3	10.0

groups was as follows: control vs. 0.50%, $P = .0063$; control vs. 1.00%, $P = .0067$; 0.25% vs. 0.50%, $P = 0.0063$; 0.25% vs. 1.00%, $P = .0067$; and 0.50% vs. 1.00%, $P = .0067$). Differences between the remaining pairs were not significant ($P > .05$). Likewise, the values of DT50 for *Mil. inceptum* between the experimental treatments were statistically significant (Kruskal–Wallis $\chi^2 = 18.722$, d.f. = 3, $P = .0003$; Fig. 7). The individual statistical significance among groups is as follows: control vs. 0.25%, $P = .027$; control vs. 0.50%, $P = .027$; control vs. 1.00%, $P = .015$; 0.25% vs. 1.00%, $P = .017$; and 0.50% vs. 1.00%, $P = .027$. Furthermore, values of DT50 for *Pam. experimentalis* between the experimental treatments were statistically significant (Kruskal–Wallis $\chi^2 = 17.938$, d.f. = 3, $P = .0005$; Table 3; Fig. 7). The individual statistical significance among groups is as follows: control vs. 1.00%, $P = .011$; 0.25% vs. 1.00%, $P = .015$; and 0.50% vs. 1.00%, $P = .015$.

Similarities and differences between species exposed to perchlorates

Large differences in activities were observed between the species after 24 h in 0.25% and 0.50% perchlorate solutions. In 0.25% solution, a high activity was observed for *Hys. exemplaris* (100%) and *A. salina* (83%), lower for other species, i.e. *Mil. inceptum*, *Pam. experimentalis* and *C. elegans* (33%–40%), and very low for *L. inermis* (only 22%) (Tables 1 and 2). After 72 h exposure to 0.25% perchlorate, differences were smaller and ranged from no active specimens observed of *Hys. exemplaris*, a few for *A. salina* and *L. inermis*, and 20%–27% for *Mil. inceptum*, *Pam. experimentalis* and *C. elegans* (Tables 1 and 2).

Large differences were also observed for 0.50% perchlorate solution, especially after 24 h exposure (Tables 1 and 2). More than 80% of *A. salina* nauplii were active, 17%–23% specimens of *Mil. inceptum*, *Pam. experimentalis* and *L. inermis*, and no active

Table 3. The median deactivation time (deactivation time 50%) of the specimens of six invertebrate species exposed to one of three concentrations of magnesium perchlorate, then transferred to distilled water (for 24 h).

Species	Median deactivation time (h)		
	0.25% perchlorate solution	0.50% perchlorate solution	1.00% perchlorate solution
Artemia salina	36–60	60	36
Caenorhabditis elegans	1.5–2.5	0.5	0.5
Lecane inermis	0.5–1.5	0.5	0.5
Hypsibius exemplaris	24	13.5	1.5–2.5
Paramacrobiotus experimentalis	13.5–24	13.5–24	1.5–2.5
Milnesium inceptum	13.5–24	2.5–24	0.5–2.5

specimens were observed for *Hys. exemplaris* and *C. elegans*. After 72 h in 0.50% perchlorate solution, a comparatively higher number of active specimens was observed for *Pam. experimentalis* (27%), a very low number of active specimens for *A. salina* and *L. inermis* (7% and 2%, respectively), and no active specimens for *Hys. exemplaris*, *Mil. inceptum* and *C. elegans* (Tables 1 and 2).

In the case of Nematoda, Rotifera and Tardigrada, almost no active specimens were observed in 1.00% perchlorate solution after 24 and 72 h. In contrast, 80% of *A. salina* specimens were active after 24 h, although after 72 h, like other species, no active specimens of *A. salina* were observed (Tables 1 and 2).

Similarities and differences between species in return to activity in culture medium

After exposure to 0.25% and 0.50% perchlorate for 24 h, the percentage of active specimens transferred to distilled water/culture medium was very high for *Hys. exemplaris*, *Mil. inceptum*, *Pam. experimentalis* and *L. inermis* (58%–100%), but slightly lower for *C. elegans* (30%–57%). The percentage of active specimens after exposure to 1% perchlorate for 24 h and subsequent transfer to distilled water/culture medium was high for *Pam. experimentalis* (86.7%), medium-high for *Hys. exemplaris* and *L. inermis* (57% and 50%, respectively) and relatively low for *Mil. inceptum* and *C. elegans* (27% and 13%, respectively). The percentage of active specimens exposed to 0.25% and 0.50% perchlorate solution for 72 h and transferred to distilled water/culture medium was very high for *Pam. experimentalis* (83%–87%) and lower for other species (27%–63%). In the case of 1.00% perchlorate, the number of active specimens was relatively high for *L. inermis* (31%) and low for *Pam. experimentalis* and *C. elegans* (10%), while no active specimens of *Hys. exemplaris* and *Mil. inceptum* were observed (Tables 1 and 2). In the case of *A. salina*, no reassessment was possible, because the species cannot enter into cryptobiosis after leaving the cyst, and all the individuals die soon after immobilization.

DISCUSSION

The present study screened the activity of different invertebrates exposed to a range of perchlorate concentrations. In general, the resistance of the tested species decreased along the concentration gradient and with exposure time, but revealed significant interspecies differences.

The results highlight the significant toxicity of perchlorate for invertebrates used in the experiments, despite the fact that they can withstand extreme conditions. In general, exposure for 72 h without transfer to a perchlorate-free environment resulted in a drastic decrease in viability, seen even at the lowest concentration (0.25%) of magnesium perchlorate. It should be underlined that the tested perchlorate levels, which reflect those that can be expected on the Martian surface, are 1000-fold higher than the highest concentration observed naturally on Earth in evaporites associated with hyperarid regions, such as the Atacama Desert (where levels ≤ 0.00025% wt were noted), and ~100- to 10 000-fold higher than those noted in associated superficially running waters (Ericksen 1983, Calderón *et al.* 2014). Therefore, no organism on Earth is challenged by conditions used in the present study to evolve specific adaptation mechanisms.

Although perchlorates are highly toxic for life on Earth, some microorganisms can sustain it to some extent through mitigation of oxidative stress linked to carotenoid production (cyanobacteria) or by utilization of perchlorate as a terminal electron acceptor and can execute its effective reduction through perchlorate reductase (e.g. *Azospirillum* spp., *Dechlorosoma* spp. and *Dechloromonas* spp.) (Bender *et al.* 2005, Nozawa-Inoue *et al.* 2005, Carlström *et al.* 2015, Rzymski *et al.* 2022). However, none of the tested animals is known to exhibit such mechanisms. Therefore, any activity, even at the lowest tested perchlorate concentration (0.25%) for ≥ 24 h is a remarkable feature revealed by all the species used in these experiments. It should also be noted that selected species (*C. elegans*, *A. salina* and *L. inermis*) could withstand the highest concentration (1.00%) for 24 h. This particularly concerned *A. salina*, for which 80% activity was observed. This is probably attributable to tolerance of this species to various salinities, resulting in its common occurrence in hypersaline lakes (Gajardo and Beardmore 2012). Addition of magnesium perchlorate results in increased electric conductivity of the solution (Rzymski *et al.* 2023); therefore, it is plausible that the highly efficient osmoregulation that allows *A. salina* to withstand high salinity (Croghan 1958a, b) also mitigates the perchlorate-induced stress. However, more prolonged exposure of 72 h decreased the activity of *A. salina* by 100%, probably resulting in the mortality of specimens owing to ClO_4^- ions preventing the coalescence of gas bubbles in solution, ultimately hampering the breathing capacity of this crustacean (Lo Nostro *et al.* 2015).

Interestingly, *L. inermis* was the only species to reveal activity after 72 h exposure to 1.00% perchlorate solution (although only at a minimal level of 0.6%) and with the highest recovery rate (~30%) when transferred to distilled water after an additional 24 h. This rotifer species is commonly associated with wastewater treatment plants and occurs in activated sludge; this can explain the elevated resistance to chloride ions. It is known that perchlorate toxicity manifests predominantly through oxidative stress and its consequences, including lipid peroxidation, protein denaturation and DNA damage (Ingram 1981, Hallsworth *et al.* 2003, Rzymski *et al.* 2022). Therefore, the adverse effects of perchlorate can be mitigated, at least to some extent, by the effective antioxidant capacity of the biological system. Further

studies would be required to understand the plasticity of *L. inermis* to respond to oxidative stress at the level of expression of antioxidative enzymes (e.g. catalase or superoxide dismutase), in addition, potentially, to other routes to mitigate perchlorate-induced toxicity.

Three species of tardigrades (*Hys. exemplaris*, *Mil. inceptum* and *Pam. experimentalis*) were used in the present study. Tardigrades are well known for their remarkable resistance to extreme abiotic conditions, such as lack of water, high ionizing and UV radiation levels, space vacuum, toxic heavy metals and metalloids, low oxygen levels, and very low and high temperatures (Ramløv and Westh 2001, Rebecchi *et al.* 2007, Jönsson *et al.* 2008, Altiero *et al.* 2011, Guidetti *et al.* 2011, 2012, Wełnicz *et al.* 2011, Rebecchi 2013, Cesari *et al.* 2022, Roszkowska *et al.* 2023). These abilities are likely to be a side effect of adaptation by the tardigrade to desiccation and the ability to enter a cryptobiotic state known as anhydrobiosis. This are mediated by various protective molecules, such as Dsup and Dsup-like proteins, cytoplasmic abundant heat-soluble, secretory abundant heat-soluble and mitochondrial abundant heat-soluble proteins, and LEA proteins (Hand *et al.* 2011, Hesgrove and Boothby 2020, Mínguez-Toral *et al.* 2020). However, it should be underlined that active specimens can also withstand relevant environmental stress, e.g. active *Hys. exemplaris* can thrive when exposed to gamma radiation [$LD_{50/48\ h}$ (median lethal dose) of ~4200 Gy], and *Halobiotus crispae* (Kristensen, 1982) resisted very low temperatures (Soemme 1996, Halberg *et al.* 2009, Beltrán-Pardo *et al.* 2015). For this reason, one would expect that these animals might also possess the extraordinary capacity to withstand perchlorate exposure, which would further advocate their use in space exploration and astrobiological studies, and they have been selected as potential candidates to survive in Martian conditions (Horikawa *et al.* 2008, Guidetti *et al.* 2012, Erdmann & Kaczmarek 2017).

Contrary to the outstanding abilities mentioned above, in the present paper we show that the resistance of tardigrades to perchlorates is limited. None of the tested species could remain active when exposed to 1.00% perchlorate for 24 h. *Paramacrobiotus experimentalis* had the highest tolerance of the three tardigrade species tested, because it was the only one to remain active after 72 h exposure to 0.50% perchlorate. Tardigrades have previously been shown to reveal the highest protection against oxidative stress (measured by superoxide dismutase activity and by glutathione peroxidase and glutathione) when entering a desiccated state compared with a hydrated state (Rizzo *et al.* 2010). It is plausible that they might be incapable of counteracting the toxic effects of perchlorates when active specimens are exposed. Therefore, it would be interesting to study survival of tardigrades when exposed to these chemicals during the cryptobiotic state. In contrast, the tested tardigrade species revealed a good recovery of activity when transferred to distilled water after exposure. This rate ranged from 93% to 100% and from 57% to 83% after 24 and 72 h exposure to 0.25% perchlorate, respectively, and from 63% to 100% and from 50% to 87% in the case of 0.50% perchlorate, respectively. After 24 h exposure to 1.00% perchlorate, the frequency of active specimens was in the range of 27%–87%, and after 72 h only 10% of *Mil. inceptum* and *Pam. experimentalis* specimens were viable. Furthermore, the median time of deactivation (DT50) for all three tardigrade species used in this study is similar; for 0.25% perchlorate exposure, the range of DT50 for *Mil. inceptum* and *Pam. experimentalis* specimens was 13.5–24 h, and for *Hys. exemplaris* it was 24 h. For 0.50% perchlorate exposure. the range of DT50 for *Pam. experimentalis* specimens was 13.5–24 h, for *Mil. inceptum* 2.5–24 h and for *Hys. exemplaris* 13.5 h. Furthermore, for 1.00% perchlorate exposure, the range of DT50 for *Hys. exemplaris* and *Pam. experimentalis* specimens was 1.5–2.5 h, and for *Mil. inceptum* it was 0.5–2.5 h. It would be of interest to address the perchlorate tolerance in tardigrades in a repeated exposure–recovery model to understand whether gradual induction of resistance is occurring and whether it might also influence the resistance in offspring, e.g. through epigenetic mechanisms.

Although the present research provides an overview of the persistence of various invertebrates in the presence of perchlorate, with potential implications for use in the exploration of Mars, study limitations must be stressed. Firstly, magnesium perchlorate was used in our experiments, although the Martian surface might also contain calcium and sodium perchlorates at varying levels (Hecht *et al.* 2009, Glavin *et al.* 2013, Hassler *et al.* 2014, Kounaves *et al.* 2014, Ojha *et al.* 2015). Some of the animals used in our experiment were exposed to perchlorates without preconditioning, although studies show that stress preconditioning (e.g. with temperature, UV and gamma radiation) can increase the tolerance of some invertebrates (e.g. *C. elegans*) to selected chemicals (Alzahrani and Ebert 2018). Moreover, the laboratory simulations demonstrated that if liquid water emerged on Mars, it would be characterized, besides perchlorates, by high electric conductivity connected by elevated concentrations of sulphur, magnesium, calcium, sodium, potassium and iron, high turbidity, pH fluctuations and highly oxidizing conditions (Rzymski *et al.* 2023). In fact, some effects of animal deactivation observed in our study might be attributable to perchlorate activity and osmotic stress induced by the high salinity of the perchlorate solution. However, the addition of 0.25%–1.00% perchlorate results in an electric conductivity > 2 mS/cm (Rzymski *et al.* 2023), which is in the low range seen for brackish ecosystems and is seen sporadically in contaminated freshwaters (Ahuja 2014, Klimaszyk *et al.* 2022). Nevertheless, the partial role of osmotic/ionic effects could be completely excluded only by experiments involving controls containing salt solutions but devoid of perchlorates. Furthermore, UVC radiation, which reaches the Martian surface, has been shown to magnify the oxidative action of perchlorate and enhance its toxicity (Wadsworth and Cockell 2017). Also, the lack of a continuous magnetic field on Mars will probably also lower the survivability of some invertebrates (e.g. tardigrades; Erdmann *et al.* 2017, 2021a, b). Recently, *Hys. exemplaris* was shown to be less resistant to lack of water (typical for Martian conditions) than previously suggested (Poprawa *et al.* 2022). Therefore, any organism from Earth would need to confront additional environmental challenges.

Our results suggest that all tested invertebrates possess some degree of resistance to extremely high concentrations of perchlorates that decrease with exposure time and doses. It is plausible that the tolerance observed in the present study is an indirect effect of adaptation of particular species to high salinity, osmotic stress and/or desiccation. Further investigations involving

solutions differing in ion concentrations and using molecular studies would be required to understand the exact mechanisms behind the observed effect. Nevertheless, the reported observations provide a basis for further research into the mechanisms behind the observed perchlorate tolerance and their potential use in developing biological systems with enhanced performance and utility on Mars using genetic engineering or synthetic biology techniques.

ACKNOWLEDGEMENTS

Studies have been conducted, in part, in the framework of activities of the Biodiversity and Astrobiology Research group (BARg). Pushpalata Kayastha holds a scholarship of Passport to the future—interdisciplinary doctoral studies at the Faculty of Biology, Adam Mickiewicz University, Poznań POWR.03.02.00-00-I006/17, and her study was also supported by Minigrant Edition II, contract number POWER6/2021/2ed, Adam Mickiewicz University, POWR.03.02.00-00-I006/17.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Paper IV

Kayastha, P., Wieczorkiewicz, F., Pujol, M., Robinson, A., Michalak, M., Kaczmarek, Ł. and Poprawa, I. Elevated external temperature affect cell ultrastructure and heat shock protein (HSP) in *Paramacrobiotus experimentalis* Kaczmarek, Mioduchowska, Poprawa & Roszkowska, 2020’ https://doi.org/10.21203/rs.3.rs-3202172/v1

Elevated external temperature affects cell ultrastructure and heat shock proteins (HSPs) in Paramacrobiotus experimentalis Kaczmarek, Mioduchowska, Poprawa, & Roszkowska, 2020

Pushpalata Kayastha (pushpalata.kayastha@gmail.com)
Adam Mickiewicz University, Poznań
Filip Wieczorkiewicz
University of Silesia in Katowice
Myriam Pujol
University of Alberta
Alison Robinson
University of Alberta
Marek Michalak
University of Alberta
Łukasz Kaczmarek
Adam Mickiewicz University, Poznań
Izabela Poprawa
University of Silesia in Katowice

Article

Keywords: heat shock proteins, storage cells, ultrastructure, tardigrades, water bears

Posted Date: August 2nd, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3202172/v1

Abstract

Increasing temperature influences the habitats of various organisms, including microscopic invertebrates. To gain insight into temperature-dependent changes in tardigrades, we isolated storage cells exposed to various temperatures and conducted biochemical and ultrastructural analysis in active and tun-state *Paramacrobiotus experimentalis*Kaczmarek, Mioduchowska, Poprawa, & Roszkowska, 2020. The abundance of heat shock proteins (HSPs) and ultrastructure of the storage cells were examined at different temperatures (20 °C, 30 °C, 35 °C, 37 °C, 40 °C, and 42 °C) in storage cells isolated from active specimens of *Paramacrobiotus experimentalis* Kaczmarek, Mioduchowska, Poprawa, & Roszkowska, 2020. In the active animals, upon increase in external temperature, we observed an increase in the levels of HSPs (HSP27, HSP60, and HSP70). Furthermore, the number of ultrastructural changes in storage cells increased with increasing temperature. Cellular organelles, such as mitochondria and the rough endoplasmic reticulum, gradually degenerated. At 42 °C, cell death occurred by necrosis. Apart from the higher electron density of the karyoplasm and the accumulation of electron-dense material in some mitochondria (at 42 °C), almost no changes were observed in the ultrastructure of tun storage cells exposed to different temperatures. We concluded that desiccated (tun-state), but not active, tardigrades are resistant to high temperatures.

