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Tardigrada 2018
14th International Symposium on Tardigrada

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FRONTPAGE
The Symposium logo shows six individuals of Isoechiniscoides sifae drawn by Stine Elle.
KEYNOTE TALKS
Another round of animal phylogenetics: known, unknowns, needs

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Background: Understanding animal phylogeny has been a hot topic through more than two centuries and a major focus for methodological developments in anatomical studies, cladistics, developmental biology, and molecular systematics. With the advent of new sequencing technologies, there has been a recent shift from using morphology or a handful of genes to genome-scale data, which can now be generated at an ever-increasing rate even from the smallest metazoans. The new data, however, come with new challenges associated with defining orthology, selecting genes and taxa for analyses, and in using automated ways to generate repeatable results.

Results: New analyses have been conducted using genomes and Illumina-based transcriptomes to assemble the largest metazoan data set ever put together. Our data show novel results with respect to the position of taxa such as Placozoa and Chaetognatha and show the roadmap for how to approach difficult questions in animal evolution, including the phylogenies of Lophotrochozoa (a subtaxon of Spiralia) and Ecdysozoa. Many of these challenges are due to data heterogeneity, including compositional bias in key lineages. The difficulty in using comparative morphology to infer phylogenies across the metazoan phyla is also discussed.

Conclusions: While the current round of analyses using improved datasets and optimized taxon sampling are able to resolve previous conflicting animal nodes, it is also clear that rate heterogeneity among members of a clade may pose a challenge for resolving certain nodes (e.g., the non-lophotrochozoan spiralian versus the lophotrochozoan ones). We also show that despite the enormous genomic resources in two lineages of Ecdysozoa (Arthropoda and Nematoda), phylogenies remain poorly resolved for this hyperdiverse lineage, especially due to limited genomic resources in most of the other ecdysozoan phyla, including Tardigrada.

Keywords: Ecdysozoa, metazoan phylogeny, phylogenomics, Spiralia
The rise of a tardigrade-unique toolbox for extremotolerance

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Background: Tardigrades are a crowned animal group renowned for their resilience against various extreme environmental stresses. Since their discovery, the amazing tolerance records have fascinated researchers, but unfortunately some intrinsic features, such as the small size of tardigrades, the difficulty in rearing to a large quantity and easy contamination of other microorganisms, hindered progress of modern molecular biological approaches. In the past decade, the situation has changed progressively, with dramatic advances in the molecular dissection of tardigrade tolerance mechanisms, which has further accelerated in recent years.

Results: Efforts to find the tolerance-related factors led to the identification of several tardigrade-unique protein families. The first category, novel heat-soluble proteins were discovered in line of searching for proteins with similar property to LEA proteins, which play important roles in desiccation-tolerant plant seeds and some anhydrobiotic animals. Till now, three classes of tardigrade-unique heat-soluble proteins, CAHS, SAHS and MAHS, have been identified and proposed to protect biomolecules from desiccation as LEA proteins do. The second category is a novel DNA protection protein dubbed Dsup. Dsup can associate with DNA in vitro and colocalize with nuclear DNA in animal cells. Human cultured cells engineered to express Dsup suffered much reduced DNA damage when exposed to X-ray irradiation or oxidative stress compared to unengineered control cells. Dsup-expressing cells also exhibited better survival and retention of proliferative ability after X-ray irradiation. Besides tardigrade-unique proteins, gene repertoire analyses revealed the specific expansion of some conserved stress-ameliorating genes and the characteristic loss of some metabolic and stress-signaling pathways. Although the proportion of foreign genes falls within the normal range of an animal genome, some of them are likely related to tolerance. Mimicking these features might be beneficial to improve the tolerance in other animals.

Conclusions: Our current knowledge place tardigrade-unique proteins as important clues supporting the tolerance ability of tardigrades, even functional in mammalian cells. Recent comparative genome/transcriptome analyses among tardigrade species revealed the presence of many tardigrade-unique genes correlated with tolerability, representing a potential bountiful resource of tolerance genes awaiting future functional analyses. Future studies using other species, especially heterotardigrades, are essential to complement our molecular understanding of tardigrade resilience. The elucidation of tardigrade tolerance mechanisms would not only satisfy our scientific curiosity, but also contribute to develop new technology for medical and industrial applications.

