Uniwersytet im. Adama Mickiewicza w Poznaniu

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Rodzaj *Paramacrobiotus* (Tardigrada): taksonomia integratywna, biogeografia i oraz wpływ czynników stresowych na wybrane gatunki.

Uniwersytet im. Adama Mickiewicza w Poznaniu

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

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Doctoral thesis

Promotor: Dr hab. Prof. UAM Łukasz Kaczmarek

Promotor pomocniczy: Dr Monika Mioduchowska

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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.

Slowa kluczowe: Tardigrada, taksonomia integratywna, hipotezy „wszystko jest wszędzie”, kosmopolityczny, misiami wodnymi

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*.

Keywords: Tardigrada, integrative taxonomy, Everything is Everywhere hypothesis, cosmopolitan, water bear

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/v2, 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., Warguła, J., Kaczmarek, Ł. (2023). A Review on the Genus *Paramacrobiotus* (Tardigrada) with a New Diagnostic Key. Diversity. 15(9):977. https://doi.org/10.3390/d15090977. 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
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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
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- 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).

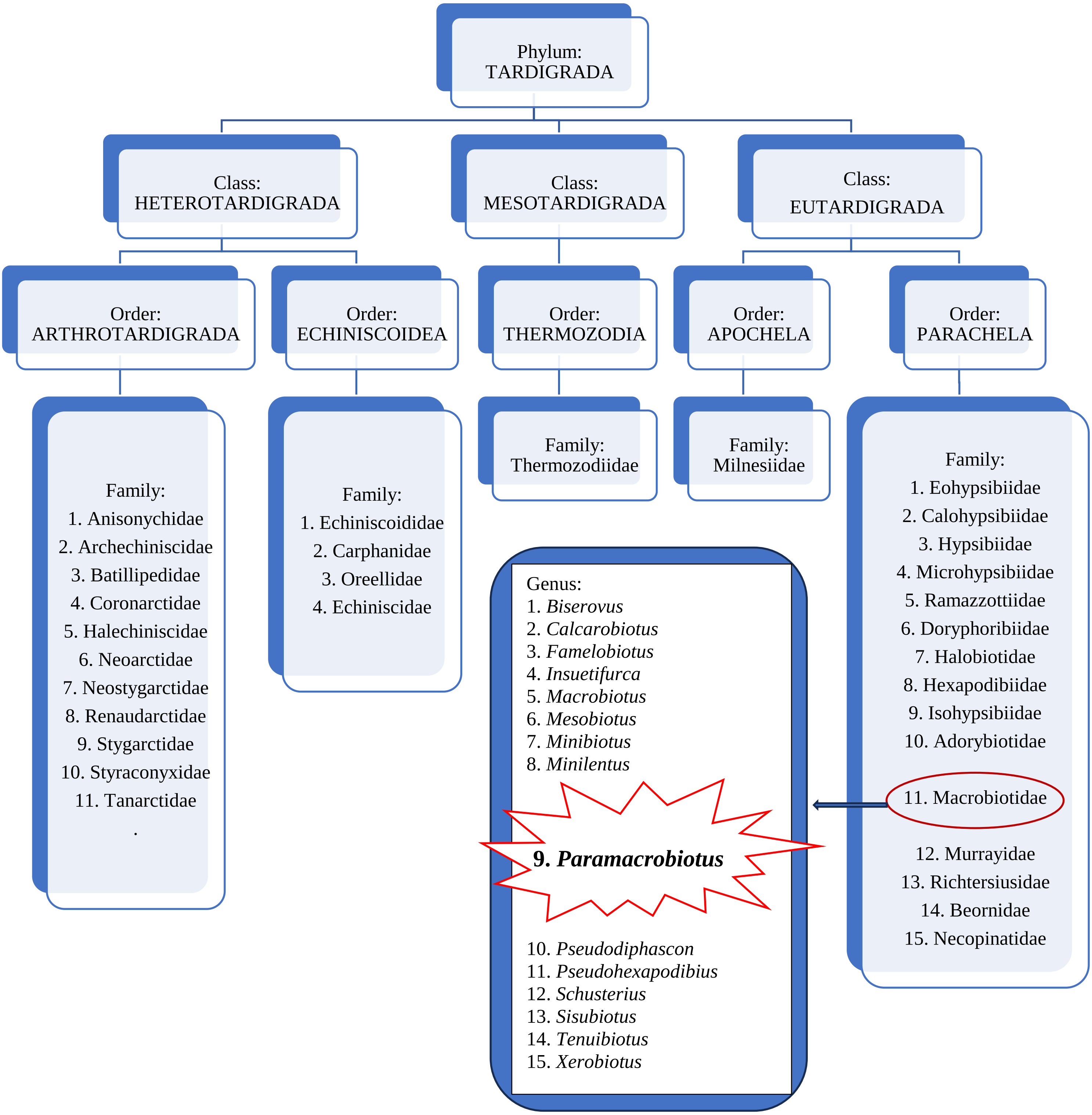

Figure 1. Taxonomic position of genus *Paramacrobiotus* within phylum Tardigrada based on Degma & Guidetti (2009) (but, not all genera and subgenera are listed).

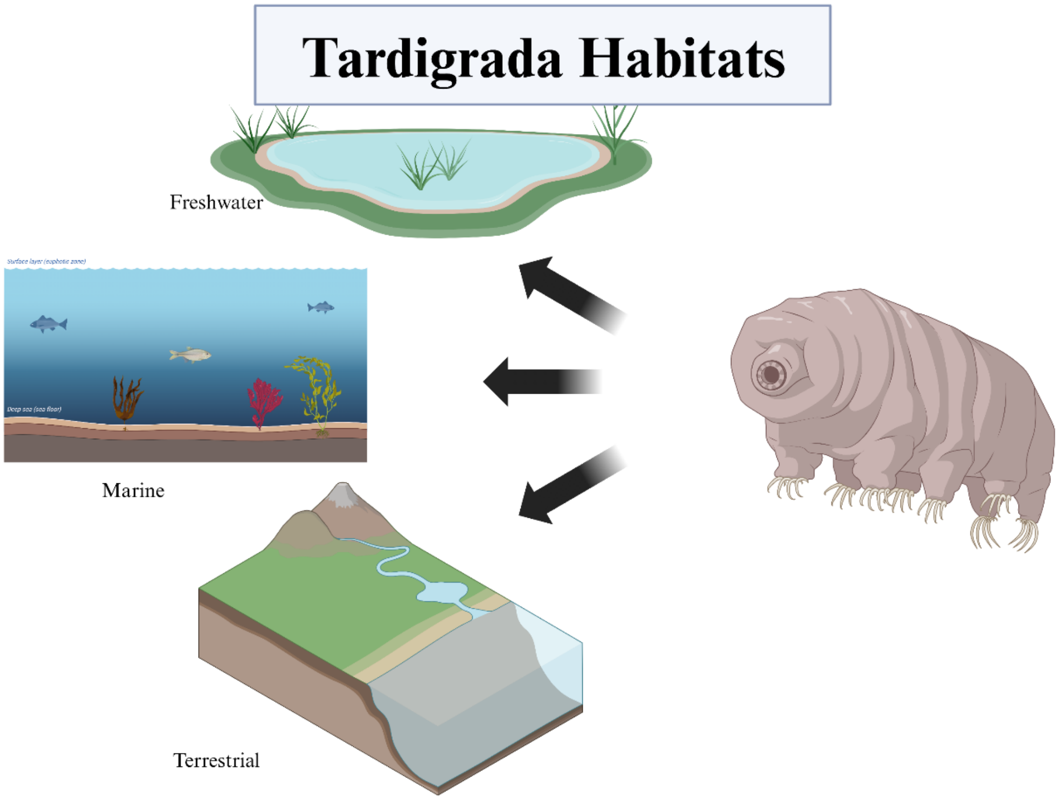

Figure 2. Habitats where tardigrades can be found (Created using BioRender.com).