Introduction

The phylum Tardigrada currently consists of *ca*. 1,500 species (33 families, 159 genera, 1,464 species, and 21 additional subspecies) [1] that inhabit most terrestrial and aquatic environments worldwide [2–4].

Tardigrades (water bears) are well-known extremophiles, organisms that are able to survive extreme conditions, due to their ability to undergo cryptobiosis, during which various proteins act as cytoprotectants [5–10]. Currently, only a few proteins, including heat shock proteins (HSPs), are recognized as cytoprotectants [11–15]. Various HSPs, including small heat shock proteins (sHSPs) and HSP70, are preserved in full range in tardigrades [16–18]. Studies of HSPs in tardigrades have primarily focused on their role in anhydrobiosis [16,19–21]. sHSPs, such as HSP27 and HSP30C, are upregulated during dehydration [22]; however, HSP70 is upregulated during recovery and rehydration [23–26]. This suggests that the upregulation of small and large HSPs is a response to different stages of stress.

Higher temperatures may damage cells and cell components, *i.e.*, cell membranes, DNA, gut, mitochondria, proteins, and storage cells. Examples of such damage have been documented in various biological systems, such as sea cucumbers [27], spermatid cells of rats [28], and tobacco (*Nicotiana sylvestris*) [29]. Doyère [30] and Pouchet [31] conducted the first studies of tardigrade thermotolerance in the early 19th century, followed by Li & Wang [32] and Rebecchi *et al*. [33], who studied the tolerance of *Borealibius zetlandicus* (Murray, 1907 [34]) and *Macrobiotus harmsworthi* (Murray, 1907 [34]) after 24 h at 36°C and 38°C. In a more recent study, Neves *et al*. [35] reported that *Ramazzottius varieornatus* exhibited a tolerance to high temperature in both its active and anhydrobiotic states.

The water bear lacks a circulatory or respiratory system, but its body cavity is filled with storage cells, which float freely in the lymph or occasionally attach to the basement membranes of other tissues [36,37]. Storage cells are responsible for both nutrient transport and storage of lipids, polysaccharides, and pigments (such as carotenoids) [2,38]. They produce protein substances that combine to form lipid globules and vitellogenins in some species [2,39,40]. Storage cells are ameboid in shape, and their numbers and size are dependent upon the nutritional state of the animal [36,37]. Each storage cell has a very large lobular nucleus with a large, non-homogenous nucleolus in the center. Intracellular organelles, such as mitochondria and rough endoplasmic reticulum cisternae, are found in the cytoplasm. The cytoplasm also includes non-homogeneous spheres of various sizes [41]. Storage cells are easy to isolate; preserve the arrangement of their organelles; and store and produce lipids, polysaccharides, and protein substances, making them ideal candidates for the study of heat-dependent structural and biochemical changes. Most importantly, both changes in storage cell ultrastructure and HSP expression as a function of increased temperature have not been reported in a single study of active and desiccated tardigrades. In the present study, we examined ultrastructural changes, as well as the abundance HSP27, HSP60, and HSP70, in storage cells isolated from heat-treated active specimens and anhydrobiotic tuns of *Paramacrobiotus experimentalis* Kaczmarek, Mioduchowska, Poprawa, & Roszkowska, 2020 [42].

Materials and Methods

Animal model

Paramacrobiotus experimentalis specimens were extracted from a sample of mosses collected near Fort-Voyron, Antananarivo, Antananarivo Province, Madagascar (18°55'35"S, 47°31'23"E, 1,340 m.a.s.l.). Fully active *Pam. experimentalis* specimens were used to establish a laboratory population in culture medium (spring water (Żywiec Zdrój):double distilled water (ddH_2O) in a 1:3 ratio) on scratched Petri dishes, following the protocol of Roszkowska *et al*. [43]. Rotifers (*Lecane inermis*) and nematodes (*Caenorhabditis elegans*) were added *ad libitum* as a food source once per week. Adult, fully active, and non-molting *Paramacrobiotus* specimens of a medium body size were selected for experiments.

Experimental design

Heat stress protocol for active animals

All experiments were performed in 1.5 mL Eppendorf tubes containing 10 specimens in culture medium. Each of the Eppendorf tubes was then placed on a heat block with an open lid for 5h at a different temperature: 20°C, 35°C, 37°C, 40°C, and 42°C. Thereafter, the specimens were used for ultrastructural and biochemical analysis.

Anhydrobiosis protocol

All experiments were performed in covered, vented plastic Petri dishes (Ø 35 mm) lined on the bottom with white filter paper (grammage 85–87, Chemland Company, Poland). The specimens were transferred

into dishes, along with 450 µL of the culture medium, using an automatic pipette. Then, the dishes were placed into a climate chamber (PolLab, Q-Cell 140), and the individuals were allowed to dry slowly in the dark for 72 h at 20°C, with 40–50% relative humidity. Once every 24 h, we monitored for tun formation using a stereomicroscope. Once tuns were formed, the specimens were subjected to 20°C, 35°C, 37°C, 40°C, and 42°C for 5 h. Then, half of the tuns were directly fixed for light and transmission electron microscopy, and the other half were left overnight to rehydrate in culture medium for anhydrobiosis success analysis.

Light and transmission electron microscopy

Active or tun-state *Pam. experimentalis* ($n = 15$ for each condition) were subjected to 20°C, 35°C, 37°C, 40°C, or 42°C for 5 h. Then, the material was fixed with 2.5% glutaraldehyde, postfixed in 2% osmium tetroxide, dehydrated, and embedded according to the protocol described by Janelt *et al*. [44]. Semi-thin (800 nm) and ultra-thin (70 nm) sections were cut using a Leica Ultracut EM UC7 ultramicrotome. Semi-thin sections were stained with 1% methylene blue in 1% borax and analyzed using an Olympus BX60 light microscope. Ultra-thin sections were mounted on copper grids and stained with uranyl acetate and lead citrate. Material was examined using a Hitachi H500 transmission electron microscope at 75 kV or a Hitachi UHR FE-SEM SU 8010 scanning electron microscope equipped with an ET (Everhart–Thornley detector) detector for imaging the sections at a low voltage of 25 kV.

Protocol for quantifying HSPs using flow cytometry

Following exposure to different temperatures, the specimens in Eppendorf tubes were transferred to a single depression concave slide with phosphate-buffered saline (PBS) and dissected using a BD precision needle (27 G × ½ in.). Post-dissection, storage cells, along with PBS, were collected and transferred to 1.5 mL Eppendorf tubes. Cells were fixed with a fixation/permeabilization buffer (eBioscience Catalog number: 88-8824-00) for 30 min at room temperature (RT), washed with a blocking buffer containing permeabilization buffer (eBioscience) with 1% BSA (Bovine serum albumin), and centrifuged at 1,500 × *g* for 5 min. Cells were incubated for 40 min at RT with 20 µL of anti-HSP27 (Rab anti-HSP27 Stress Marq SPC-106A & Ms anti-HSP27 Stress Marq SMC-161b), anti-HSP60, or anti-HSP70 (Rab anti-HSP70 Stress Marq SPC-103D; 1:25 dilution) in the blocking buffer (*i.e.*, permeabilization buffer (eBioscience) with 1% BSA). Ten µL of 488 anti-mouse antibodies (1:40 dilution in blocking buffer) was added to each tube, incubated for 1 h, and then washed in blocking buffer by centrifugation at 4,000 RPM for 5 min. Single events were recorded using an LSRFortessa™ X-20 flow cytometer (BD Biosciences Pharmingen). Data analysis was performed using FlowJo™ v10 for PC (TreeStar). Three to five independent experiments were conducted for each experimental condition.

Statistical analysis for HSP data

The mean fluorescent intensity observed was normalized using control (20°C) data. We analyzed the differences between treatments using a nonparametric Kruskal–Wallis test, followed by Dunn's test for multiple comparisons. A value of $p < 0.05$ was deemed statistically significant.

Results

Ultrastructural analysis of active specimens

At 35°C, active individuals of *Pam. experimentalis* shrunk, tucking their legs and head, but did not form tuns, as in anhydrobiosis. After being transferred to RT, they resumed activity. However, active individuals incubated at 37°C, 40°C, or 42°C had firmly erect bodies, and after being transferred to RT, they did not resume activity.

At 20°C, isolated storage cells of the control group remained in the body cavity among the internal organs (Fig. 1A, sc). The cells had amoeboid or spherical shapes (Figs. 1A–B). A lobular nucleus with a heterogeneous nucleolus occupied the central part of each cell. The internal region of the nucleolus had a lower electron density than the external region. The nucleolus was surrounded by clumps of heterochromatin (Fig. 1B). The cytoplasm was rich in cisternae of the rough endoplasmic reticulum, mitochondria with visible cristae and low electron density in the matrix, ribosomes, and spheres of the reserve material (Fig. 1B). These spheres varied in size and electron density.

Similar to the storage cells isolated from control specimens, storage cells isolated from individuals subjected to 35°C for 5 h had amoeboid or spherical shapes (Figs. 1C–D). Additionally, each cell's nucleus had a prominent heterogeneous nucleolus that occupied the central part of the cell (Fig. 1D). However, we observed changes in mitochondria morphology in cells from specimen exposed to 35°C. Several mitochondria degenerated, gradually losing cristae. Their matrices had higher electron densities than those of undamaged mitochondria (Fig. 1D).

The storage cells isolated from the specimens subjected to 37°C for 5 h retained amoeboid or spherical shapes (Figs. 1E–F), but some of the ultrastructural features were altered. There were numerous degenerating mitochondria, which showed a loss of cristae and a matrix with higher electron density, and autophagic structures in the cell cytoplasm (Fig. 1F). The cisternae of the rough endoplasmic reticulum exhibited fragmentation. Moreover, we observed areas of the cytoplasm with low electron density (Fig. 1F). The nucleus and the reserve material sphere in the cytoplasm showed no visible changes. The shapes of the storage cells of the specimens subjected to 40 °C for 5 h were highly irregular (Figs. 2A–D). Many storage cells were shrunken, with many degenerated mitochondria with lost cristae. The mitochondrial matrix showed medium electron density or was electron lucent (Fig. 2C–D). Numerous autophagic structures were present in the cytoplasm of the cells (Figs. 2B–D). No changes were observed in cell nuclei or spheres of the reserve material (Figs. 2B–D).

The most extensive changes were observed in storage cells isolated from active individuals exposed to 42 °C (Figs. 2E–H). In these animals, cells and all organs degenerated and were difficult to distinguish (Figs. 2F–H). The midgut was identifiable only in that it retained the basal lamina (Figs. 2F–G). The storage cells had spherical shapes (Figs. 2G–H) and showed features typical of necrosis. The cytoplasm became electron lucent, and the cell membranes ruptured (Fig. 2H), releasing the cell contents into the body cavity (Figs. 2F–H). The cell nucleus had distended and assumed a spherical shape, but the

nucleolus was still visible (Figs. 2G–H). Mitochondria and the cisternae of the rough endoplasmic reticulum were no longer evident, while spheres of the reserve material were still visible (Fig. 2H).

Ultrastructural analysis of desiccated specimens (tun stage)

The survival rates of tuns after 24 h of rehydration were as follows: 93.3% at 20 °C, 60.0% at 35 °C, 33.3% at 37 °C, 33.3% at 40 °C, and 20.0% at 40 °C. Storage cells occupied all free spaces in each tun's body cavity. The analysis of the ultrastructure of the storage structures of tuns subjected to the experimental temperatures (35 °C, 37 °C, 40 °C, and 42°C) showed no differences compared to cells isolated from control tuns (20 °C) (Figs. 3A–F, 4A–D). These storage cells had shrunk and had amoeboid shapes. The cytoplasm had a high electron density (Figs. 3F, 4B). Frequently, lower electron density was observed in the cytoplasm (Figs. 3B, D, 4D). In the central part of the cell, there was an irregularly shaped nucleus (Figs. 3B, F, 4B, D) with a nucleolus (Figs. 4B, D). The nucleolus was surrounded by clumps of heterochromatin-like structures (Fig. 4B). In the storage cells of tuns exposed to 42 °C, karyolymph had a higher electron density, and large nucleolar vacuoles were visible (Fig. 4D). In the mitochondria, due to the density of the matrix, the cristae were hardly visible (Figs. 3B, D, F; 4B, D). Electron-dense material had accumulated in the few remaining mitochondria of storage cells exposed to 42 °C (Fig. 4D). In the cytoplasm of the storage cells of both the control group and all experimental groups, distended cisternae of the rough endoplasmic reticulum and spheres of reserve material were visible. These spheres varied in size and had medium electron densities (Figs. 3B, D, F; 4B, D).

Abundance of HSPs in storage cells of active animals

The changes in the abundance of HSPs were examined using flow cytometry in active animals at three different temperatures: 20 °C (control group, optimal temperature for tardigrade survival), 35 °C (highest temperature at which active animals survived), and 42 °C (all animals died). Histograms (Fig. 5A), percentages (Fig. 5B), and mean fluorescent intensity (MFI) values (Fig. 5C) correspond only to the HSP^+ cell population. We observed a significant increase in the percentage of HSP^+ storage cells for HSP27 and HSP70 at 35 °C and for HSP60 at 42 °C, compared to controls (Fig. 5B). $HSP27^+$ and $HSP70^+$ cells showed increased values at 35 °C, while $HSP60^+$ cells exhibited increased MFI at 42 °C (Fig. 5C). There was an increase in the percentage of HSP27 (Kruskal–Wallis test: $p = 0.0116$). All experimental treatments were significantly different from the control group (Dunn's multiple comparisons test: $p < 0.05$ in all cases). Similarly, for HSP60, we observed statistically significant upregulation (Kruskal–Wallis test: $p = 0.0018$). Both experimental treatments were significantly different from the control group (Dunn's multiple comparisons test: 20 °C *vs* 35 °C, $p = 0.0235$; 20 °C *vs* 42 °C, $p = 0.0079$). Furthermore, HSP70 showed statistically significant upregulation with temperature (Kruskal–Wallis test: $p = 0.0036$). However, only the comparison between 20 °C and 35 °C was statistically significant (Dunn's multiple comparisons test: $p = 0.0127$). We concluded that there was a parallel increase in the abundance of all three heat shock proteins in animals exposed to increased external temperatures, compared to controls.

Discussion

Water is essential for tardigrade survival because it keeps them physiologically active. However, as they desiccate [45] and form tuns, by constricting and retracting their head and legs to produce a barrel-shaped, quiescent body, their bodies lose roughly 95% of their water content [46–48]. To survive the extreme environmental conditions they experience, many species enter cryptobiosis, a state in which no metabolic activity occurs, preventing reproduction and development [6,49]. Cryptobiosis is a widespread and reversible state caused by, for example, desiccation, particularly in limno-terrestrial animals [50]. Different types of cryptobiosis are caused by various environmental stressors and include anhydrobiosis (caused by lack of water), anoxybiosis (lack of oxygen), cryobiosis (low temperature), and osmobiosis (change in osmotic conditions) [6,51]. Although tardigrades are known for their ability to survive highly unfavorable environmental conditions in a state of anhydrobiosis, relatively little is known about their resistance to heat stress [38,49-51], especially in active individuals [35].

In the present study, we compared temperature-induced ultrastructural changes (at 20 °C, 35 °C, 37 °C, 40 °C, and 42 °C) in both active *Pam. experimentalis* and those in anhydrobiosis. Active specimens were susceptible to elevated temperatures. No individual survived 5 h at 37 °C, 40 °C, or 42 °C. This species was much more sensitive than *Ramazzottius varieornatus*, which exhibited 50% mortality at 37.1 °C [35].

Storage cells typically float freely in the fluid in the body cavity of tardigrades, but they occasionally attach to the internal organs. Storage cells contain reserve material (lipids, polysaccharides, and proteins) and pigments [2,36,37,39,40,52–54]. The reserve material is an energy reservoir that allows tardigrades to survive unfavorable environmental conditions, including by entering a state of anhydrobiosis and returning to an active state [19,36,39–41,53,55,56].

In the species studied, initial changes in the ultrastructure of storage cells were observed in the active individuals subjected to 35 °C. In these individuals, degeneration of few mitochondria was evident by loss of their cristae. Mitochondria play an important role in cell physiology, aging, and cell death; therefore, they are among the first organelles to be affected by changes in environmental conditions [57]. Mitochondrial changes similar to those we observed in *Pam. experimentalis* have also been described for *Hypsibius exemplaris* [58] individuals incubated in paracetamol for 7 days at a concentration of 1 mg/L and for 28 days at concentrations of 0.2 µg/L, 230 µg/L, and 1 mg/L [54]. Although this was due to chemical stress, the result was similar to that induced by heat stress in our experiment. Loss of mitochondrial cristae has also been observed in somatic and germline cells of various invertebrates exposed to toxic metals [57,59,60] and in the midgut cells of starved shrimp (*Neocaridina davidi*) [61]. Because mitochondria play a crucial role in the activation of cell death [62], changes in both mitochondrial ultrastructure and mitochondrial membrane potential are expected to activate cell death [61,63–66]. Here, we showed that increased environmental temperature also has profound effects on mitochondrial architecture.