Keywords: DNA protection, Dsup, genome, heat soluble protein, tolerance mechanism
Phylogenomics, gene families and the phylogenetic position of Tardigrada within Ecdysozoa

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Background: The superphylum Ecdysozoa includes species-rich (Nematoda, Arthropoda) and species poor (Tardigrada, Nematomorpha, Kinorhyncha and others) phyla, but collectively includes most of animal diversity. Morphological analyses and some molecular datasets place Tardigrada comfortably with Arthropoda and Onychophora within Panarthropoda, with Nematoida (Nematoda plus Nematomorpha) sister to Panarthropoda. However, several phylogenomic analyses using multi-locus datasets have resolved Tardigrada as sister to Nematoida. Whether this is an artefact of poor modelling of sequence change or a reflection of biological truth is unclear. Results: We have used genome-scale data from a range of ecdysozoans, including new genome and transcriptome data from many tardigrades, a kinorhynch and a nematomorph, to explore this question. We have used single-organism sequencing, or sequencing of populations of wild-isolated individuals to generate draft genome assemblies for previously unsampled taxa, in particular using advanced tools to separate contaminants (food, microbiome) from the target species. As an example, using these approaches we have identified putative alphaproteobacterial symbionts of the kinorhynch Semnoderes armiger. From the proteomes predicted from the genomes and transcriptomes, we defined orthologue groups, and explored the patterns of protein family birth and loss between the phyla. Sequence-based phylogenetic analyses using single-copy orthologues yielded heterodox phylogenies associating Tardigrada with Nematoida. However, gene family presence-absence analyses supported the traditional Tardigrada plus Arthropoda relationship. This pattern was not universal: for example, Tardigrada have lost some of the same core HOX genes that have also been lost in Nematoda. Conclusions: Analyses based on whole genomes disagree with phylogenetic analyses based on morphology alone. Whether this is because of inadequacies in our models of sequence evolution, because of unusual convergent patterns of gene gain, loss and change in different lineages, or because the true phylogeny differs from expectations, it is clear that the internal structure of Ecdysozoa, and the phylogenetic position of the Tardigrada, remains an exciting area for investigation. Keywords: Comparative genomics, genome sequencing, Kinorhyncha, Nematomorpha, phylogenomics
Phylum-wide genome sequencing of Tardigrada

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**Background:** Tardigrades remain relatively unexplored by genomics, in spite of the enigmatic phylogenetic position of the phylum that, if identified, is likely to be the key to resolve the ecdysozoan phylogeny, and albeit the many unique adaptations exemplified by anhydrobiosis. Previous reports of the genome sequencing of two eutardigrades, *Ramazzottius varieornatus* and *Hypsibius exemplaris*, revealed a multitude of genes related to anhydrobiosis, and provided novel data for phylogenomics. While a more comprehensive exploration of the phylum is necessary, limited amount of samples that can be obtained from the wild, and non-negligible effects of contamination have been major hurdles for such studies.

**Results:** Using ultra-low input sequencing from individual tardigrades, we have so far sequenced 50 tardigrade genomes, including 11 heterotardigrade species, both marine and terrestrial (Batillipedidae, Halechiniscidae, Echiniscoididae, Echiniscidae), and 39 eutardigrade species (Milnesiidae, Hypsibiidae, Macrobiotidae, Murrayidae). Although the coverage within the phylum is still limited, we are beginning to see a rather diverse evolution of anhydrobiosis. Whereas several components, such as the duplication of oxidative stress response genes, are shared between Eutardigrada and Heterotardigrada, many of the tardigrade-specific anhydrins identified so far seem to be clade-specific and non-conserved.

**Conclusions:** Our ongoing effort of this “phylome” study is hoped to provide a fundamental resource for tardigradology. We also hypothesise about convergent evolution of anhydrins that appear to have evolved independently at least twice in the two classes of Tardigrada.