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.

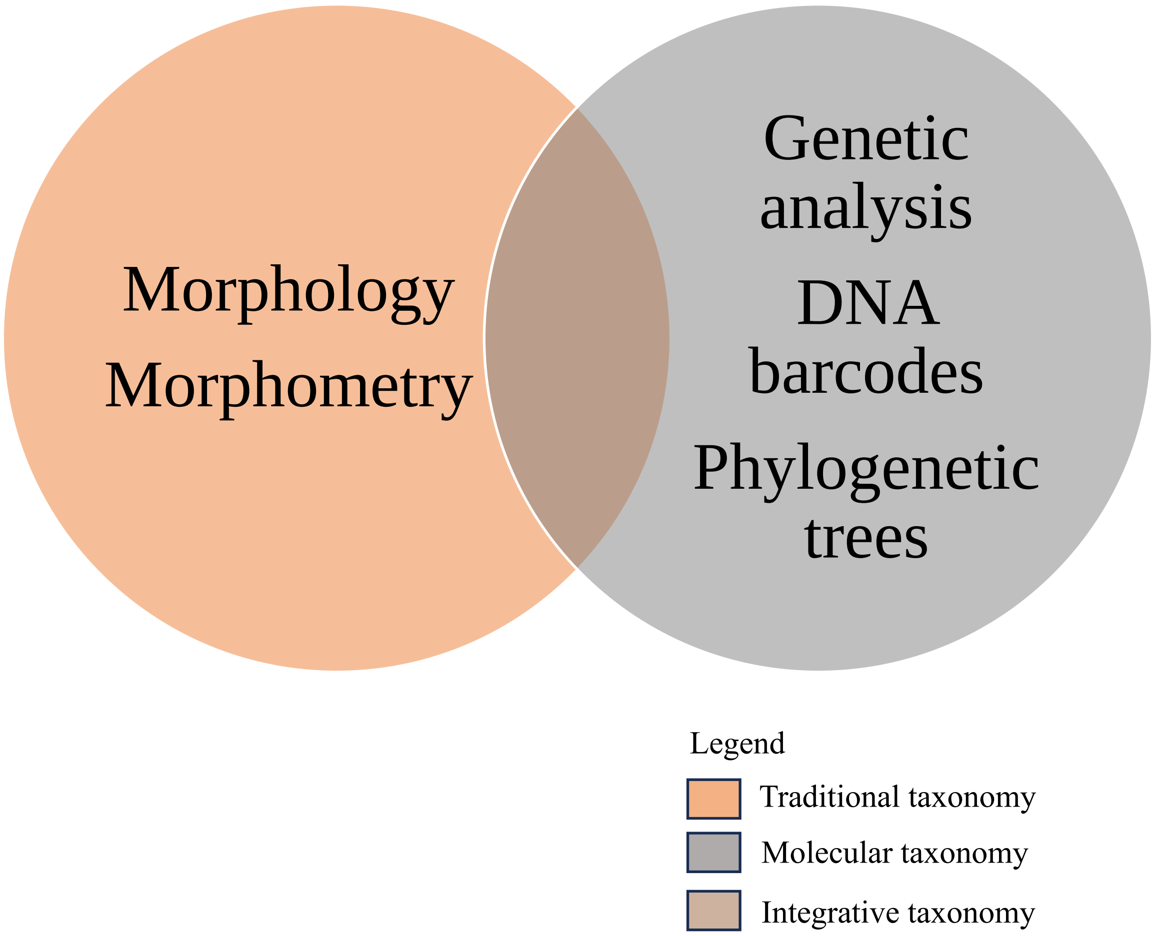

Figure 3. Schematic representation of the integrative taxonomy approach.