In the storage cells of active *Pam. experimentalis* incubated at 37 °C, mitochondria degenerated, exhibiting lost cristae in addition to numerous autophagic structures. More striking changes occurred in

the storage cells of individuals incubated at 40 °C. Autophagy is the process during which damaged organelles, such as mitochondria, cisternae of the endoplasmic reticulum, or structures accumulating xenobiotics, are neutralized inside autophagosomes. Autophagy is aimed at protecting the cell from death [60,67–69]. An increase in autophagic structures in tardigrade storage cells incubated at higher temperatures indicated activation of the cells' defense mechanisms. Increased autophagy has also been observed in the storage cells of *Hys. exemplaris* treated with paracetamol [54] and in midgut cells of *Grevenius granulifer* infected with microsporidia [70]. In the former case, autophagy was responsible for eliminating damaged cell organelles, and, in the latter, for removing parasites. Accumulating too many autophagic structures in the cell activates the cell death pathway. Here, we showed that dramatic changes in storage cells, caused by high temperature, induced autophagy.

Exposure to 42 °C caused the most damage to active individuals. The cytoplasm of *Pam. experimentalis* storage cells became electron lucent, the cell membranes broke, and the degraded cells and organs displayed classic symptoms of necrosis. Necrosis is a type of cell death that can be induced by various stressors, such as external factors, mechanical trauma, or even intensive autophagy [66,71,72]. The interaction between autophagy and necrosis has been documented in the midgut cells of tardigrades [72] and the fat bodies of myriapods [73]. Again, we showed that high temperature induced these changes.

Active individuals incubated at a higher temperature (37 °C, 40 °C, or 42 °C) did not survive the experiment. An approximate lifespan under experimental conditions can be estimated by considering the ultrastructural changes in their storage cells. Specimens incubated at 37 °C showed relatively minor structural change, so they probably died toward the end of the 5 h experiment. By contrast, specimens incubated at 42 °C died at the beginning of the experiment, as evidenced by the advanced necrosis of their internal organs and storage cells.

As in previous work on *Richtersius coronifer* [41], the storage cells of anhydrobiotic specimens of *Pam. experimentalis* were smaller than those of active individuals and had an amoeboid shape. Due to cell shrinkage caused by water loss, the cytoplasm was electron dense. Similar changes were observed in the storage cells of anhydrobiotic tuns of *Pam. experimentalis* in the present study.

Ultrastructural analysis of storage cells in tuns of *Pam. experimentalis* showed no differences between the cells exposed to certain elevated temperatures (35 °C, 37° C, or 40 °C) and a control group (20 °C). However, in the tun storage cells incubated at 42 °C, the karyolymph became denser, and electron-dense material accumulated in the mitochondria. Czerneková *et al*. [41], in their study on the effect of temperature on 6-month-old tuns of *R. coronifer*, also found no ultrastructural changes in storage cells of tuns incubated for 24 h at 50 °C. They reported that heating the *R. coronifer* tuns reduced survival significantly. Additionally, animals subjected to more intense heating generally required more time to rehydrate before being revived [41]. Interestingly, Ramløv and Westh [74], who analyzed the effect of temperature on tuns of *R. coronifer*, found no effect on animal survival at 50–70 °C. By contrast, survival fell to 20% at 80 °C and 0% at 100 °C. It is difficult to explain these discrepancies. It is likely that, in the study by Czernekova *et*

al. [41], the 6 months of desiccation prior to exposure to 50 °C may have rendered the specimens more susceptible to heat stress. Because repair mechanisms are not operating during anhydrobiosis, damage from oxidative interactions with the surrounding air accumulates over time [33]. The non-heated animals did not show reduced survival after 6 months. The reason for this difference might be that the body may have been more sensitive to heat injury or less able to mend the combined damage from long-term desiccation and heating. It appears that the chemical elements required for cell survival, rather than overall cell architecture, were damaged, causing these harmful effects. In the present study, survival fell to 60.0% at 35 °C, 33.3% at both 37 °C and 40 °C, and 20.0% at 42 °C. At temperatures between 42 °C and 45 °C, protein denaturation occurs in cellular organelles [75–77], which might be another reason for reduced survivability at higher temperatures. Since the durations of both anhydrobiosis and higher temperature exposure in the present study were minimal, changes in the ultrastructure of the storage cells were unlikely to significantly affect animal survival. Additional events, including damage to proteins and other chemical compounds, may play a significant role in the proper functioning of the cell. This is supported by our analysis of the expression of HSPs in heat-treated organisms.

Three different HSPs (HSP27, HSP60, and HSP70) in active specimens of *Pam*. *experimentalis* at 20 °C, 37 °C, and 42 °C were analyzed using flow cytometry.

Even in cells that are not under stress, HSPs and their molecular partners have been shown to play a variety of roles in the folding, assembly, intracellular localization, secretion, regulation, and destruction of other proteins [78,79]. However, for cytoprotection from extreme cellular stress, including increased temperature, the rapid expression of inducible HSP70 is essential. Due to their rapid and substantial upregulation in response to a variety of environmental stressors, proteins in the HSP70 family frequently have been employed as biomarkers [80]. HSP70 expression appears to be elevated in active specimens following the induction of heat stress. HSP70 chaperones the refolding of heat-denatured peptides to reduce proteolytic destruction, a conserved process in eukaryotes known as the heat shock response [81]. Several heat-soluble protein families, also called the "tardigrade-unique proteins," as well as the highly conserved heat-inducible HSP70 family, are among the proteins suspected to play a role in tardigrade thermotolerance. They function as molecular barriers during anhydrobiosis [11,13,82]. Their function may be related to mechanisms of repair following stress, such as desiccation [24].

Likewise, under heat stress, the mitochondrial HSP60 has been shown to form complexes with a number of polypeptides. HSP60 is a well-known chaperone and is stress inducible [83]. HSP60 is necessary to stop native dihydrofolate reductase (DHFR) imported into mitochondria from becoming thermally inactive *in vivo*. When DHFR was being thermally denatured *in vitro*, HSP60 linked to it, preventing it from aggregating and mediating its adenosine triphosphate–dependent refolding at higher temperatures. This is a generic mechanism by which proteins in the HSP60 family stabilize pre-existing proteins under stressful circumstances [84,85]. In the present study, the expression of HSP60 was also upregulated with heat stress. Because of their constant exposure to temperature fluctuations, cells increase their resistance to stress by upregulating the expression of HSP60 when necessary [83].

HSP27, a sHSP, also showed increased expression at higher temperatures. In both prokaryotic and eukaryotic cells, sHSPs were identified as a collection of proteins with a molecular mass ranging from 15 to 42 kDa [86]. Through their involvement in the modulation of cellular redox states, sHSPs were recognized to play a role in the regulation of other cellular functions, such as apoptosis and differentiation, in mammalian cells, in addition to promoting cell survival in response to stress [87]. Furthermore, two crystallin sHSPs (Mt-sHSP17.2 and Mt-sHSP19.5) from the tardigrade *Milnesium inceptum* [88] were reported to form sizable complexes under heat stress conditions, potentially stabilizing the structure of other proteins [19]. Interestingly, despite not being regulated during anhydrobiosis, the expression of one of these HSPs (Mt-sHSP17.2) is elevated by heat shock treatment of active specimens [19]. Recently, Hibshman *et al*. [89] discovered that sHSPs were among the most abundant transcripts recorded in two separate RNA-seq datasets of *Hys. exemplaris*, which implies that sHSPs may be more transcriptionally responsive to temperature changes than large HSPs.

Declarations

Acknowledgements

Studies have been partially conducted in the framework of activities of BARg (Biodiversity and Astrobiology Research group). PK is scholarship holder of Passport to the future - Interdisciplinary doctoral studies at the Faculty of Biology, Adam Mickiewicz University, Poznań, Poland POWR.03.02.00-00-I006/17. Paul LaPoint-antibodies for providing HSP antibodies. The authors also wish to thank Cambridge Proofreading LLC (http://proofreading.org) for their linguistic assistance.

Author contributions

Conceptualization, P.K. and Ł.K.; data curation, P.K., M.P. and I.P.; investigation, P.K., M.P. and I.P.; methodology, P.K., M.P., M.M., F.W., I.P. and Ł.K.; statistical analysis, P.K. and M.P.; validation, all authors; supervision, M.M., I.P. and Ł.K.; writing—original draft, P.K. and I.P.; writing—review and editing, all authors. All authors accepted the final version of the manuscript.

Data Availability Statement

All relevant data are within the paper.

Ethical approval

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

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figures

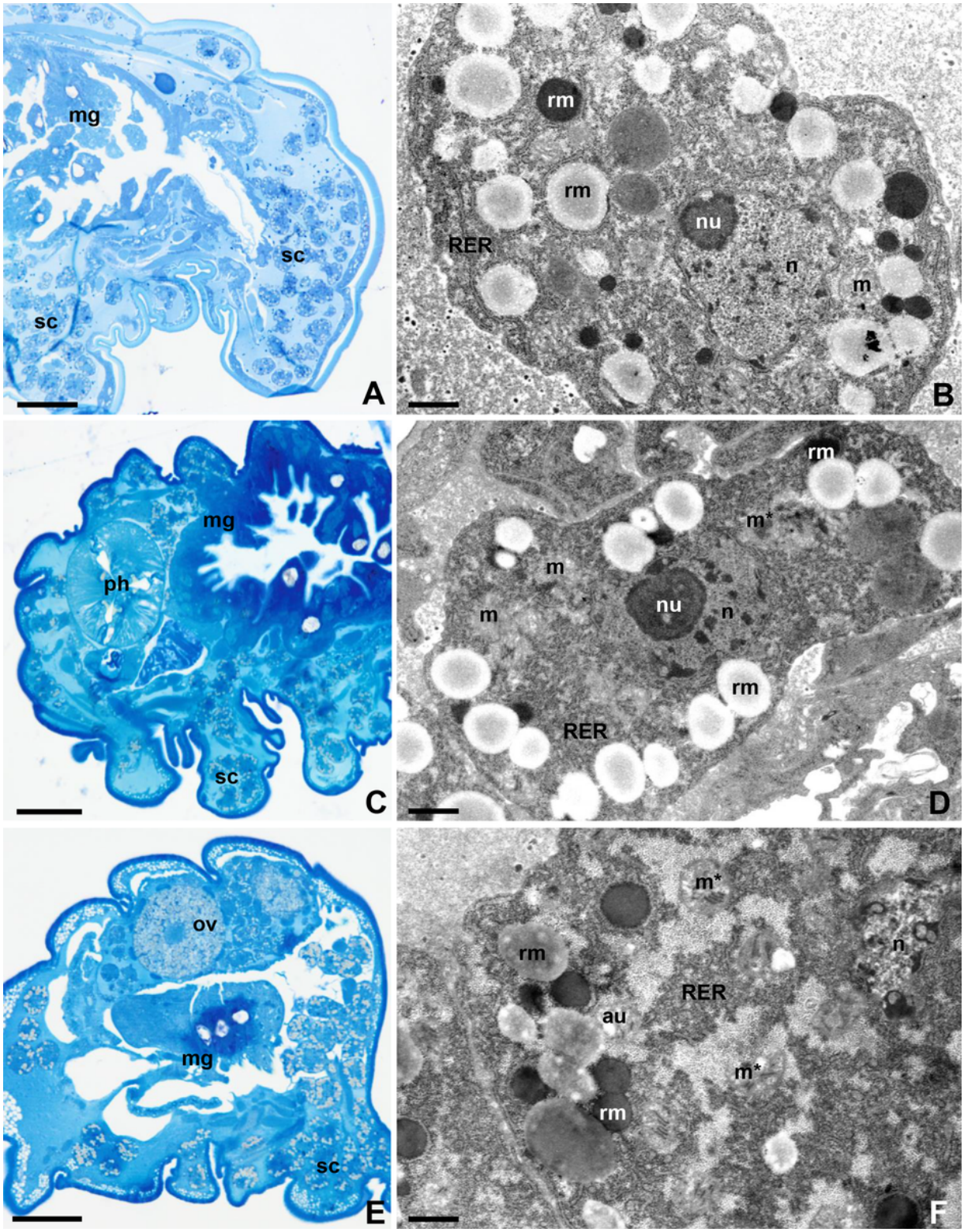

Figure 1

The storage cells of the active specimens of *Paramacrobiotus experimentalis*.

A. The storage cells of a control animal (20 °C), LM, scale bar = 20 μm, **B.** The ultrastructure of a storage cell of a control animal (20 °C), TEM, scale bar = 0.83 μm, **C.** The storage cells of an animal exposed to 35 °C, LM, scale bar = 20 μm, **D.**The ultrastructure of a storage cell of an animal exposed to 35 °C, TEM,

scale bar = 0.88 µm, **E.** The storage cells of an animal exposed to 37 °C, LM, scale bar = 20 µm, **F.** The ultrastructure of a storage cell of an animal exposed to 37 °C, TEM, scale bar = 0.68 µm.

Abbreviations: autophagic structure (au), midgut (mg), mitochondrion (m), damaged mitochondrion (m*), nucleus (n), nucleolus (nu), ovary (ov), pharynx (ph), reserve material (rm), rough endoplasmic reticulum (RER), storage cells (sc).

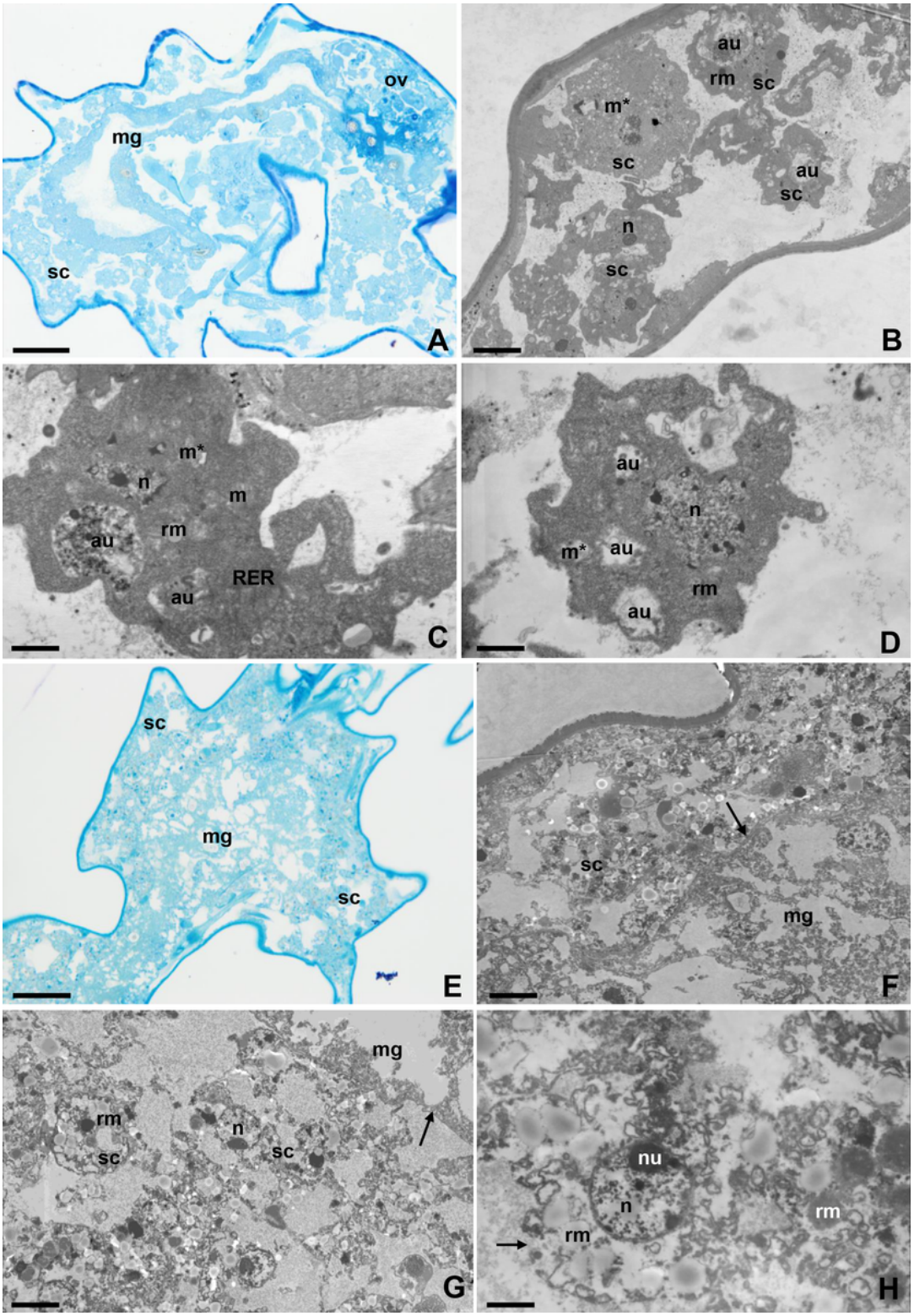

Figure 2

The storage cells of active specimens of *Paramacrobiotus experimentalis*.

A. The storage cells of an animal exposed to 40 °C, LM, scale bar = 20 μm, **B–D.** The ultrastructure of storage cells of animals exposed to 40 °C, TEM, **B.** scale bar = 4 μm, **C.** scale bar = 1.1 μm, **D.** scale bar = 1.25 μm, **E**. The storage cells of an animal exposed to 42 °C, LM, scale bar = 20 μm, **F–H.**The ultrastructure of storage cells of animals exposed to 42 °C, TEM, **F.** Basal lamina (arrow), scale bar = 4.2 μm, **G.** Basal lamina (arrow), scale bar = 4 μm, **H.** Damaged cell membrane (arrow), scale bar = 1.2 μm.

Abbreviations: autophagic structure (au), midgut (mg), mitochondrion (m), damaged mitochondrion (m*), nucleus (n), nucleolus (nu), ovary (ov), pharynx (ph), reserve material (rm), rough endoplasmic reticulum (RER), storage cells (sc).