**Keywords:** anhydrobiosis, comparative genomics, genome sequencing, phylogenomics
ORAL PRESENTATIONS
New evolutionary insights from the tardigrade fossil record

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Background: Tardigrades are a diverse and ubiquitous group of microscopic panarthropods with an extensive worldwide distribution in aquatic environments. Despite substantial advances towards understanding the phylogenetic relationships and morphology of extant representatives, the deep evolutionary history of the group remains largely unexplored due to the difficulty of recognizing tardigrades preserved in the rock record. Currently, there are only three legitimate fossil tardigrades, including a phosphatized putative larval form from the upper Cambrian of Sweden, *Beorn leggi* Cooper, 1964, from Cretaceous Canadian amber, and *Milnesium swolenskyi* Bertolani and Grimaldi, 2000, from Upper Cretaceous (Turonian) amber of New Jersey. Although these forms provide reliable fossil calibration points for molecular clock analyses, their morphology closely resembles that of extant representatives and thus offer limited information on early tardigrade evolution.

Results: Restudy of extensive fossil collections of the lobopodian *Aysheaia pedunculata* from the middle Cambrian (ca. 508 million years old) Burgess Shale deposits in British Columbia reveal new morphological characters that allow to establish a link with tardigrades. These include the presence of oral lamellae, a short eversible proboscis, an anterior-facing mouth opening that continues into a voluminous pharyngeal cavity and narrowed esophagus, and the rotation of the posterior-most leg pairs. Critically, *Aysheaia* uniquely shares the presence of numerous (i.e. more than five) scythe-like claws per limb with members of the extant Echiniscoididae (Heterotardigrada), for example the genus *Echiniscoides*, which suggests that this claw configuration may be an ancestral feature that evolved in stem-group Tardigrada. In a follow up study, the application of phase contrast micro computed tomography has produced new data on the preserved morphology of *Beorn leggi*, including exceptional details of the muscular organization that enable comparisons with the myoanatomy of extant Heterotardigrada.

Conclusions: Despite a scant representation in the rock record, the application of new imaging techniques and fossil material provide new insights on the early evolution of tardigrades, and allow to polarize major morphological characters to better reconstruct the evolutionary history of this major clade.

Keywords: *Aysheaia pedunculata*, *Beorn leggi*, Cretaceous, Heterotardigrada, middle Cambrian
Comparative morphological study between tardigrades and Cambrian lobopodians

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\textbf{Background:} Order Arthrotardigrada includes marine heterotardigrades, which have been known to retain more plesiomorphic conditions than the Eutardigrada. Especially, \textit{Parastygarctus} was considered as the most primitive genus based on the morphological comparison with a Cambrian lobopodian, \textit{Aysheaia pedunculata} from the Burgess Shale, Canada, which was one of the two known Cambrian lobopodian until the 1980’s. However, since the 90’s thirteen more Cambrian lobopodian species have been discovered from the Chengjiang, China, which have significantly promoted our knowledge on Cambrian lobopodian morphology, necessitating novel morphological comparison between tardigrades and the Cambrian lobopodians.

\textbf{Results:} We have compared morphological characters between tardigrades and the Cambrian lobopodians. Among several species of Cambrian lobopodians, \textit{Aysheaia pedunculata} displays six peri-buccal papillae around the mouth opening, which are reminiscent of those of the apochelan \textit{Milnesium tardigradum}. Eutardigrades and the Cambrian lobopodians have a terminally-opened mouth, which is an important plesiomorphic character of panarthropods, whereas most heterotardigrades show a ventrally-opened mouth. The Cambrian lobopodians had claws at the end of their limbs, lacking digits or toes, which are present in heterotardigrades. The Cambrian lobopodians and eutardigrades show lobe-like limbs, while heterotardigrades have telescopic limbs. Presence of circum-oral elements and pharyngeal teeth are plesiomorphic for Ecdysozoa, but only eutardigrades have these structures. In addition, the elongated pharyngeal bulb and parallel piercing stylets of apochelan tardigrades reminds one of the elongated pharynx and parallel rostral spines of \textit{Kerygmachela kierkegaardii}.

\textbf{Conclusions:} Based on the morphological comparison with the Cambrian lobopodians, it is suggested that eutardigrades retain more plesiomorphic morphologies than heterotardigrades. Particularly, apochelans display many similarities with Cambrian lobopodians.