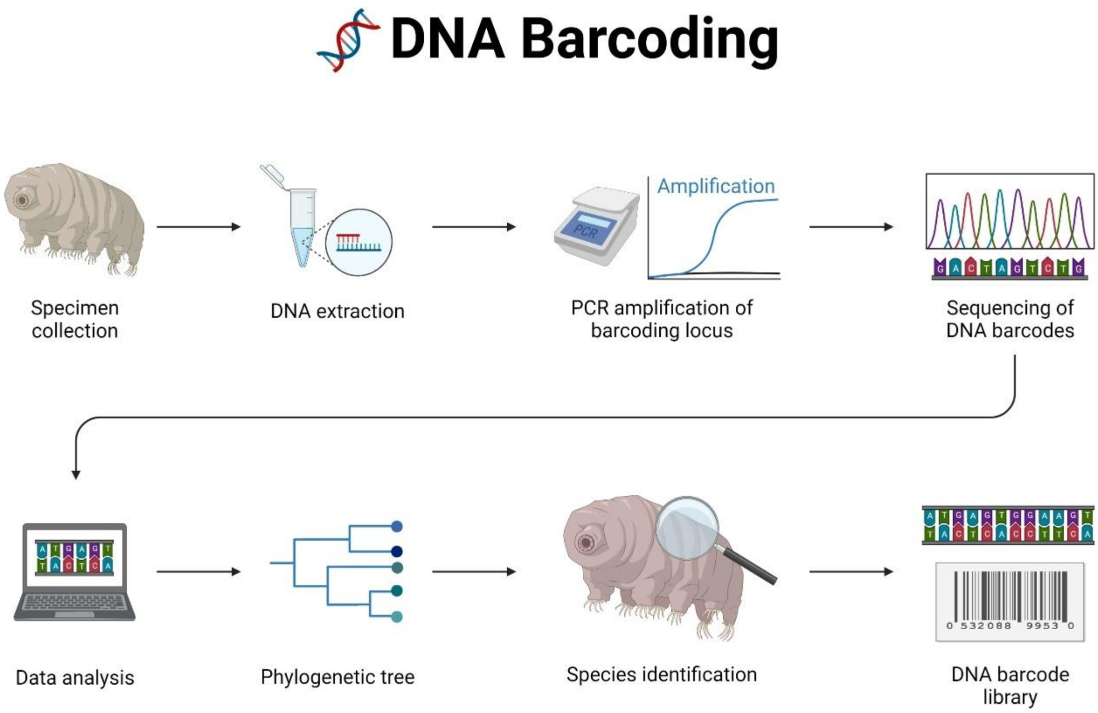

Figure 4. Schematic representation of the DNA barcoding methodology for tardigrades (Created using BioRender.com).