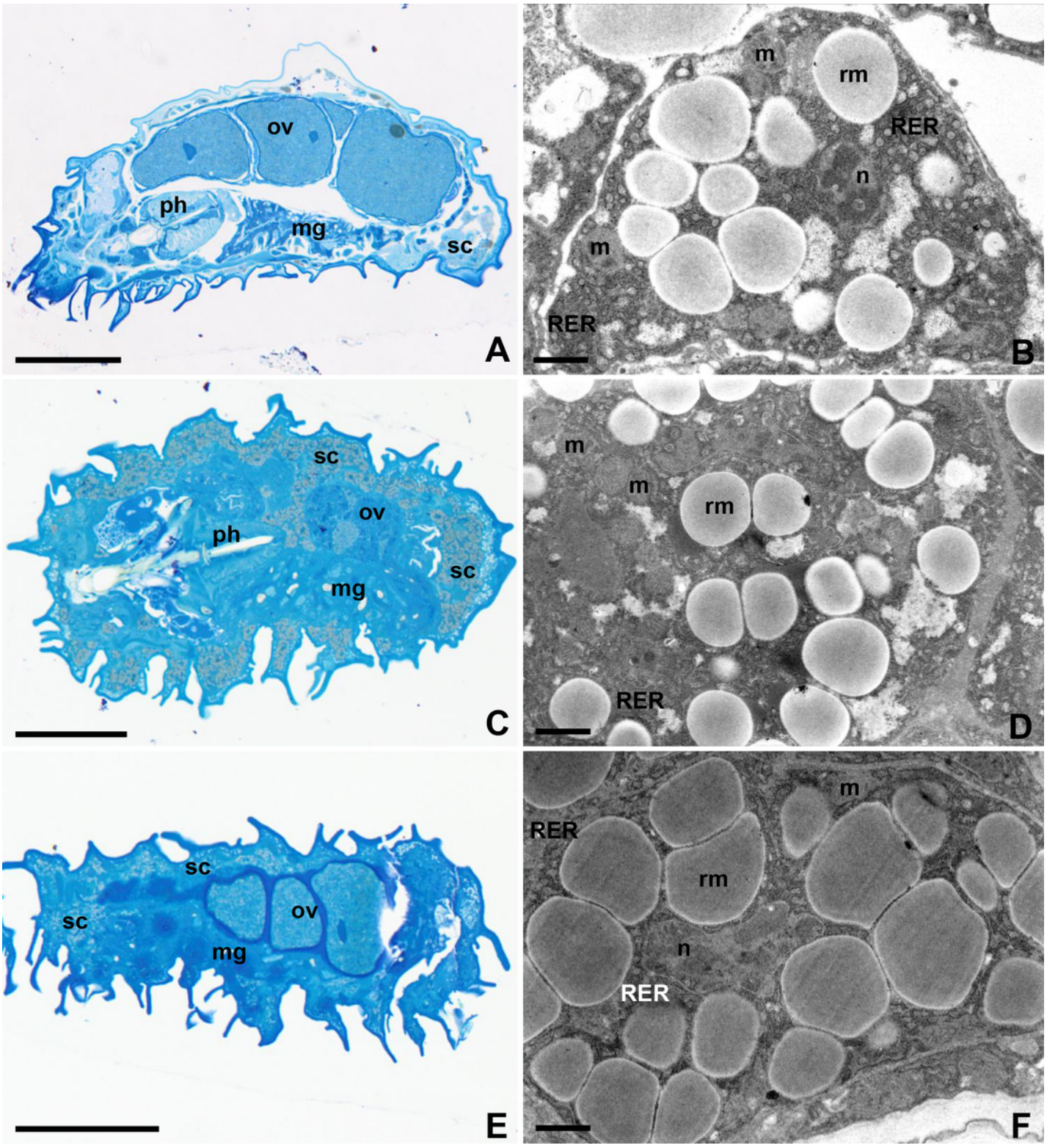

Figure 3

The storage cells of tuns of *Paramacrobiotus experimentalis*.

A. The storage cells of a tun-state control animal (20 °C), LM, scale bar = 50 μm, **B.** The ultrastructure of a storage cell of a tun-state control animal (20 °C), TEM, scale bar = 0.63 μm, **C.** The storage cells of a tun exposed to 35 °C, LM, scale bar = 50 μm, **D.** The ultrastructure of a storage cell of a tun exposed to 35 °C,

TEM, scale bar = 0.8 μm, **E.** The storage cells of a tun exposed to 37 °C, LM, scale bar = 50 μm, **F.** The ultrastructure of a storage cell of a tun exposed to 37 °C, TEM, scale bar = 0.6 μm.

Abbreviations: midgut (mg), mitochondrion (m), nucleus (n), ovary (ov), pharynx (ph), reserve material (rm), rough endoplasmic reticulum (RER), storage cells (sc).

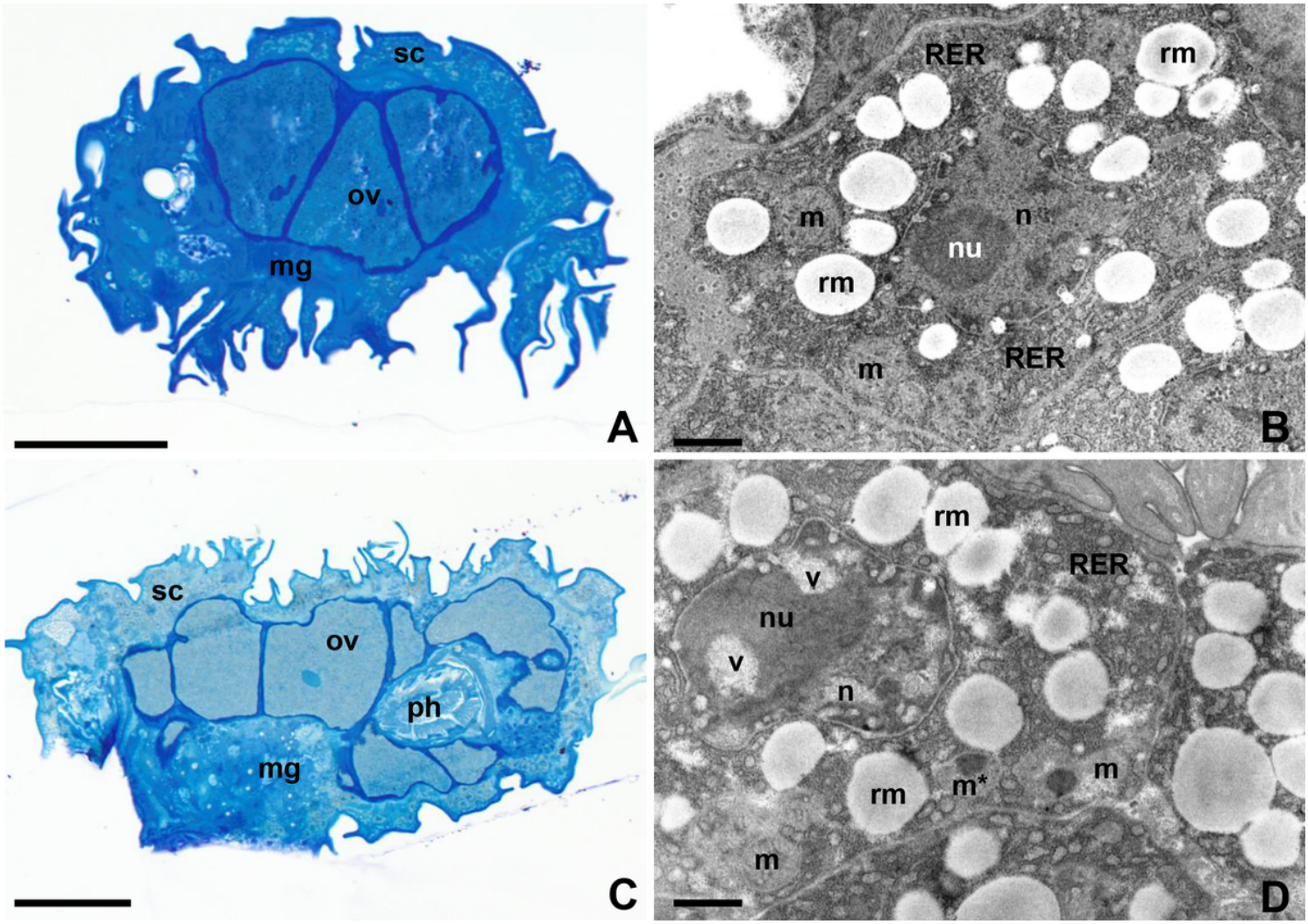

Figure 4

The storage cells of tuns of *Paramacrobiotus experimentalis*.

A. The storage cells of a tun exposed to 40 °C, LM, scale bar = 50 μm, **B.** The ultrastructure of a storage cell of a tun exposed to 40 °C, TEM, scale bar = 0.9 μm, **C.** The storage cells of a tun exposed to 42 °C, LM, scale bar = 50 μm, **D.**The ultrastructure of a storage cell of a tun exposed to 42 °C, TEM, scale bar = 0.8 μm.

Abbreviations: midgut (mg), mitochondrion (m), damaged mitochondrion (m*), nucleus (n), nucleolus (nu), ovary (ov), pharynx (ph), reserve material (rm), rough endoplasmic reticulum (RER), storage cells (sc), nucleolar vacuole (v).

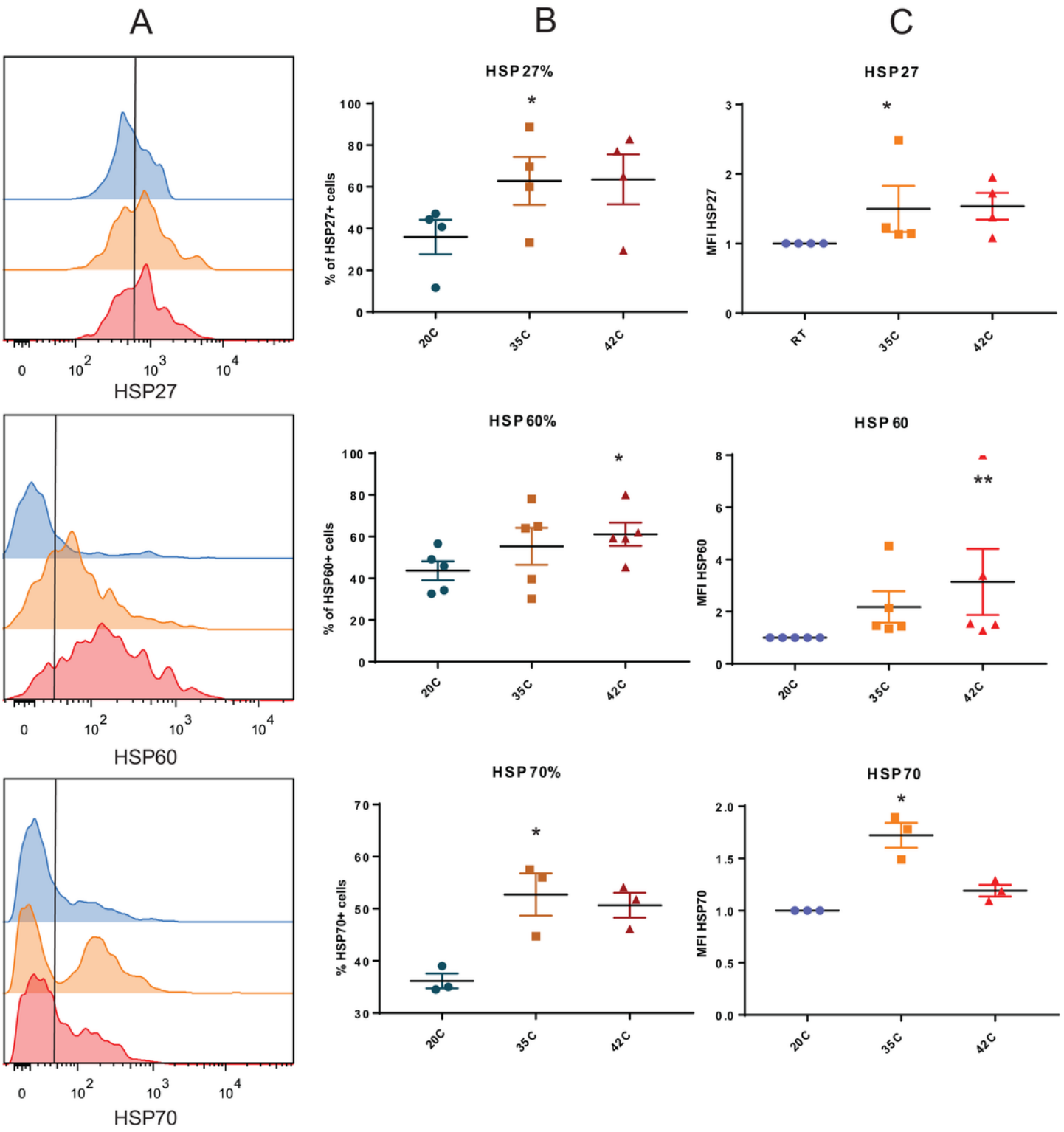

Figure 5

Levels of heat shock proteins (HSPs) in *Pam. experimentalis*.

A. Representative histograms of the flow cytometry analysis of HSP27, HSP60, and HSP70 in storage cells from tardigrades incubated at 20 °C, 35 °C, and 42 °C; **B.** Percentage of $HSP27^{+}$, $HSP60^{+}$, and $HSP70^{+}$ storage cells from tardigrades at 20 °C, 35 °C, and 42 °C; **C.** Mean fluorescence intensity (MFI)

values for $HSP27^{+}$, $HSP60^{+}$, and $HSP70^{+}$ storage cells from tardigrades at 20 °C, 35 °C, and 42 °C. Levels of significance are represented as: * – $p \leq 0.05$, ** – $p \leq 0.01$, *** – $p \leq 0.001$, and **** – $p \leq 0.0001$. Data are from three independent experiments.

Paper V

A review on genus Paramacrobiotus. Preprints.org 2023, 2023071250.

https://doi.org/10.20944/preprints202307.1250.v1

Review

A Review on Genus Paramacrobiotus

Pushpalata Kayastha *, Monika Mioduchowska, Łukasz Kaczmarek

Posted Date: 18 July 2023

doi: 10.20944/preprints202307.1250.v1

Keywords: tardigrade; reproduction; taxonomy; distribution; microbiome

Review

A Review on Genus *Paramacrobiotus*

Pushpalata Kayastha [1,*], Monika Mioduchowska [2] and Łukasz Kaczmarek [1]

[1] Department of Animal Taxonomy and Ecology, Faculty of Biology, Adam Mickiewicz University, Poznań, Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland; kaczmar@amu.edu.pl
[2] Department of Evolutionary Genetics and Biosystematics, Faculty of Biology, University of Gdańsk, Poland; monika.mioduchowska@ug.edu.pl
* Correspondence: pushpalata.kayastha@gmail.com

Abstract: *Paramacrobiotus* species has been described from almost every corner of the world. To date 45 species have been reported from this genus. The species' presence in different climatic conditions and habitat provides evidence of their adaptation to various harsh environments. In this review, we provide a concise summary of changes observed due to various cryptobiotic conditions in many species of this genus, geographical distribution of all the species, feeding behaviour, life history, microbiome community, *Wolbachia* endosymbiont identification, reproduction, phylogeny and general taxonomy of the species from genus *Paramacrobiotus*. Furthermore, we provide a new diagnostic key to the genus *Paramacrobiotus* based on the morphological and morphometric characters of adults and eggs.

Keywords: tardigrade; reproduction; taxonomy; distribution; microbiome

1. Introduction

Tardigrade, also called water bears, is a phylum consisting of *ca*. 1,500 species [1–4] that inhabit terrestrial and aquatic environments throughout the world[5]. They are mostly found in mosses, lichens, soil, leaf litter, sediments and on aquatic plants[5–7]. The phylum consists of two classes, i.e., Heterotardigrada and Eutardigrada[5]. Eutardigrada is further divided into two limnoterrestrial orders, i.e., Apochela and Parachela. Moreover, order Parachela consists of various superfamilies and families, one of them being Macrobiotidae (Thulin, 1928)[8] with genus *Paramacrobiotus* Guidetti, Schill, Bertolani, Dandekar and Wolf, 2009[9]. The genus was erected in 2009 from the genus *Macrobiotus* and till date 45 species have been described: *Paramacrobiotus alekseevi* (Tumanov, 2005)[10], *Pam. arduus* Guidetti, Cesari, Bertolani, Altiero & Rebecchi, 2019[11], *Pam. areolatus* (Murray, 1907)[12], *Pam. beotiae* (Durante Pasa & Maucci, 1979)[13], *Pam. celsus* Guidetti, Cesari, Bertolani, Altiero & Rebecchi, 2019[11], *Pam. centesimus* (Pilato, 2000)[14], *Pam. chieregoi* (Maucci & Durante Pasa, 1980)[15], *Pam. corgatensis* (Pilato, Binda & Lisi, 2004)[16], *Pam. csotiensis* (Iharos, 1966)[17], *Pam. danielae* (Pilato, Binda, Napolitano & Moncada, 2001)[18], *Pam. danielisae* (Pilato, Binda & Lisi, 2006)[19], *Pam. depressus* Guidetti, Cesari, Bertolani, Altiero & Rebecchi, 2019[11], *Pam. derkai* (Degma, Michalczyk & Kaczmarek, 2008)[20], *Pam. experimentalis* Kaczmarek, Mioduchowska, Poprawa & Roszkowska, 2020[21], *Pam. fairbanksi* Schill, Förster, Dandekar & Wolf, 2010[22], *Pam. filipi* Dudziak, Stec & Michalczyk 2020[23], *Pam. gadabouti* Kayastha, Stec, Mioduchowska and Kaczmarek 2023[24], *Pam. garynahi* (Kaczmarek, Michalczyk & Diduszko, 2005)[25], *Pam. gerlachae* (Pilato, Binda & Lisi, 2004)[16], *Pam. halei* (Bartels, Pilato, Lisi & Nelson, 2009)[26], *Pam. hapukuensis* (Pilato, Binda & Lisi, 2006)[19], *Pam. huziori* (Michalczyk & Kaczmarek, 2006)[27], *Pam. intii* Kaczmarek, Cytan, Zawierucha, Diduszko & Michalczyk, 2014[28], *Pam. kenianus* Schill, Förster, Dandekar & Wolf, 2010[22], *Pam. klymenki* Pilato, Kiosya, Lisi & Sabella, 2012[29], *Pam. lachowskae* Stec, Roszkowska, Kaczmarek & Michalczyk, 2018[30], *Pam. lorenae* (Biserov, 1996)[31], *Pam. magdalenae* (Michalczyk & Kaczmarek, 2006)[27], *Pam. metropolitanus* Sugiura, Matsumoto & Kunieda, 2022[32] *Pam. palaui* Schill, Förster, Dandekar & Wolf, 2010[22], Pam. peteri (Pilato, Claxton & Binda, 1989)[33], *Pam. pius* Lisi, Binda & Pilato, 2016[34], *Pam. priviterae* (Binda, Pilato, Moncada & Napolitano, 2001)[35], *Pam. richtersi* (Murray, 1911)[36], *Pam. rioplatensis* (Claps & Rossi, 1997)[37], *Pam. sagani* Daza, Caicedo, Lisi & Quiroga, 2017[38], *Pam. savai* (Binda & Pilato, 2001)[39], *Pam. sklodowskae* (Michalczyk, Kaczmarek & Węglarska, 2006)[40], *Pam. spatialis* Guidetti, Cesari, Bertolani, Altiero & Rebecchi, 2019[11], *Pam. spinosus*

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Kaczmarek, Gawlak, Bartels, Nelson & Roszkowska, 2017[41], *Pam. submorulatus* (Iharos, 1966)[17], *Pam. tonollii* (Ramazzotti, 1956)[42], *Pam. vanescens* (Pilato, Binda & Catanzaro, 1991)[43], *Pam. walteri* (Biserov, 1997/98)[44] and *Pam. wauensis* (Iharos, 1973)[45]. Furthermore, the genus is divided into two species groups, i.e., *richtersi* group with presence of microplacoid within the pharynx, and *areolatus* group without microplacoid within the pharynx. In turn, Kaczmarek *et al.*[41] proposed to separate subgenera for which specific names were clarified by Marley *et al.*[46]. However, the two subgenera are not valid according to Guidetti *et al.*[11] and Stec *et al.*[47].