\textbf{Keywords:} Apochela, Cambrian lobopodian, comparative morphology, paleontology
Progress in the integration of morphological and molecular investigations in tardigrade taxonomy and phylogeny

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Background: Until the 20th century, tardigrade taxonomy and phylogeny were investigated only with a morphological approach, based almost entirely on sclerified structures. This led to very different conclusions by various authors. With regard to taxonomy, the concept of very high variability within the species was accepted until the 1970s, when Pilato introduced new characters and evaluated their intraspecific variability [1], and later he presented a morphometric approach [2]. Recent studies with a molecular approach have revealed unsuspected and very high species diversity. Regarding phylogeny, the first plausible study was by Thulin [3], but unfortunately it was not followed for many years. Consequently alphabetical order of the genera and species was widely used in the past and continues in some cases even today. Pilato [4] formulated new proposals for the evolution of Eutardigrada, partly using Thulin’s studies. Kristensen [5] first applied a cladistic approach to the heterotardigrades, analyzing the family Echiniscidae. More recently the molecular approach has allowed us to construct robust phylogenetic trees and erect superfamilies and new families [6-7]. Results: At the species level, a more in depth morphological analysis combined with the molecular approach has led to a large increase of the number of described species and the identification of cryptic species. Comparisons between traditional data and molecular data have led to the erection of new higher-level taxa, such as superfamilies and families, in addition to several genera. Conclusions: Comparisons between traditional and molecular data have revealed several overlaps in phylogenetic lineages, but also important differences. The combination of morphological and molecular approaches provides verification of phylogenetic hypotheses. Nevertheless, without morphological data, the molecular approach alone can produce major errors. Therefore, an integrative approach to taxonomy and phylogeny is essential.


Keywords: molecular approach, morphology, Tardigrada phylogeny, taxonomy
A new comprehensive phylogeny of the Tardigrada may alter the hypotheses for their colonisation of Antarctica

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Background: Internal phylogenetic relationships within the Tardigrada are poorly resolved, and the studies that have been undertaken in this area are generally restricted to certain groups within the phylum, as well as rarely including molecular analyses of divergence times. In this ongoing study, we infer the phylogeny of the phylum using 18S, 28S and CO1 sequences obtained from GenBank. To investigate the colonisation history of tardigrades in Antarctica, further newly-available sequences for Acutuncus antarcticus and Mesobiotus furciger for different geographical regions were added. Trees were created using Maximum Likelihood and Bayesian methods, with divergence times estimated using a relaxed clock model.

Results: Ongoing analyses suggest that the biogeographic history of A. antarcticus and M. furciger may be different to that previously proposed. Divergence times indicate that the origin of M. furciger is older than previously thought, with the Antarctic lineage of Mesobiotus diverging approximately 50 mya from other non-Antarctic lineages of Macrobiotidae, well before the geographical isolation of Antarctica. The data also indicate that within the species’ clade a significant radiation event took place approximately 10 mya, implying that M. furciger is in fact a species complex with at least three distinct haplotypes existing. Conversely, A. antarcticus is inferred to have a much more recent origin, rather than the previously hypothesised ancient Gondwanan relict status. With a divergence time from related Antarctic species of approximately 10 mya, our data identify little difference between different populations within the continent.

Conclusions: Preliminary data from this study challenge previous interpretations of the biogeography of these two tardigrade species in Antarctica. M. furciger is probably a species complex with ancient origins within the Antarctic continent, while A. antarcticus is a relatively younger species, even though it is thought to be endemic to the continent.

Keywords: Acutuncus antarcticus, Antarctica, divergence times, Mesobiotus furciger, phylogeny
Combined morphological and molecular phylogeny of *Echiniscus* C.A.S. Schultze, 1840

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**Background:** Since the times of early tardigradologists, such as Thulin or Murray, armoured echiniscid genera have been distinguished on the basis of dorsal plate morphology. However, more recently erected genera were defined using additional criteria, such as the presence of ventral plates or dorsolateral body appendages. Nevertheless, the largest polyphyletic tardigrade genus *Echiniscus* C.A.S. Schultze, 1840 has not undergone a thorough phylogenetic and taxonomic revision, despite that the polyphyletic character of the genus has been demonstrated several times.