"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

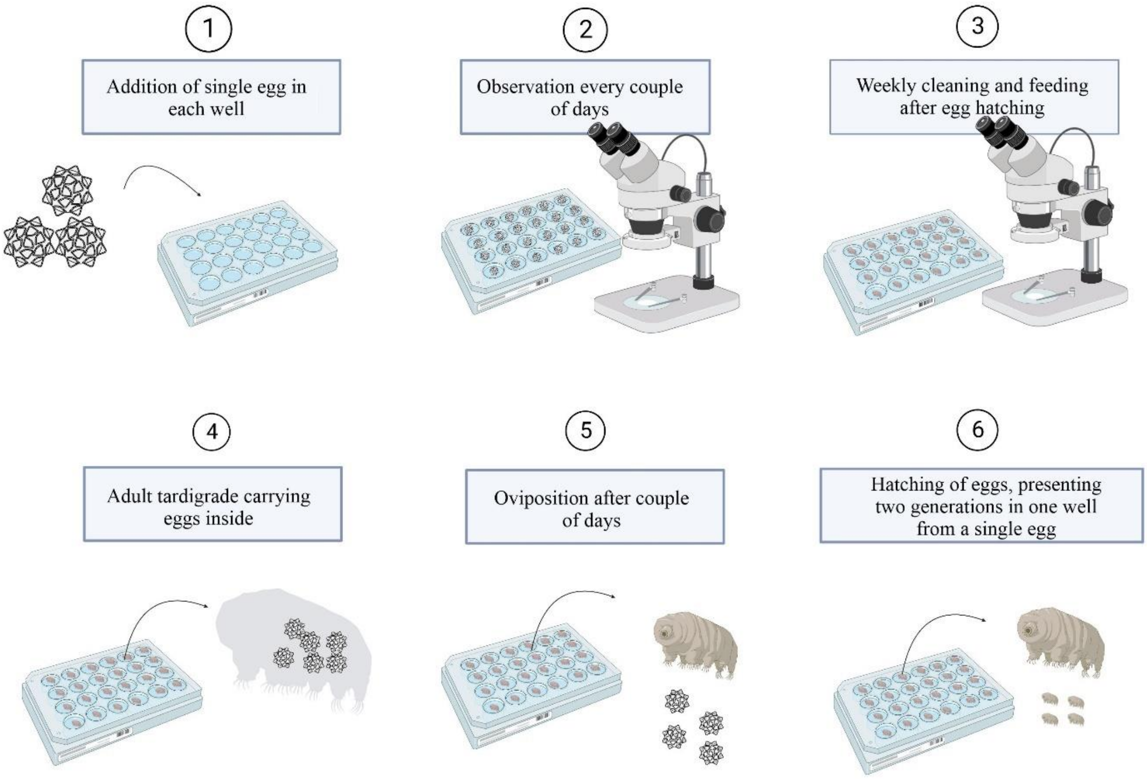

Figure 5. Workflow used to determine of the parthenogenetic reproduction mode in *Pam. gadabouti* (Created using BioRender.com).

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 6). 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).