In this paper we summarise the data on taxonomy, distribution, mode of reproduction, microbiome study, feeding behaviour, life history, morphological taxonomy, phylogeny and cryptobiotic studies along with new key for species identification in genus *Paramacrobiotus*.

2. Cryptobiosis

A stage of an organism's life known as cryptobiosis is one in which no activity is apparent[48]. Many organisms go through cryptobiosis to survive the harsh environmental conditions they encounter [49–51]. These conditions can include anhydrobiosis (lack of water), anoxybiosis (lack of oxygen), cryobiosis (low temperature), or osmobiosis (change in osmotic conditions). Tardigrades have a remarkable capacity for undergoing and surviving several types of cryptobiosis [48,52]. The majority of anhydrobiosis, or absence of water, has been studied in the species of the genus *Paramacrobiotus*, although there has also been research on famine, freezing, and bet-hedging[53–57]. Reuner *et al.*[53] studied how the influence of starvation and anhydrobiosis affects the size and number of storage cells in *Paramacrobiotus tonollii* to understand the energetic side of anhydrobiosis. Starving *Pam. tonollii* for seven days led to reduction in storage cell size by 46.41% but no significant reduction in storage cell number was observed. Furthermore, when storage cells size and number were investigated after inducing anhydrobiosis for seven days where no significant changes in storage cell size and number of *Pam. tonollii* was observed. Also, the mortality was checked using prolonged starvation and *Pam. tonollii* reached 50% mortality after 30 days. Likewise, Rizzo *et al.*[54] investigated antioxidant defenses (capable of counteracting reactive oxygen species (ROS)) in *Pam. richtersi* in both active and dehydrated states. Activity of several antioxidant enzymes, the fatty acid composition and heat shock protein (Hsp) expression were compared in these two states. The increase in both antioxidant enzyme (superoxide dismutase due to induction of both glutathione peroxidase and glutathione during desiccation) and the fatty acid composition (polyunsaturated fatty acids and the amount of thiobarbituric acid reactive substances) were observed in desiccated *Pam. richtersi* specimens but no significant differences in the relative level of heat shock proteins were observed (Hsp70 and Hsp90). In addition, Tsujimoto *et al.*[55] performed a study where the production of reactive oxygen species and involvement of bioprotectants during anhydrobiosis in *Pam. spatialis* was investigated. The study provides evidence of increase in ROS production relative to time spent in anhydrobiosis which is due to oxidative stress in the animals. Using RNA interference, involvement of bioprotectants, including those combating ROS was assessed. As Rizzo *et al.*[54] concluded the role of glutathione peroxidase in desiccation in *Pam. richtersi*, this gene was targeted and what was observed is that glutathione peroxidase gene compromised survival during drying and rehydration of *Pam. spatialis*. This furthermore strengthened the evidence that glutathione reductase and catalase play important roles during desiccation and rehydration. Also, involvement of aquaporins 3 and 10 during rehydration of *Pam. spatialis* was observed. And recently Roszkowska *et al.*[57] study the length that different tardigrades survive in the anhydrobiotic state including *Pam. experimentalis*. The study concludes that anhydrobiotic competence is dependent on habitat instead of nutritional behavior and the time taken to return to activity after anhydrobiosis is dependent upon the length of the anhydrobiosis.

3. Distribution

The distribution of species from this genus shows worldwide distribution. The distribution of all 45 species in genus *Paramacrobiotus* till date is presented in SM.01 and Figure 1.

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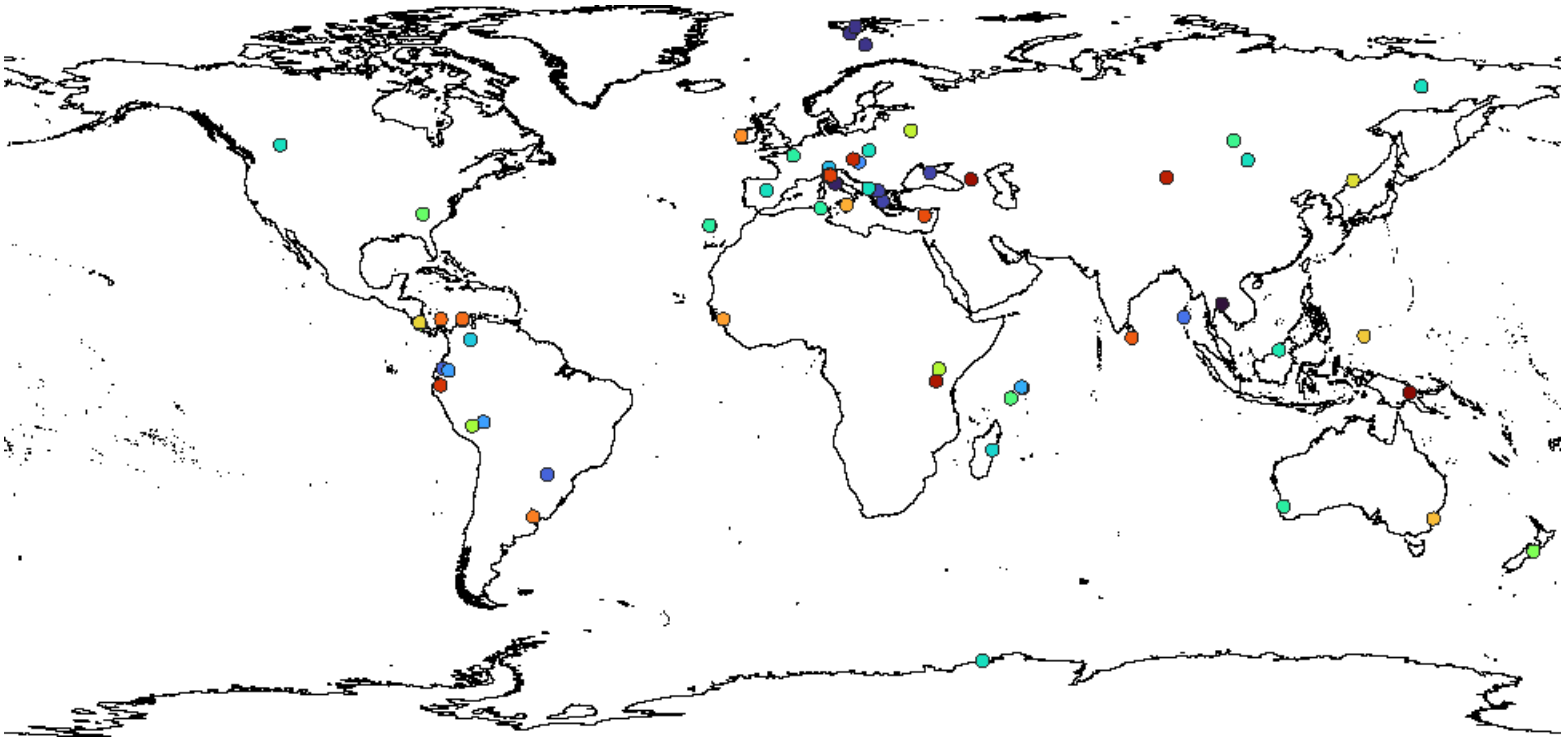

Figure 1. Distribution of all the species in genus *Paramacrobiotus* (co-ordinates present in SM.01). (Map prepared using QGIS ver. 3.28.0-Firenze).

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4. Feeding behaviour

The tardigrade species *Paramacrobiotus* are omnivorous, consumes a variety of organisms, including certain cyanobacteria, algae, and fungi, as well as the rotifer *Lecane*, the nematode *Caenorhabditis*, and small juvenile tardigrades. Additionally, the diet of adults and juveniles eat is different: adults favour rotifers and nematodes, whereas juveniles favour unicellular green algae. Moreover, juveniles suck out all of them, including algal cells, animal food, and fungal cells, in contrast to adults who only consume entire fungal and algal cells[58].

5. Life history

Life history refers to total life span, development, reproduction and death of an organism[59]. The life history list in case of tardigrades consists of age at first oviposition, clutch size, fecundity, hatching percentage, hatching success, lifespan, moulting number and total number of ovipositions[60,61]. The lifespan differs from species to species in case of tardigrades[62]. The life history of only a few *Paramacrobiotus* species have been reported till date. Namely, *Pam. fairbanski* with an average lifespan of 137.3 ± 136.4 days and 194.9 ± 164.4 days respectively and age at first oviposition of 70.7 ± 19.4 days and 76.9 ± 16.4 days respectively[63]; *Pam. kenianus* with average lifespan of 125 ± 35 days and 141 ± 54 days respectively, maximum life span of 204 days and 212 days respectively and age at first oviposition of 10 days and10 days respectively[60]; *Pam. metropolitanus* with juveniles hatching in 12–20 days, first oviposition in 11–13 days after hatching[64]; *Pam. palaui* with average lifespan of 97 ± 31 days, maximum life span of 187 days and age at first oviposition of 10 days[60]; *Pam. richtersi* with age at first oviposition of 64.2 ± 1.7 days[65]; *Pam. tonollii* with average lifespan of 69.0 ± 45.1 days and maximum life span of 237 days and age at first oviposition 24.4 ± 4.4 days[62].

6. Microbiome

The microbiome represents the entire community of microorganisms, including fungi, protists, bacteria, archaea, as well as viruses, that inhabit all known metazoan species. The bacterial component of the microbiome community plays crucial roles in multiple aspects of ecdysozoan host life, such as behavior, metabolism, development, immunity, or pathogen defense, thereby regulating the functioning of the entire organism[66,67]. Conversely, it has also been demonstrated that the host's phylogeny[68] and diet[69] have significant impacts on the overall microbial composition. Indeed, many metazoan species appear to harbor their own specific microbiome community[70]. However, our understanding of the microbiome composition of Tardigrada, based on next-generation sequencing methods (NGS) targeting the standard 16S rRNA bacterial barcoding gene fragment, is limited to a very small number of published articles[71–77].

In the case of species from the genus *Paramacrobiotus,* the microbiomes of a few species have been studied to date. In 2018, Vecchi *et al.*[71] described the bacterial communities associated with six limno-terrestrial tardigrade taxa, one of which was *Pam. areolatus*. The study revealed that the microbial community was mainly composed of Proteobacteria and Bacteroidetes. Interestingly, certain classified Operational Taxonomic Units (OTUs) showed variations among species from geographically distant samples, indicating the presence of specific bacterial communities in each species. However, in all the investigated species' microbiome profiles, the order Rickettsiales was consistently identified. This order belongs to the class Alphaproteobacteria and is characterized by both pathogens and intracellular mutualists[78]. There were two distinct patterns in the diversity observed between tardigrades and their substrates, indicating significantly less microbial diversity in tardigrades compared to their substrates. This phenomenon may be attributed to tardigrades selectively associating with specific microbial communities that promote the growth of certain bacterial species while inhibiting others. Another hypothesis suggests that substrates, being complex matrices with wide surface areas and volumes, can support a high bacterial biomass, resulting in a vast and complex microbial community.

Similarly, Kaczmarek *et al.*[21] conducted a microbiome analysis on two populations of *Pam. experimentalis* from Madagascar and their laboratory culture environment. These populations of *Pam.*

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experimentalis had been maintained in laboratory culture for two years. The most abundant phylum in all samples was Proteobacteria. Firmicutes was the second most dominant phylum in both *Pam. experimentalis* populations, while Bacteroides was the second most dominant phylum in the laboratory habitat. With the exception of the phyla Verrucomicrobia and Saccharibacteria, which were not found in the tardigrade microbiome, all identified taxa in the *Pam. experimentalis* microbiome community and laboratory culture environment were widespread and had comparable abundances. This confirms that the tardigrade microbiome significantly differs in composition from the bacteria inhabiting their environment. Moreover, within the microbiome of *Pam. experimentalis*, Operational Taxonomic Units (OTUs) classified as potential endosymbionts belonging to the order Rickettsiales were identified. The absence of Rickettsiales OTUs in the environment of the studied species further supports the close association of these bacteria with their host.

Furthermore, Mioduchowska *et al.*[73] conducted a study to investigate whether tardigrade species are infected with bacterial endosymbionts belonging to the genus *Wolbachia*. The analysis included *Pam. fairbanksi* and *Paramacrobiotus* sp. In the study Proteobacteria, Firmicutes, and Actinobacteria as the three most prevalent phyla among the analyzed tardigrades, including species outside the genus *Paramacrobiotus*, were identified. However, the focus of the study was on potential tardigrade endosymbionts, particularly Operational Taxonomic Units (OTUs) from the order Rickettsiales and the genus *Wolbachia*. Both Rickettsiales and *Wolbachia* were detected in adult *Paramacrobiotus* sp., while only Rickettsiales were found in *Pam. fairbanksi* eggs. Adult *Pam. fairbanksi* did not have either *Wolbachia* or Rickettsiales infections. The genus *Wolbachia* is an intracellular bacterium belonging to the order Rickettsiales and it infects various invertebrates, particularly terrestrial insects[79]. However, recent studies have identified infections of this bacterial endosymbiont in various freshwater invertebrate species[77,80,81]. Generally, this bacterium is transmitted vertically from mother to offspring and/or through horizontal transfer directly from the environment or between different hosts[82]. Subsequently, *Wolbachia* manipulates host reproduction by inducing parthenogenesis, feminization, male killing, or cytoplasmic incompatibility[83,84].

In 2023, Mioduchowska *et al.*[77] described new molecular and bioinformatic tools for detecting *Wolbachia* in freshwater invertebrates. In this study, *Wolbachia* was detected in *Pam. experimentalis*, which were the same isolates analysed by Kaczmarek *et al.*[72]. Phylogenetic analysis of the obtained bacterial sequences allowed for their classification within the differentiated supergroup A of the genus *Wolbachia*. The discovery of *Wolbachia* in tardigrades opens new frontiers in understanding the *Wolbachia*-driven biology and ecology of Tardigrada.

7. Reproduction

Reproduction refers to the process where every organism known produces offspring either sexually or asexually. In case of tardigrades, they reproduce only through gametes via many different patterns i.e. dioecious (separate male and female), hermaphroditic (single animal with both male and female reproductive parts) or parthenogenetic (form of asexual reproduction)[85]. The genus *Paramacrobiotus* consists of both bisexual and unisexual species/populations. *Pam. richtersi* is both bisexual and unisexual from Italy; according to modern taxonomy they probably constitute a distinct species, *Pam. areolatus* population from Italy is bisexual and population from Svalbard is unisexual, *Pam. tonolli* from the USA is bisexual, *Pam. fairbanksi* is unisexual from various locations as Antarctic, Italy, Poland, Spain and USA, *Pam. kenianus* from Kenya is unisexual and *Pam. palaui* from Micronesia is unisexual, *Pam. depressus* from Italy is bisexual, *Pam. celsus* from Italy is bisexual, *Pam. spatialis* from Italy is bisexual, *Pam. arduus* from Italy is bisexual, *Pam. experimentalis* from Madagascar is bisexual and *Pam. gadabouti* is unisexual from various locations in Portugal, Australia, France and Tunisia. Out of 45, mode of reproduction for only 18 species are known (SM.01). Also, Guidetti *et al.*[11] suggests the mode of reproduction being related to constrained or wide distribution of the species. The amphimictic species displays a very constrained or punctiform distribution, in contrast to the parthenogenetic species' extremely extensive spread and presence over multiple continents. The difference in the ability for dispersal linked to the two modes of reproduction can be used to explain why apomictic and amphimictic populations are distributed differently.

 doi:10.20944/preprints202307.1250.v1

8. Morphological taxonomy

The genus *Paramacrobiotus* is divided into two morphologically distinct species groups: *areolatus* (species without a microplacoid or with rudimentary structures in the place of microplacoid in the pharynx) and *richtersi* (species with a microplacoid in the pharynx)(e.g.[23,28]). It was suggested that initially the microplacoid was present, however it was lost in some species from the *areolatus* group. But, the opposite situation, in which the microplacoid gradually appears, is also possible[41]. For example, in Pam vanescens the microplacoid suggests a gradual reduction. In turn, in *Pam. areolatus* and *Pam. centesimus* the microplacoid is generally absent, but a thin cuticular thickening is present in the place where microplacoid should be normally present and can be considered as rudimentary microplacoid[14,47]. Although, the presence or absence of microplacoid seems to be a clear morphological character dividing genus *Paramacrobiotus* into two separate phylogenetic lineages (which was suggested by Kaczmarek *et al.*[41]) the genetic studies did not confirm this[11,47].