**Results:** The aim of this research was to show that the genus *Echiniscus* comprises evident groups that are genetically and morphologically distinct, which justifies their elevation to the rank of genera. Phylogenies, both morphological and molecular, imply that the so-called “arctomys group” (consisting of species with only trunk cirri A) is paraphyletic with respect to the “cirrous” *Echiniscus s.s.* (with single possible cases of the secondary loss of trunk cirri). Species in both groups cluster within a number of uniform clades characterised by similar plate sculpturing and claw shape. Moreover, the Neotropical *bigranulatus* group appears as the sister group to all other *Echiniscus*-like taxa. Finally, a new genus is established for the *reticulatus* group, a clade characterised by apomorphic honeycomb-like dorsal ornamentation and ventral plates.

**Conclusions:** Increased taxon sampling allowed to partially resolve entangled phyletic affinities in the largest heterotardigrade genus. Future directions, regarding the phylogenetics of *Echiniscus*, are proposed, with an increased effort in obtaining rare and/or poorly examined morphological groups being the priority.

**Keywords:** cirri, Echinisciidae, Heterotardigrada, phylogeny, plate sculpturing
Analysis of non-morphometric morphological characters used in the taxonomy of the genus *Pseudechiniscus*

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**Background:** *Pseudechiniscus* is the second largest genus within the family Echiniscidae. After the separation of the genera *Multipseudechiniscus* and *Acantechiniscus* based on results of morphological and molecular analyses, species belonging to the former *suillus/conifer* complex are the only ones that remain within the nominal genus. Taxonomic work within *Pseudechiniscus* is complicated by the presence of a large number of poorly and inadequately described species and frequent absence of type material. Another problem is that *Pseudechiniscus* species are usually present in samples in low numbers, which impedes the parallel investigation of the morphology of the given population with different methods.

**Results:** During the investigation of moss samples collected in Croatia, a new species within the *Pseudechiniscus* genus, closely related to *Pseudechiniscus ramazzottii* was found. Relatively large numbers of specimens (more than 40) provided a possibility to investigate the morphology of the new species with Light Microscopy (LM) and Scanning Electron Microscopy (SEM) simultaneously. The following character complexes were analyzed: 1) cephalic and leg sensory structures, 2) dorsal cuticular plate arrangement and structure, 3) details of the cuticular sculpture. The investigation revealed that some statements, based on the LM observations, traditionally used in the descriptions of *Pseudechiniscus* species do not reflect the real morphology of structures, observed by SEM. I discuss the apparent indistinctness in traditional terminology used in the descriptions of *Pseudechiniscus* (and all Echiniscidae). This investigation revealed the presence of two different types of organization of cephalic papillae within the genus *Pseudechiniscus* and obvious sexual dimorphism of the ventral sculpture pattern.

**Conclusions:** Complex external morphology of Echiniscidae needs to be investigated using SEM in addition to LM methods to avoid misinterpretation of anatomical details, caused by the limitations of the observation of flattened specimens, usually fixed in the dorso-ventral position. The terminology used in the descriptions of the Echiniscidae cuticular plates should be corrected to exclude the misunderstanding and ambiguous interpretation. Several new characters, which could be useful for differentiation of similar species belonging to the genus *Pseudechiniscus* are supposed.

**Keywords:** Echiniscidae, Heterotardigrada, morphology, Scanning Electron Microscopy, taxonomy
A new interstitial species and genus of Echiniscoididae with unique anal system structures

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Background: Marine heterotardigrades of the family Echiniscoididae have gained increased interest in recent years due to their adaptations to marine tidal habitats, and their associated unique evolutionary position bridging marine and limno-terrestrial heterotardigrade taxa. The marine tidal genus Echiniscoides was established in the late 19th century by Plate, and for nearly 100 years it was constituted solely by Echiniscoides sigismundi (M. Schultze, 1865). We recently split Echiniscoides into Isoechiniscoides, comprising six clawed interstitial species, and Echiniscoides, which remains a ragbag of species complexes and undescribed genera. Currently many new species and genera of Echiniscoididae are in the process of description.