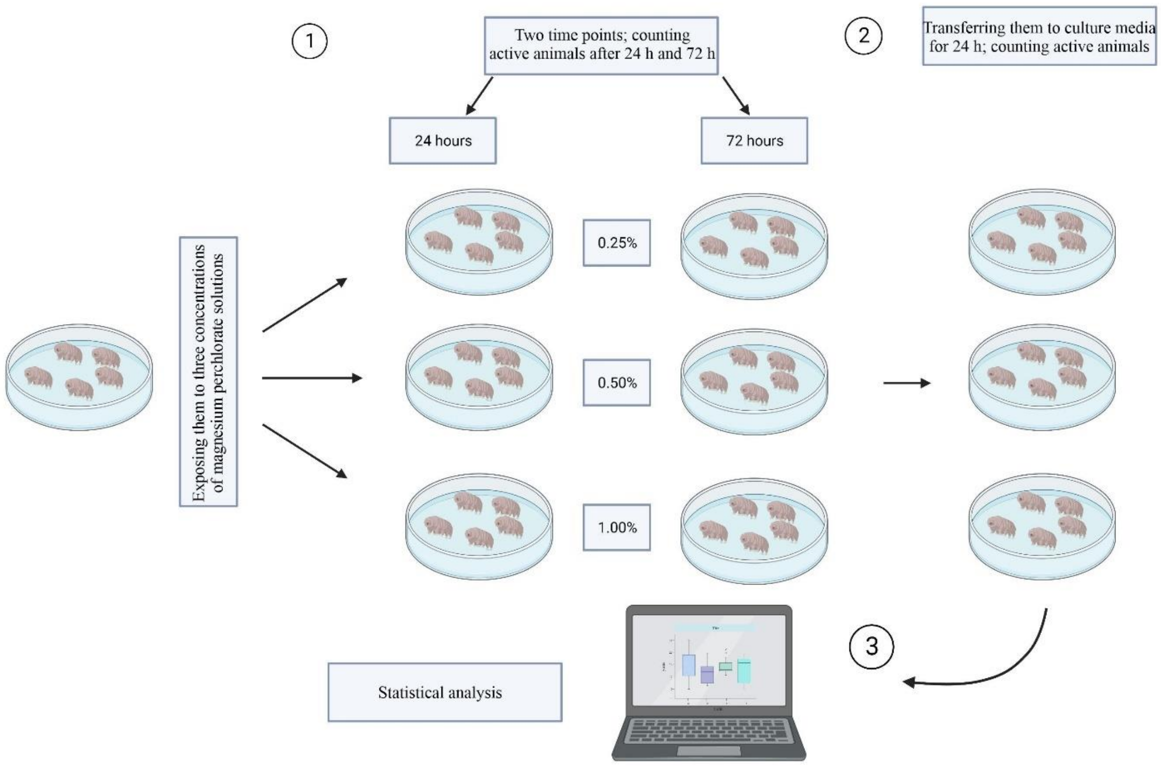

Figure 6. Protocol for testing *Paramacrobiotus experimentalis* tolerance against exposure to magnesium perchlorate solution (Created using BioRender.com).

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 7). 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 8).