At present 45 species are formally attributed to the genus *Paramacrobiotus* and 13 belong to *areolatus* group and 32 *richtersi* group. They can be further divided into smaller groups based on egg types. In total, seven types of eggs were identified. However, two of them (*areolatus* and *richtersi* types) are the most common and occur in 37 species (*ca*. 82%). In the next two species *huziori* type of eggs are present (*ca*. 5%). The other types of eggs (i.e. *beotiae*, *chieregoi*, *csotiensis*, *tonollii* and *submorulatus*) were identified only in single taxa (for details of egg morphology see Kaczmarek *et al.*[41]). What is more, eggs are unknown for one species i.e. *Pam. wauensis*.

In recent years two very important for taxonomy of the entire genus, species *Pam. areolatus* and *Pam. richtersi* were integratively redescribed[11,47]. Another species *Pam. fairbanksi* described based, mostly, on genetic data was also morphometrically well characterized few years ago[21]. However, a few *Paramacrobiotus* species still need a redescription based on type material or on additional material from type localities. Descriptions of *Pam. beotiae, Pam. chieregoi, Pam. csotiensis, Pam. rioplatensis*, *Pam. submorulatus*, *Pam. tonollii* and *Pam. wauensis* are inaccurate and some important morphological information are lacking.

Another two species, i.e., *Pam. kenianus* and *Pam. palaui* are cryptic taxa described mostly based on genetic data without morphological differential diagnosis[22].

Descriptions of the other *Paramacrobiotus* species more or less complete, but in most of them exact morphometric data of claws, buccal tubes placoids and above all genetic data are lacking (see Table 1 and SM.01). Based on all the abovementioned doubts, 3 species, i.e., *Pam. kenianus*, *Pam. palaui* and *Pam. wauensis* were not included to the key.

Table 1. Selected morphological characters of the known species of genus *Paramacrobiotus*.

Species	Cuticle	Oral Cavity Armature	Eyes	Lunules IV	Granulation on Legs	Type of Egg	Egg process height (min. and max. values in µm)	Egg process base width (min. and max. values in µm)	Egg process shape	Number of processes on circumference
Paramacrobiotus alekseevi	smooth	I–III	absent	dentate	IV	*richtersi*	11.8–21.8	13.3–22.9	with cap	10–12
Paramacrobiotus arduus	smooth	I–III	absent	smooth	I–IV	*richtersi*	12.1–18.3	10.4–16.3	conical	16–21
Paramacrobiotus areolatus	smooth	I–III	present	crenate	I–IV	*areolatus*	20.0–28.0	19.0–22.0	conical	?
Paramacrobiotus beotiae	smooth	I–III	absent	dentate	?	*beotiae*	up to 16.0	?	spines	?
Paramacrobiotus celsus	smooth	I–III	absent	smooth	I–IV	*richtersi*	15.2–19.1	14.3–18.2	conical (jagged)	15–19
Paramacrobiotus centesimus	smooth	I–III	absent	smooth	I–IV	*areolatus*	7.0–11.0	?	conical	11–12
Paramacrobiotus chieregoi	smooth	I–III	absent	smooth	?	*chieregoi*	?	?	elongated	14
Paramacrobiotus corgatensis	sculptured	I–III	present	dentate	?	*richtersi*	20.0–25.0	18.0–24.0	conical (jagged)	8–11
Paramacrobiotus csotiensis	smooth	II–III	present	?	?	*csotiensis*	?	?	blunt	?
Paramacrobiotus danielae	sculptured	I–III	present	smooth	?	*areolatus*	14.5	24.7	conical	?

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Paramacrobiotus danielisae	sculptured	I–III	absent	smooth	?	*richtersi*	17.3–23.0	17.5–20.0	conical	9–10
Paramacrobiotus depressus	smooth	I–III	absent	smooth	IV	*richtersi*	9.3–12.4	12.4–15.2	conical	16–23
Paramacrobiotus derkai	smooth	I–III	present	smooth	I–IV	*huziori*	8.0–17.1	12.5–28.3	conical	12–16
Paramacrobiotus experimentalis	smooth	I–III	absent	smooth	IV	*areolatus*	10.3 – 14.9	13.8 – 19.4	conical	10–12
Paramacrobiotus fairbanksi	smooth	I–III	absent	smooth	I–IV	*richtersi*	10.9 – 14.9	10.9 – 20.8	conical (jagged)	?
Paramacrobiotus filipi	granulation	I–III	absent	smooth	I–IV	*richtersi*	17.8–25.2	11.7–21.7	elongated with disc	10–11
Paramacrobiotus gadabouti	smooth	I–III	absent	smooth	IV	*richtersi*	12.1–23.7	15.0–25.5	truncated cones	11–13
Paramacrobiotus garynahi	with pores	I–III	absent	smooth	I-IV	*areolatus*	18.0–30.0	20.0–42.0	with cap	10–13
Paramacrobiotus gerlachae	smooth	I–III	absent	smooth	IV	*richtersi*	11.8–14.5	16.8–18.7	blunt	?
Paramacrobiotus halei	sculptured	I–III	absent	?	I-IV	*richtersi*	11.0–14.0	22.0–23.5	blunt	11
Paramacrobiotus hapukuensis	smooth	I–III	absent	smooth	absent	*areolatus*	15.7–21.1	14.8–16.6	elongated	10
Paramacrobiotus huziori	smooth	I–III	present	smooth	I–IV	*huziori*	20.0–33.0	20.0–30.0	conical	9–11

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Paramacrobiotus intii	smooth	II–III	present	dentate	I–IV	*areolatus*	15.4–24.4	22.0–34.0	conical	9–10
Paramacrobiotus kenianus	smooth	?	present	?	?	*richtersi*	13.5 ± 1.9	19.7 ± 2.7	conical	17.7 ± 3.6
Paramacrobiotus klymenki	smooth	I–III	absent	dentate	I–IV	*areolatus*	14.5–18.5	16.4–18.2	conical	10–11
Paramacrobiotus lachowskae	smooth	I–III	present	smooth	I–IV	*areolatus*	17.6–32.1	8.1–17.7	dome with filaments	8–14
Paramacrobiotus lorenae	smooth	I–III	absent	smooth	I-IV	*richtersi*	25.0–42.2	17.8–23.0	elongated	?
Paramacrobiotus magdalenae	smooth	I–III	present	smooth	IV	*richtersi*	13.0–25.0	16.2–21.0	conical	10–12
Paramacrobiotus metropolitanus	smooth	I–III	absent	smooth	IV	*areolatus*	7.4–14.6	9.8–21.1	conical	10–15
Paramacrobiotus palaui	smooth	?	present	?	?	*richtersi*	10.2 ± 1.3	13.4 ± 1.3	conical	15.4 ± 1.4
Paramacrobiotus peteri	smooth	I–III	absent	smooth	?	*areolatus*	10.0–14.0	9.0–12.0	conical (jagged)	?
Paramacrobiotus pius	smooth	I–III	absent	smooth	I-IV	*richtersi*	up to 12.3	19.5–24.7	conical (jagged)	10
Paramacrobiotus priviterae	smooth	I–III	present	smooth	I-IV	*richtersi*	11.8–15.0	12.9–16.3	conical (jagged)	?
Paramacrobiotus richtersi	smooth	I–III	absent	smooth	I-IV	*richtersi*	17.1–22.1	17.2–22.2	conical	13–17

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Paramacrobiotus rioplatensis	smooth	I–III	present	smooth	?	*areolatus*	ca. 4.6	?	elongated	17-19
Paramacrobiotus sagani	granulation	I–III	present	smooth	I-IV	*richtersi*	9.4–13.2	14.6–22.4	cylindrical, indented apices	10–13
Paramacrobiotus savai	smooth	I–III	present	smooth	IV	*areolatus*	12.0–18.0	16.7–18.5	blunt	?
Paramacrobiotus sklodowskae	smooth	I–III	present	smooth	I-IV	*richtersi*	16.0–17.5	20.5–23.5	blunt	10
Paramacrobiotus spatialis	smooth	I–III	absent	smooth	I-IV	*richtersi*	13-16	15.2–20.4	conical	15–23
Paramacrobiotus spinosus	smooth	I–III	absent	smooth	I–IV	*richtersi*	22.1–42.2	17.3–26.0	elongated (jagged)	10–11
Paramacrobiotus submorulatus	smooth	II–III	present	?	?	*submorulatus*	7.0–8.3	17.5–20.4	blunt	13
Paramacrobiotus tonollii	smooth	?	present	smooth	?	*tonollii*	32.0–35.0	?	conical	8–10
Paramacrobiotus vanescens	smooth	I–III	absent	?	I-IV	*richtersi*	16.0–17.0	24.0–25.0	blunt (jagged)	9–12
Paramacrobiotus walteri	smooth	I–III	present	dentate	I–IV	*areolatus*	10.0–17.0	9.0–20.0	conical (jagged)	?
Paramacrobiotus wauensis	smooth	I– III	absent	?	?	?	?	?	?	?

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9. Molecular taxonomy

Molecular markers serve as valuable tools for species identification. In the integrative taxonomy of Tardigrada, four DNA fragments with different mutation rates are commonly used: two conservative nuclear ribosomal subunit genes, namely 18S rRNA (the small ribosome subunit) and 28S rRNA (the large ribosome subunit), the noncoding nuclear ITS2 fragment (the internal transcribed spacer-2) with high evolution rates, and the protein coding mitochondrial COI barcode gene (the cytochrome oxidase subunit I) with an intermediate effective mutation rate (e.g., Kaczmarek *et al.*[72]). The COI mtDNA molecular marker, in particular, has been recommended for DNA barcoding purposes (http://www.barcodinglife.org), such as rapid species identification, discrimination between cryptic species, and resolving phylogenetic relationships among closely related species[86,87]. To gain additional insights into the phylogenetic relationships within the genus *Paramacrobiotus*, an analysis based on COI mtDNA was conducted. This analysis was performed to supplement the information obtained from previous studies using four molecular markers[24].

Due to ongoing revisions and redescriptions of *Paramacrobiotus* species, studies are becoming more accessible, leading us to anticipate that the species diversity within the genus is greatly underestimated[11,23]. One significant challenge that needs to be addressed in future studies is the lack of available barcodes. Despite the designation of 45 species to the genus *Paramacrobiotus*, not all species have available barcode sequences. In this study, we aimed to estimate the phylogenetic relationships among all *Paramacrobiotus* species (including taxa designated as "cf." – meaning "compare with" and "aff." – meaning "similar to") for which COI barcode sequences are available in the GenBank database. We used the COI sequence of *Milnesium berladnicorum* Ciobanu, Zawierucha, Moglan & Kaczmarek, 2014[88] as outgroups to construct the most reliable evolutionary tree. To determine the most appropriate model of sequence evolution, we applied jModelTest v. 2.1.4[89] with both the Bayesian Information Criterion (BIC) and the Akaike Information Criterion (AIC)[90]. The GTR + G (Time Reversible model with gamma distributed rate heterogeneity) was selected as the best-fit evolutionary model. The phylogenetic tree was constructed using Bayesian inference (BI) analysis with the program MrBayes 3[91], following the settings described by Mioduchowska *et al.*[92]. The alignment of COI barcode sequences resulted in 574 characters, with 270 variable sites and 241 parsimony informative sites. Uncorrected pairwise distances (p-distances) were calculated using MEGA X[93].

The binary model of phylogenetic relationships, which involves reconstructing gene trees from sequence data, allows us to gain insights into the speciation history of species[94]. However, in our analysis of barcode sequences, we observed speciation events that resulted in polytomies within the phylogeny of the genus *Paramacrobiotus* (Figure 2). This means that more than two descendants were observed from certain nodes[95]. The presence of unresolved nodes in a polytomic multifurcating tree indicates a lack of signal in the data to resolve relationships within the genus *Paramacrobiotus*. This observation is partially consistent with previous studies, where both groups of *richtersi* and *areolatus* were described as polyphyletic[11,47]. However, in the work by Kayastha *et al.*[24], the interrelationships of the genus *Paramacrobiotus* were not depicted as a polytomy when two conservative coding nuclear molecular markers (18S rRNA and 28S rRNA) and a noncoding nuclear marker with high evolution rates (ITS2) were included in the analysis. As a result, the phylogenetic relationships within the genus *Paramacrobiotus* were resolved. Interestingly, other examples of polytomies in Tardigrada gene trees based on nuclear molecular markers have also been observed[96].

The genetic p-distances between the analyzed COI barcode sequences of *Paramacrobiotus* species ranged from 16% to 27%, indicating different species (Table 2). However, it was shown that there are very low genetic differences, i.e., a p-distance of 0.3%, between *Pam*. aff. *richtersii* from Tunisia (GenBank: MH676016) and *Pam. gadabouti* from Portugal (GenBank: OP394113), suggesting they belong to the same species (Table 2). This finding is consistent with the work by Kayastha *et al.* [24], where both species were described as *Pam. gadabouti*. No genetic differences were found between *Pam*. aff. *richtersi* from Madagascar (GenBank: MH676008) and *Pam. experimentalis* from Madagascar (GenBank: MN097836) (Table 2). Both sequences represented *Pam. experimentalis*, which is also

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consistent with the previous study (Kayastha *et al.*[24]). Moreover, we found very low genetic differences, i.e., a p-distance of 2.1%, between *Pam. arduus* from Italy (GenBank: MK041020) and *Pam.* aff. *arduus* from Italy (GenBank: MK041022), indicating the same species (Table 2).

The text continues here (Figure 2 and Table 2).

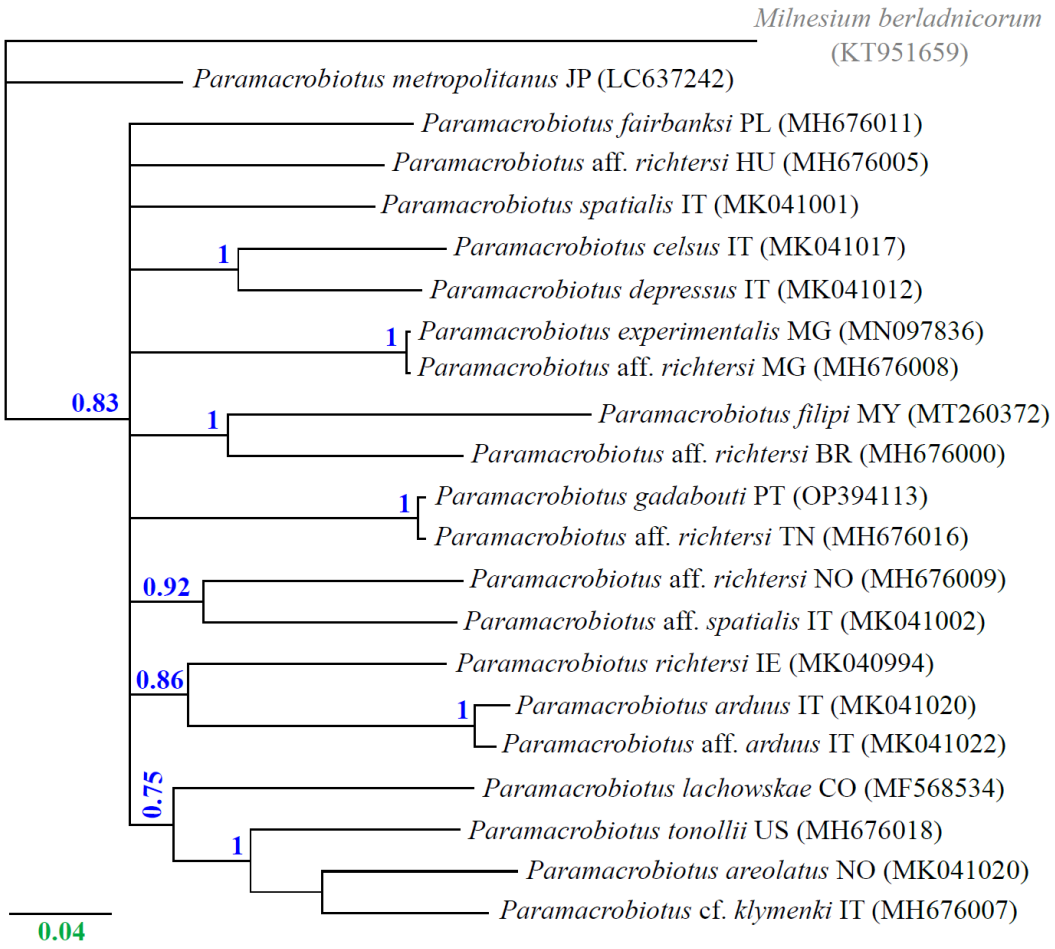

Figure 2. Phylogenetic relationships of the genus *Paramacrobiotus* constructed based on the COI barcode sequences obtained from the GenBank database. The GenBank accession numbers are given in parentheses. In turn, locations of identified species given in abbreviations: JP – Japan; PL – Poland; HU – Hungary; IT – Italy; MG – Madagascar; MY – Malaysia; BR – Brazil; PT – Portugal; TN – Tunisia; NO – Norway; IE – Ireland; CO – Colombia; US – United States. The numbers at the branches represent Bayesian posterior probabilities. The COI sequence of *Milnesium berladnicorum* was used as an outgroup.

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Table 2. Estimates of evolutionary divergence between COI barcode sequences based on p-distances.

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.
1. *Pam. arduus* (IT; MK041020)																					
2. *Pam.* aff. *arduus* (IT; MK041022)	**0,021**																				
3. *Pam. areolatus* (NO; MK041020)	0,244	0,240																			
4. *Pam. celsus* (IT; MK041017)	0,206	0,206	0,232																		
5. *Pam. depressus* (IT; MK041012)	0,209	0,204	0,228	0,141																	
6. *Pam. experimentalis* (MG; MN097836)	0,253	0,249	0,207	0,239	0,218																
7. *Pam. fairbanksi* (PL; MH676011)	0,218	0,213	0,207	0,186	0,179	0,213															
8. *Pam. filipi* (MY; MT260372)	0,268	0,270	0,253	0,228	0,258	0,232	0,260														
9. *Pam. gadabouti* (PT; OP394113)	0,207	0,207	0,220	0,195	0,192	0,239	0,195	0,240													
10. *Pam.* cf. *klymenki* (IT; MH676007)	0,226	0,223	0,176	0,242	0,223	0,211	0,233	0,230	0,235												
11. *Pam. lachowskae* (CO; MF568534)	0,226	0,221	0,199	0,242	0,233	0,199	0,216	0,267	0,237	0,192											
12. *Pam. metropolitanus* (JP; LC637242)	0,233	0,232	0,192	0,228	0,230	0,190	0,204	0,228	0,221	0,223	0,204										
13. *Pam. richtersi* (IE; MK040994)	0,199	0,192	0,216	0,192	0,186	0,204	0,188	0,237	0,214	0,213	0,226	0,209									
14. *Pam.* aff. *richtersi* (HU; MH676005)	0,206	0,193	0,211	0,193	0,183	0,225	0,192	0,240	0,186	0,200	0,190	0,209	0,200								
15. *Pam.* aff. *richtersi* (NO; MH676009)	0,199	0,193	0,232	0,186	0,176	0,225	0,188	0,268	0,214	0,230	0,237	0,230	0,181	0,211							
16. *Pam.* aff. *richtersi* (BR; MH676000)	0,225	0,218	0,239	0,202	0,193	0,202	0,214	0,225	0,202	0,230	0,226	0,207	0,190	0,204	0,216						
17. *Pam.* aff. *richtersi* (TN; MH676016)	0,209	0,207	0,220	0,195	0,192	0,239	0,195	0,240	**0,003**	0,235	0,237	0,221	0,214	0,186	0,214	0,202					
18. *Pam.* aff. *richtersi* (MG; MH676008)	0,253	0,249	0,207	0,239	0,218	**0,000**	0,213	0,232	0,239	0,211	0,199	0,190	0,204	0,225	0,225	0,202	0,239				
19. *Pam. spatialis* (IT; MK041001)	0,214	0,216	0,228	0,195	0,193	0,197	0,178	0,240	0,206	0,213	0,209	0,213	0,190	0,185	0,197	0,230	0,207	0,197			
20. *Pam.* aff. *spatialis* (IT; MK041002)	0,193	0,183	0,225	0,202	0,176	0,206	0,195	0,263	0,197	0,221	0,211	0,221	0,206	0,192	0,176	0,233	0,197	0,206	0,185		
21. *Pam. tonollii* (US; MH676018)	0,221	0,214	0,179	0,213	0,207	0,185	0,207	0,230	0,218	0,157	0,206	0,186	0,204	0,202	0,216	0,207	0,218	0,185	0,185	0,213	

10. Key for species identification

1. Microplacoid present (*richtersi* group) **2**

–. Microplacoid absent (*areolatus* group) **31**

2. Cuticular pattern on dorsal side of the body present and visible in LM (PCM and/or DIC) **3**

–. Cuticle on dorsal side of the body smooth or cuticular pattern not visible in LM (PCM and/or DIC) **7**

3. Eggs of *areolatus* type. . . . . ***Pam. danielae***

–. Eggs of *richtersi* type **4**

4. Eyes present, lunules under claws IV dentate . . . . . ***Pam. corgatensis***

–. Eyes absent, lunules under claws IV smooth **5**

5. Dorsal cuticle covered with very small circular or elongated tubercles, egg processes less than 14.5 µm height . . . . . ***Pam. halei***

–. Dorsal cuticle covered with small dots (granules) or small polygons, egg processes more than 15.5 µm height **6**

6. Dorsal cuticle covered with small dots (granules) . . . . . ***Pam. vanescens***

–. Dorsal cuticle covered with small polygons . . . . . ***Pam. danielisae***

7. Areolation between egg processes absent **8**

–. Areolation between egg processes present **9**

8. Lunules under claws IV dentate, eggs of *beotiae* type . . . . . ***Pam. beotiae***

–. Lunules under claws IV smooth, egg of *chieregoi* type . . . . . ***Pam. chieregoi***

9. Eggs of *submorulatus* type . . . . . ***Pam. submorulatus***

–. Eggs of *richtersi* or *areolatus* type **10**

10. Eggs of *richtersi* type **11**

–. Eggs of *areolatus* type **25**

11. Only five or six areoles present around each egg process **12**

–. The number of areoles around each egg process larger than six **18**

12. Eyes present. . . . . ***Pam. priviterae***

–. Eyes absent. **13**

13. Granulation on leg I-III present. **14**

–. Granulation on legs I-III absent. . . . . ***Pam. depressus***

14. The *pt* values of the macroplacoid length less than *43.5*. . . . . ***Pam. pius***

–. The *pt* values of the macroplacoid length more than *49.0*. **15**

15. Egg process jagged **16**

–. Egg process not jagged **17**

16. Egg processes height less than 15.0 µm and parthenogenetic mode of reproduction. . . . . ***Pam. fairbanksi***

–. Egg processes height more than 15.1 µm and bisexual mode of reproduction. . . . . ***Pam. celsus***

17. Egg diameter without processes less than 62.5 µm. . . . . ***Pam. arduus***

–. Egg diameter without processes more than 65.0 µm. . . . . ***Pam. spatialis***

18. Eyes present **19**

–. Eyes absent **21**

19. Granulation on legs I-III present **20**

–. Granulation on legs I-III absent . . . . . ***Pam. magdalenae***

20. Egg bare diameter less than 87.9 µm, egg process height more than 15 µm, egg processes hemispherical with blunt terminal part . . . . . ***Pam. sklodowskae***

–. Egg bare diameter more than 92.0 µm, egg process height less than 13.5 µm, egg processes hemispherical with cylindrical indented apices . . . . . ***Pam. sagani***

21. Lunules under claws IV dentate. . . . . ***Pam. alekseevi***

–. Lunules under claws IV smooth. **22**

22. Egg processes with cap-like vesicular structures on the top **23**

– Egg processes without cap-like vesicular structures on the top. **24**

23. Egg processes with elongated terminal portion, second macroplacoid length less than 6.5 µm, *pt* values of second macroplacoid length less than *14.0*, *pt* values of macroplacoid row length less than *59.0*, placoid row length less than 34.5 µm and *pt* values of placoid row length less than *74.0*. . . . . ***Pam. filipi***

– Egg processes without elongated terminal portion, second macroplacoid length 7.0 µm or more, *pt* values of second macroplacoid length more than *15.0*, *pt* values of macroplacoid row length more than *60.0*, placoid row length more than 34.9 µm and *pt* values of placoid row length more than *77.5*. . . . . ***Pam. gadabouti***

24. Egg processes with long, thin and flexible terminal portion and egg process height more than 24.5 µm . . . . . ***Pam. lorenae***

– Egg processes without long, thin and flexible terminal portions and egg process height less than 22.5 µm . . . . . ***Pam. richtersi***

25. Cuticle with oval pores, egg processes with cap-like structure on the top and clearly narrower under caps . . . . . ***Pam. garynahi***

–. Cuticle without oval pores, egg processes without cap-like structure on the top and without narrowing at the top **26**

26. Egg processes hemispherical with blunt apex not divided and without elongated terminal part . . . . . ***Pam. savai***

–. Egg processes different **27**

27. Egg processes with long flexible spines on the top . . . . . ***Pam. rioplatensis***

–. Egg processes without long flexible spines on the top **28**

28. Egg processes base width less than 12.5 µm. . . . . ***Pam. peteri***

–. Egg processes base width more than 13.0 µm. **29**

29. Granulation on IVth pair of legs absent and egg processes height more than 15.5 µm . . . . . ***Pam. hapukuensis***

–. Granulation on IVth pair of legs present and egg processes height less than 15.0 µm . **30**

30. Presence of wrinkled surface on the egg areolae and the absence of cuticular bulge on inner surface of claws I–III. . . . . ***Pam. experimentalis***

–. Lack of wrinkled surface on the egg areolae and the presence of cuticular bulge on inner surface of claws I–III. . . . . ***Pam. metropolitanus***

31. Egg of *csotiensis* type . . . . . ***Pam. csotiensis***

–. Eggs of *areolatus*, *huziori, tonollii* or *richtersi* type **32**

32. Eggs of *tonollii* type . . . . . ***Pam. tonollii***

–. Eggs of *areolatus*, *huziori* or *richtersi* type **33**

33. The egg areolation of the *huziori* type **34**

–. Eggs of *richtersi* or *areolatus* type **35**

34. Only one row of larger teeth present in the second band in the oral cavity, the distances between all macroplacoids are approximately the same, accessory points well developed but not protruding high above the primary branch, diameter of bases of egg processes approximately equal to or slightly smaller than their height, 9–11 processes on egg circumference . . . . . ***Pam. huziori***

–. A row of larger teeth and a posterior band of small granules/conical teeth present in the second band of teeth in the oral cavity, the second macroplacoid situated closer to the first than to the third macroplacoid, accessory points extremely well developed, protruding high above the primary branch, diameter of bases of egg processes greater than their height, 12–16 processes on egg circumference . . . . . ***Pam. derkai***

35. Eggs of *richtersi* type . . . . . ***Pam. spinosus***

–. Eggs of *areolatus* type **36**

36. The first/anterior band of teeth visible under PCM **37**

–. The first/anterior band of teeth absent or not visible under PCM . . . . . ***Pam. intii***

37. Lunules under claws IV smooth. **38**

–. Lunules under claws IV dentate **39**

38. Eyes present, macroplacoid length sequence 2<3<1, full egg diameter more than 93.0 µm and egg process height more than 17.5 µm. . . . . ***Pam. lachowskae***

–. Eyes absent, macroplacoid length sequence 2<1<3, full egg diameter less than 92.0 µm and egg process height less than 11.5 µm . . . . . ***Pam. centesimus***

39. Eyes present, macroplacoid length sequence 2<1<3 and egg processes elongated **40**

–. Eyes absent, macroplacoid length sequence 2<3<1 and egg processes short. . . . . ***Pam. klymenki***

40. Egg process height more than 26.5 µm and egg process surface smooth . . . . . ***Pam. areolatus***

–. Egg process height less than 17.5 µm and egg process surface apically covered by irregular granulation . . . . . ***Pam. walteri***

5. Conclusions

The genus *Paramacrobiotus* shows cosmopolitan distribution with presence of both bisexual and parthenogenetic species. Although the integrative descriptions and redescriptions are improving the overall situation and allowing for fresh opportunities for detailed study, the phylogeny of the genus *Paramacrobiotus* seems to be unresolved. Also, there are many other studies regarding life-history, cryptobiotic abilities and microbiome community as well as bacterial endosymbiont infections identification, which are lacking, and such studies are required for the advancement of tardigrade knowledge in general.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. SM.01 Locations, mode of reproduction and presence of genetic data for all the *Paramacrobiotus* species.

Author Contributions: Conceptualization, P.K. and Ł.K.; methodology, P.K. and M.M.; formal analysis, P.K. and M.M.; investigation, P.K.; data curation, P.K.; writing—original draft preparation, P.K.; writing—review and editing, P.K., M.M. and Ł.K; visualization, P.K. and M.M.; supervision, Ł.K. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement: All the DNA sequences data are from GenBank.

Acknowledgments: Studies have been partially conducted in the framework of activities of BARg (Biodiversity and Astrobiology Research group). PK is scholarship holder of Passport to the future - Interdisci-plinary doctoral studies at the Faculty of Biology, Adam Mickiewicz University, Poznań POWR.03.02.00-00-I006/17. The work of MM was supported by National Science Centre, Poland, grant no. 2021/43/D/NZ8/00344 and grant no. 1220/146/2021 from the Small Grants Pro-gramme of the University of Gdansk (i.e., Ugrants-first competition). Tomasz Bartylak for helping with QGIS.

Conflicts of Interest: The authors declare no competing interests.

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Authorship Statements of the PhD candidate

AUTHOR STATEMENT

for the research article 'Integrative taxonomy reveals new, widely distributed tardigrade species of the genus *Paramacrobiotus* (Eutardigrada: Macrobiotidae). Sci Rep 13, 2196 (2023).'

I declare that the research article 'Kayastha *et al*. **Integrative taxonomy reveals new, widely distributed tardigrade species of the genus *Paramacrobiotus* (Eutardigrada: Macrobiotidae). Sci Rep 13, 2196 (2023).** **https://doi.org/10.1038/s41598-023-28714-w** is part of my PhD dissertation. I, Pushpalata Kayastha along with my supervisor Łukasz Kaczmarek conceptualized the idea, curated the data, performed formal analysis, investigated the research study, prepared tables and figures, wrote the original drafts and performed all editorial work.

Date: 03.07.2023

Name: Pushpalata Kayastha (P.K.)

Signature: 

AUTHOR STATEMENT

for the research article 'Morphological and genetic variability in cosmopolitan tardigrade species - *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf, 2010'

I declare that the research article 'Kayastha *et al*. **Morphological and genetic variability in cosmopolitan tardigrade species - *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf, 2010'** https://doi.org/10.21203/rs.3.rs-2736709/v1 is part of my PhD dissertation. I, Pushpalata Kayastha along with my supervisor Łukasz Kaczmarek conceptualized the idea, curated the data, performed formal analysis, investigated the research study, prepared tables and figures, wrote the original drafts and performed all editorial work.

Date: 30.07.2023

Name: Pushpalata Kayastha (P.K.)

Signature: 

AUTHOR STATEMENT

for the research article 'Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates'

I declare that the research article 'Kayastha *et al*. **Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates.** Zoological Journal of the Linnean Society, zlad060. https://doi.org/10.1093/zoolinnean/zlad060' is part of my PhD dissertation. I, Pushpalata Kayastha along with my supervisor Łukasz Kaczmarek and Dr. Piotr Rzymski conceptualized the idea, performed all the experiments for tardigrade and nematodes, performed statistical analysis, prepared all the figures, wrote the original drafts and performed all editorial work.

Date: 30.07.2023

Name: Pushpalata Kayastha (P.K.)

Signature: 

AUTHOR STATEMENT

for the research article 'Elevated external temperature affects cell ultrastructure and heat shock protein (HSP) in *Paramacrobiotus experimentalis* Kaczmarek, Mioduchowska, Poprawa & Roszkowska, 2020.'

I declare that the research article 'Kayastha *et al*. **Elevated external temperature affect cell ultrastructure and heat shock protein (HSP) in *Paramacrobiotus experimentalis* Kaczmarek, Mioduchowska, Poprawa & Roszkowska, 2020**' https://doi.org/10.21203/rs.3.rs-3202172/v1 is part of my PhD dissertation. I, Pushpalata Kayastha along with my supervisor Łukasz Kaczmarek conceptualized the idea, performed all the experiments for heat stress, performed statistical analysis, prepared all the figures, wrote the original drafts and performed all editorial work.

Date: 30.07.2023

Name: Pushpalata Kayastha (P.K.)

Signature: 

AUTHOR STATEMENT

for the review article 'A review on genus *Paramacrobiotus*'

I declare that I am aware of that the work in the research article 'Kayastha *et al*. **A review on genus *Paramacrobiotus*. Preprints.org 2023, 2023071250.** https://doi.org/10.20944/preprints202307.1250.v1' of which I am co-author is a part to PhD dissertation by Pushpalata Kayastha.

Conceptualization, P.K. and Ł.K.; methodology, P.K. and M.M.; formal analysis, P.K. and M.M.; investigation, P.K.; data curation, P.K.; writing—original draft preparation, P.K.; writing—review and editing, P.K., M.M. and Ł.K; visualization, P.K. and M.M.; supervision, Ł.K. All authors have read and agreed to the published version of the manuscript.

Date: 30.07.2023

Name: Pushpalata Kayastha (P.K.)

Signature: 

Co-authors Statements

CO-AUTHOR STATEMENT

for the research article 'Integrative taxonomy reveals new, widely distributed tardigrade species of the genus *Paramacrobiotus* (Eutardigrada: Macrobiotidae)'

I declare that I am aware that the work in the research article 'Kayastha et al. '**Integrative taxonomy reveals new, widely distributed tardigrade species of the genus *Paramacrobiotus* (Eutardigrada: Macrobiotidae)**. *Sci Rep* **13**, 2196 (2023). https://doi.org/10.1038/s41598-023-28714-w of which I am co-author is a part to PhD dissertation by Pushpalata Kayastha.

Conceptualization, P.K. and Ł.K.; data curation, P.K.; sample collection, Ł.S.; formal analysis, P.K., D.S., M.M. and Ł.K.; investigation, P.K., D.S., Ł.S., M.M., M.G. and Ł.K.; methodology, P.K., D.S., M.M. and Ł.K.; supervision, Ł.K.; validation, P.K., D.S., M.M. and Ł.K.; visualization, P.K. and M.G.; writing—original draft, P.K., D.S., M.M. and Ł.K.; writing—review and editing, All authors reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

Date: 01.06.2023

Name: Daniel Stec (D.S.)
Address: Institute of
Systematics and
Evolution of Animals,
Polish Academy of
Sciences, Slawkowska 17, 31-016,
Krak6w, Poland

Signature• Daniel Stec
.....