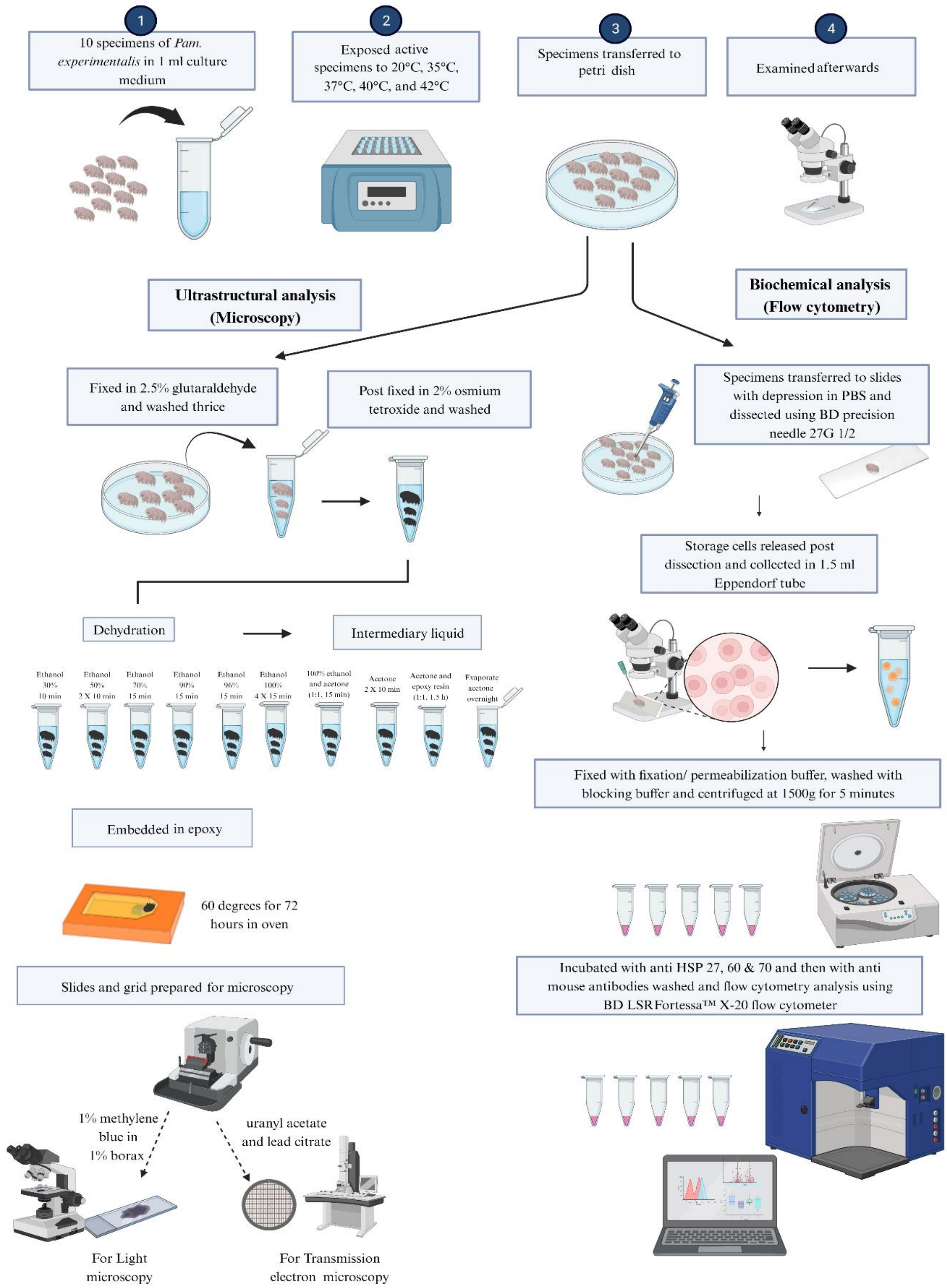

Figure 7. Protocol for testing effects of heat stress on active specimens of the *Paramacrobiotus experimentalis* (Created using BioRender.com).

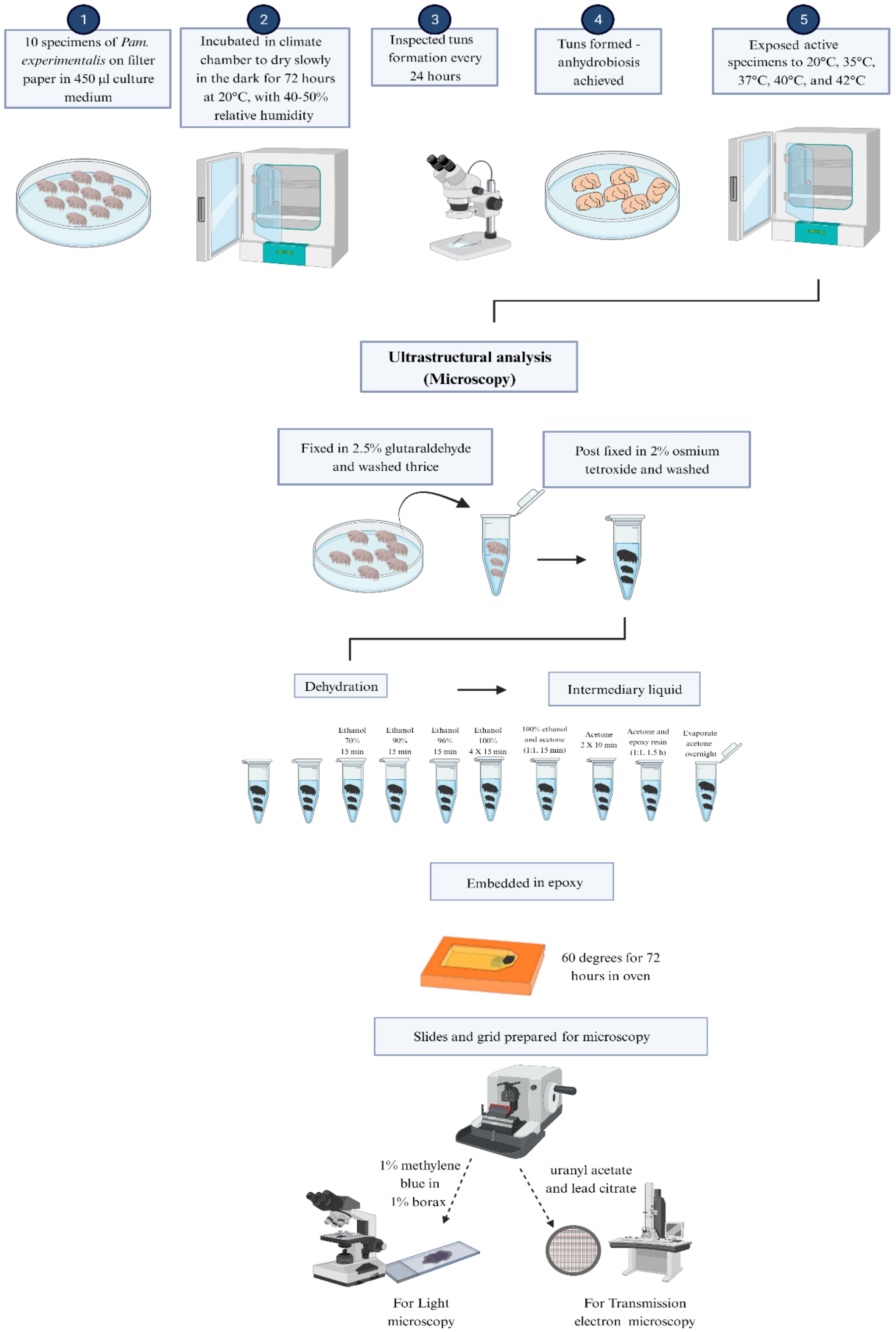

Figure 8. Protocol for anhydrobiosis and testing effects of heat stress on anhydrobiotic tuns of the *Paramacrobiotus experimentalis* (Created using BioRender.com)

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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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/v2

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Paper III

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Paper IV

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Paper V

Kayastha, P., Mioduchowska, M., Warguła, J., Kaczmarek, Ł. A Review on the Genus *Paramacrobiotus* (Tardigrada) with a New Diagnostic Key. *Diversity* 15(9):977 (2023).
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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.

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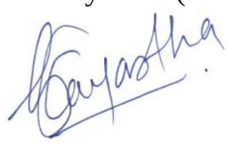
Signature: 

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/v2 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.

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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.

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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.

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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.

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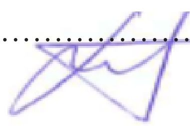

CO-AUTHOR STATEMENT

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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/v2 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.

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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: 18.07.2023

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Signature: Monika Mioduchowska

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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

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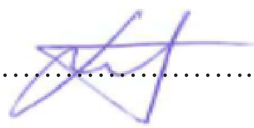

CO-AUTHOR STATEMENT

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,
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Poznań, Poland

Signature:... ....

CO-AUTHOR STATEMENT

for the research article ‘Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates’

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My contribution are as follows: Artemia experiment and reviewed the manuscript.

Date: 18.07.2023

Name: Bartłomiej Gołdyn

Address: Department of General Zoology,
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Adam Mickiewicz University,
Uniwersytetu Poznańskiego 6,
61-614 Poznań, Poland

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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
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61-614 Poznań, Poland

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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

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CO-AUTHOR STATEMENT

for the research article 'Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates'

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My contribution are as follows: rotifera experiments and reviewed the manuscript.

Date: 18.07.2023

Name: Agnieszka Pajdak-Stós

Address: Institute of Environmental Sciences,
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CO-AUTHOR STATEMENT

for the research article 'Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates'

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My contribution are as follows: provided nematodes for experiments.

Date: 18.07.2023

Name: Robert Sobkowiak

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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
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61-614 Poznań, Poland

Signature: Gracjan Robotnikowski

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

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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.

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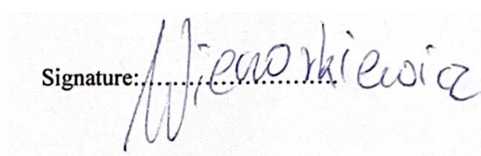

CO-AUTHOR STATEMENT

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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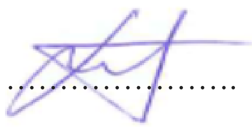
Signature:… …………………..

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.

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I declare that I am aware of that the work in the research article 'Kayastha *et al*. **A Review on the Genus Paramacrobiotus (Tardigrada) with a New Diagnostic Key. Diversity. 15(9):977.** https://doi.org/10.3390/d15090977' of which I am co-author is a part to PhD dissertation by Pushpalata Kayastha.

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