CO-AUTHOR STATEMENT

for the research article 'Integrative taxonomy reveals new, widely distributed tardigrade species of the genus *Paramacrobiotus* (Eutardigrada: Macrobiotidae)'

I declare that I am aware that the work in the research article 'Kayastha et al. **'Integrative taxonomy reveals new, widely distributed tardigrade species of the genus *Paramacrobiotus* (Eutardigrada: Macrobiotidae)**. *Sci Rep* **13**, 2196 (2023). https://doi.org/10.1038/s41598-023-28714-w of which I am co-author is a part to PhD dissertation by Pushpalata Kayastha.

Conceptualization, P.K. and Ł.K.; data curation, P.K.; sample collection, Ł.S.; formal analysis, P.K., D.S., M.M. and Ł.K.; investigation, P.K., D.S., Ł.S., M.M., M.G. and Ł.K.; methodology, P.K., D.S., M.M. and Ł.K.; supervision, Ł.K.; validation, P.K., D.S., M.M. and Ł.K.; visualization, P.K. and M.G.; writing—original draft, P.K., D.S., M.M. and Ł.K.; writing—review and editing, All authors reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

Date: 01.06.2023

Name: Łukasz Sługocki (Ł.S.)
Address: Department of Hydrobiology, Institute of Biology, University of Szczecin, Szczecin, Poland

Signature:……

CO-AUTHOR STATEMENT

for the research article 'Integrative taxonomy reveals new, widely distributed tardigrade species of the genus *Paramacrobiotus* (Eutardigrada: Macrobiotidae)'

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Date: 01.06.2023

Name: Magdalena Gawlak (M.G.)
Address: The Institute of Plant ProtectionNational Research Institute, Wegorka 20, 60-318, Poznan, Poland

Signature: Magdalena Gawlak

CO-AUTHOR STATEMENT

for the research article 'Integrative taxonomy reveals new, widely distributed tardigrade species of the genus *Paramacrobiotus* (Eutardigrada: Macrobiotidae)'

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Date: 18.07.2023

Name: Monika Mioduchowska (M.M.)
Address: Department of Evolutionary Genetics and Biosystematics,
Faculty of Biology, University of Gdańsk, Gdańsk,
Wita Stwosza 59, 80-308, Gdańsk, Poland

Signature:…….

CO-AUTHOR STATEMENT

for the research article 'Integrative taxonomy reveals new, widely distributed tardigrade species of the genus *Paramacrobiotus* (Eutardigrada: Macrobiotidae)'

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Date: 31.07.2023

Name: Łukasz Kaczmarek (Ł.K.)
Address: Department of Animal Taxonomy and Ecology,
Faculty of Biology, Adam Mickiewicz University in Poznań, Poznań,
Uniwersytetu Poznańskiego 6, 6

Signature:…….

CO-AUTHOR STATEMENT

for the research article 'Morphological and genetic variability in cosmopolitan tardigrade species - Paramacrobiotusfairbanksi Schill, Förster, Dandekar & Wolf, 2010'

I declare that I am aware of that the work in the research article 'Kayastha et al. 'Morphological and genetic variability in cosmopolitan tardigrade species Paramacrobiotus fairbanksi Schill, Förster, Dandekar & Wolf, 2010 https://doi.org/10.21203/rs.3.rs-2736709/vl of which I am co-author is a part to PhD dissertation by Pushpalata Kayastha.

Conceptualization, P.K. and L.K.; data curation, P.K.; formal analysis, PK.; investigation, P.K., M.M., and L.K.; methodology, P.K., M.M. and L.K.; supervision, L.K.; validation, P.K., W.S. M.M. and L.K.; visualization, P.K.; writing—original draft, P.K., M.M. and L.K.; writing— review and editing, P.K., W.S., M.M. and L.K.

Date: 18.07.2023

Name: Wiktoria Szydlo (W.S.)
Address: Center for Advanced Technology,
Adam Mickiewicz University in Poznafi,
Uniwersytetu Poznafiskiego 10, 61-614 Poznafi, Poland

Signature:. 

CO-AUTHOR STATEMENT

for the research article 'Morphological and genetic variability in cosmopolitan tardigrade species - *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf, 2010'

I declare that I am aware of that the work in the research article 'Kayastha *et al*. '**Morphological and genetic variability in cosmopolitan tardigrade species - *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf, 2010** https://doi.org/10.21203/rs.3.rs-2736709/v1 of which I am co-author is a part to PhD dissertation by Pushpalata Kayastha.

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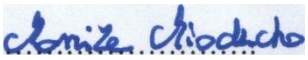
Date: 18.07.2023

Name: Monika Mioduchowska (M.M.)
Address: Department of Evolutionary Genetics and Biosystematics,
Faculty of Biology, University of Gdańsk, Gdańsk,
Wita Stwosza 59, 80-308, Gdańsk, Poland



Signature:…….

CO-AUTHOR STATEMENT

for the research article 'Morphological and genetic variability in cosmopolitan tardigrade species - *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf, 2010'

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Conceptualization, P.K. and Ł.K.; data curation, P.K.; formal analysis, P.K.; investigation, P.K., M.M., and Ł.K.; methodology, P.K., M.M. and Ł.K.; supervision, Ł.K.; validation, P.K., W.S., M.M. and Ł.K.; visualization, P.K.; writing—original draft, P.K., M.M. and Ł.K.; writing—review and editing, P.K., W.S., M.M. and Ł.K.

Date: 31.07.2023

Name: Łukasz Kaczmarek (Ł.K.)
Address: Department of Animal Taxonomy and Ecology,
Faculty of Biology, Adam Mickiewicz University in Poznań, Poznań,
Uniwersytetu Poznańskiego 6, 61-614, Poznań, Poland

Signature:…….

CO-AUTHOR STATEMENT

for the research article 'Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates'

I declare that I am aware of that the work in the research article 'Kayastha *et al*. **Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates.** Zoological Journal of the Linnean Society, zlad060. https://doi.org/10.1093/zoolinnean/zlad060' which is a part to PhD dissertation by Pushpalata Kayastha. I declare that Pushpalata Kayastha was a leading author of this work, while I served an accessory role in data discussion.

Date: 18.07.2023

Name: Piotr Rzymski

Address: Department of Environmental Medicine,

Poznan University of Medical Sciences,

Poznań, Poland

Signature:...  .

CO-AUTHOR STATEMENT

for the research article 'Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates'

I declare that I am aware of that the work in the research article 'Kayastha *et al*. **Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates.** Zoological Journal of the Linnean Society, zlad060. https://doi.org/10.1093/zoolinnean/zlad060' of which I am co-author is a part to PhD dissertation by Pushpalata Kayastha. I declare that Pushpalata Kayastha was a leading author of this work.

My contribution are as follows: Artemia experiment and reviewed the manuscript.

Date: 18.07.2023

Name: Bartłomiej Gołdyn
Address: Department of General Zoology,
Faculty of Biology,
Adam Mickiewicz University,
Uniwersytetu Poznańskiego 6,
61-614 Poznań, Poland

Signature:……

CO-AUTHOR STATEMENT

for the research article 'Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates'

I declare that I am aware of that the work in the research article 'Kayastha *et al*. **Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates.** Zoological Journal of the Linnean Society, zlad060. https://doi.org/10.1093/zoolinnean/zlad060' of which I am co-author is a part to PhD dissertation by Pushpalata Kayastha. I declare that Pushpalata Kayastha was a leading author of this work.

My contribution are as follows: one tardigrade species experiment and reviewed the manuscript.

Date: 18.07.2023

Name: Amit Kumar Nagwani
Address: Department of Bioenergetics,
Faculty of Biology,
Adam Mickiewicz University,
Uniwersytetu Poznańskiego 6,
61-614 Poznań, Poland

Signature:……

CO-AUTHOR STATEMENT

for the research article 'Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates'

I declare that I am aware of that the work in the research article 'Kayastha *et al*. **Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates.** Zoological Journal of the Linnean Society, zlad060. https://doi.org/10.1093/zoolinnean/zlad060' of which I am co-author is a part to PhD dissertation by Pushpalata Kayastha. I declare that Pushpalata Kayastha was a leading author of this work.

My contribution are as follows: rotifera experiments and reviewed the manuscript.

Date: 18.07.2023

Name: Edyta Fiałkowska
Address: Institute of Environmental Sciences,
Faculty of Biology, Jagiellonian University,
Gronostajowa 7, 30-387 Kraków, Poland

Signature: Edyta Fiałkowska

CO-AUTHOR STATEMENT

for the research article 'Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates'

I declare that I am aware of that the work in the research article 'Kayastha *et al*. **Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates.** Zoological Journal of the Linnean Society, zlad060. https://doi.org/10.1093/zoolinnean/zlad060' of which I am co-author is a part to PhD dissertation by Pushpalata Kayastha. I declare that Pushpalata Kayastha was a leading author of this work.

My contribution are as follows: rotifera experiments and reviewed the manuscript.

Date: 18.07.2023

Name: Agnieszka Pajdak-Stós
Address: Institute of Environmental Sciences,
Faculty of Biology, Jagiellonian University,
Gronostajowa 7, 30-387 Kraków, Poland

Signature:……

CO-AUTHOR STATEMENT

for the research article 'Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates'

I declare that I am aware of that the work in the research article 'Kayastha *et al*. **Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates.** Zoological Journal of the Linnean Society, zlad060. https://doi.org/10.1093/zoolinnean/zlad060' of which I am co-author is a part to PhD dissertation by Pushpalata Kayastha. I declare that Pushpalata Kayastha was a leading author of this work.

My contribution are as follows: provided nematodes for experiments.

Date: 18.07.2023

Name: Robert Sobkowiak

Address: Institute of Environmental Sciences,
Faculty of Biology, Jagiellonian University,
Gronostajowa 7, 30-387 Kraków, Poland

Signature: Robert Sobkowiak .....

CO-AUTHOR STATEMENT
for the research article 'Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates'

I declare that I am aware of that the work in the research article 'Kayastha *et al*. **Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates** https://doi.org/10.1093/zoolinnean/zlad060' of which I am co-author is a part to PhD dissertation by Pushpalata Kayastha.

My contribution are as follows: Artemia experiment.

Date: 18.07.2023
Name: Gracjan Robotnikowski
Address: Department of General Zoology,
Faculty of Biology,
Adam Mickiewicz University,
Uniwersytetu Poznańskiego 6,
61-614 Poznań, Poland

Signature:.....

CO-AUTHOR STATEMENT

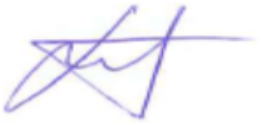
for the research article 'Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates'

I declare that I am aware of that the work in the research article 'Kayastha *et al*. **Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates.** Zoological Journal of the Linnean Society, zlad060. https://doi.org/10.1093/zoolinnean/zlad060' of which I am a co- author is a part to PhD dissertation by Pushpalata Kayastha. I declare that Pushpalata Kayastha was a leading author of this work, while I served an accessory role in data discussion and supervision.

Date: 18.07.2023

Name: Łukasz Kaczmarek

Address: Department of Animal Taxonomy and Ecology,
Faculty of Biology, Adam Mickiewicz University in Poznań, Poznań,
Uniwersytetu Poznańskiego 6, 61-614, Poznań, Poland



Signature:…….

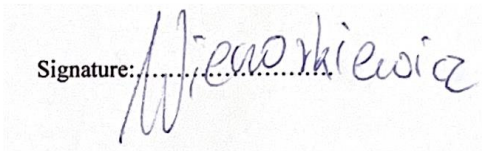
CO-AUTHOR STATEMENT

for the research article 'Elevated external temperature affects cell ultrastructure and heat shock protein (HSP) in *Paramacrobiotus experimentalis* Kaczmarek, Mioduchowska, Poprawa & Roszkowska, 2020.'

I declare that the research article 'Kayastha *et al*. **Elevated external temperature affect cell ultrastructure and heat shock protein (HSP) in Paramacrobiotus experimentalis Kaczmarek, Mioduchowska, Poprawa & Roszkowska, 2020**' https://doi.org/10.21203/rs.3.rs-3202172/v1 of which I am co-author is a part of PhD dissertation by Pushpalata Kayastha.

Conceptualization, P.K. and Ł.K.; data curation, P.K., M.P. and I.P.; investigation, P.K., M.P. and I.P.; methodology, P.K., M.P., M.M., F.W., I.P. and Ł.K.; statistical analysis, P.K. and M.P.; validation, all authors; supervision, M.M., I.P. and Ł.K.; writing—original draft, P.K. and I.P.; writing—review and editing, all authors. All authors accepted the final version of the manuscript.

Date: 18.07.2023
Name: Filip Wieczorkiewicz (F.W.)
Address: Institute of Biology,
Biotechnology and Environmental Protection,
Faculty of Natural Sciences,
University of Silesia in Katowice,
Bankowa 9, Katowice 40-007, Poland

Signature: 

CO-AUTHOR STATEMENT

for the research article 'Elevated external temperature affects cell ultrastructure and heat shock protein (HSP) in *Paramacrobiotus experimentalis* Kaczmarek, Mioduchowska, Poprawa & Roszkowska, 2020.'

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Date: 18.07.2023

Name: Myriam Pujol (M.P.)
Address: Department of Biochemistry,
Faculty of Medicine & Dentistry,
University of Alberta, Edmonton,
Alberta, Canada

Signature:…..

CO-AUTHOR STATEMENT

for the research article 'Elevated external temperature affects cell ultrastructure and heat shock protein (HSP) in *Paramacrobiotus experimentalis* Kaczmarek, Mioduchowska, Poprawa & Roszkowska, 2020.'

I declare that the research article 'Kayastha *et al*. **Elevated external temperature affect cell ultrastructure and heat shock protein (HSP) in *Paramacrobiotus experimentalis* Kaczmarek, Mioduchowska, Poprawa & Roszkowska, 2020**' https://doi.org/10.21203/rs.3.rs-3202172/v1 of which I am co-author is a part of PhD dissertation by Pushpalata Kayastha.

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Date: 18.07.2023

Name: Alison Robinson (A.R.)
Address: Department of Biochemistry,
Faculty of Medicine & Dentistry,
University of Alberta, Edmonton,
Alberta, Canada

Alison Robinson

Signature:…….

CO-AUTHOR STATEMENT

for the research article 'Elevated external temperature affects cell ultrastructure and heat shock protein (HSP) in *Paramacrobiotus experimentalis* Kaczmarek, Mioduchowska, Poprawa & Roszkowska, 2020.'

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Date: 18.07.2023

Name: Marek Michalak (M.M.)
Address: Department of Biochemistry,
Faculty of Medicine & Dentistry,
University of Alberta, Edmonton,
Alberta, Canada

Signature: .....

CO-AUTHOR STATEMENT

for the research article 'Elevated external temperature affects cell ultrastructure and heat shock protein (HSP) in *Paramacrobiotus experimentalis* Kaczmarek, Mioduchowska, Poprawa & Roszkowska, 2020.'

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Date: 18.07.2023

Name: Łukasz Kaczmarek (Ł.K.)
Address: Department of Animal Taxonomy and Ecology,
Faculty of Biology, Adam Mickiewicz University in Poznań, Poznań,
Uniwersytetu Poznańskiego 6, 61-614, Poznań, Poland

Signature:……

CO-AUTHOR STATEMENT

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Date: 18.07.2023
Name: Izabela Poprawa (I.P.)
Address: Institute of Biology,
Biotechnology and Environmental Protection,
Faculty of Natural Sciences,
University of Silesia in Katowice,
Bankowa 9, Katowice 40-007, Poland

Signature:  ……

CO-AUTHOR STATEMENT

for the review article 'A review on genus *Paramacrobiotus*'

I declare that I am aware of that the work in the research article 'Kayastha *et al*. **A Review on Genus *Paramacrobiotus*. Preprints.org 2023, 2023071250.** https://doi.org/10.20944/preprints202307.1250.v1' of which I am co-author is a part to PhD dissertation by Pushpalata Kayastha.

Conceptualization, P.K. and Ł.K.; methodology, P.K. and M.M.; formal analysis, P.K. and M.M.; investigation, P.K.; data curation, P.K.; writing—original draft preparation, P.K.; writing—review and editing, P.K., M.M. and Ł.K; visualization, P.K. and M.M.; supervision, Ł.K. All authors have read and agreed to the published version of the manuscript.

Date: 22.07.2023

Name: Monika Mioduchowska (M.M.)
Address: Department of Evolutionary Genetics and Biosystematics,
Faculty of Biology, University of Gdańsk, Gdańsk,
Wita Stwosza 59, 80-308, Gdańsk, Poland

Signature 

CO-AUTHOR STATEMENT

for the review article 'A review on genus *Paramacrobiotus*'

I declare that I am aware of that the work in the research article 'Kayastha *et al*. **A Review on Genus *Paramacrobiotus*. Preprints.org 2023, 2023071250.** https://doi.org/10.20944/preprints202307.1250.v1' of which I am co-author is a part to PhD dissertation by Pushpalata Kayastha.

Conceptualization, P.K. and Ł.K.; methodology, P.K. and M.M.; formal analysis, P.K. and M.M.; investigation, P.K.; data curation, P.K.; writing—original draft preparation, P.K.; writing—review and editing, P.K., M.M. and Ł.K; visualization, P.K. and M.M.; supervision, Ł.K. All authors have read and agreed to the published version of the manuscript.

Date: 22.07.2023

Name: Łukasz Kaczmarek (Ł.K.)
Address: Department of Animal Taxonomy and Ecology,
Faculty of Biology, Adam Mickiewicz University in Poznań, Poznań,
Uniwersytetu Poznańskiego 6, 61-614, Poznań, Poland

Signature:… ……