Results: Here we erect a new interstitial species and genus within the cryptic E. sigismundi species complex. The new genus includes the former E. pollocki from Rhode Island, USA, E. horningi from Macquarie Island, Antarctica and a new species from Roscoff, France. The genus is characterized by a unique anal complex constituted by wing-like, swollen structures associated the lateral valves of the anus. The dorsal cuticle of the new species from Roscoff is strongly sculptured, the epicuticle is without pillars and it has between 7 and 9 (most often 8) claws on all leg pairs. Interestingly, its movements are more similar to slowly moving eutardigrades than to other members of the Echiniscoididae. Females of the new species have two lateral pockets associated with the gonopore and a small plate anterior to the gonopore. A similar plate has been observed in females of E. horningi. Phylogenetic analysis based on COI sequences infers a close relationship between the new species from Roscoff, another undescribed species from Roscoff and undetermined species from Maine, USA.

Conclusions: We erect a new interstitial species from Roscoff, France and a new cosmopolitan genus within the Echiniscoides complex. Importantly, the diversity within Echiniscoides is of a magnitude that warrants erection of new species, genera and even higher taxon levels calling for an increased attention to this large group of tardigrades.

Keywords: Echiniscoides, DNA sequence data, Heterotardigrada, marine, morphology
Multilocus molecular phylogeny of the genus *Milnesium* Doyère, 1840

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**Background:** The largest apochelan genus, *Milnesium* Doyère, 1840, is continuously gaining more and more attention, with as many as half of the known species described only within the last decade. However, because of the exceedingly small number of taxonomically meaningful morphological and morphometric traits, the taxonomy of milnesiids is challenging. Moreover, our knowledge of the phylogeny of Milnesiidae, including both the relationships between species and genera, is strongly limited. In fact, apochelan species usually serve only as an outgroup for phylogenetic analyses of remaining tardigrade groups. Thus, the goal of this study was to reconstruct the first multilocus molecular phylogeny of the genus *Milnesium* based on four DNA markers (nuclear 18S rRNA, 28S rRNA, ITS-2 and mitochondrial COI) and propose a hypothesis on the evolution of morphological traits.

**Results:** We generated molecular phylogenetic trees based on concatenated nuclear and mitochondrial markers for more than twenty-five *Milnesium* species, collected throughout the World. Both Maximal likelihood and Bayesian Inference methods gave generally congruent results. The trees revealed distinct molecular lineages within the genus, but rather than by morphological characters, species clustered by climate zones. In fact, no obvious patterns of phenotypic traits could be observed, which might indicate high levels of convergent evolution in morphology throughout the genus.

**Conclusions:** The first multilocus molecular phylogeny of *Milnesium* provides an insight into relationships among species and lineages. The inferred phylogenetic relationships between the analysed species suggest adaptations to climate conditions and may render allegedly cosmopolitan distributions of particular *Milnesium* species unlikely. The lack of congruence between morphology and phylogeny does not allow splitting this largest apochelan genus into separate taxonomic units such as subgenera or genera. The addition of more *Milnesium* species, especially from poorly sampled regions, as well as sequencing the remaining milnesiid genera are hoped to elucidate the phylogenetic relationships within this unique group of exclusively carnivore tardigrades.

**Keywords:** Apochela, geographic distribution, Milnesiidae, phylogeny, trait evolution
Batillipes pennaki Marcus, 1946
(Arthrotardigrada: Batillipedidae): deciphering a species complex

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Background: Batillipes pennaki Marcus, 1946 is a common and apparently cosmopolitan marine tardigrade species. However, the original diagnosis of this species is very incomplete, and consequently, there is a high probability of incorrect records. Therefore, a comparative analysis of quantitative and qualitative morphological characters among eight different populations from the Atlantic basin was performed in this study to investigate the suspicion that B. pennaki is a complex of similar species, each with a more restricted distribution range.

Results: The result of a discriminant analysis showed morphometric differences among populations that were arranged into three main clusters, distinguishing Western Atlantic populations (S. Sebastián, Brazil, very near the type locality, Warm Temperate Southwestern Atlantic biogeographic province; Itamaracá, Brazil, Tropical Southwestern Atlantic biogeographic province; and South Carolina, U.S.A, Warm Temperate Northwest Atlantic biogeographic province); Eastern Atlantic populations (Tavira, Oliveirainha and Moledo in Portugal, and Galicia in Spain, all in the Lusitian biogeographic province), and a Mediterranean population (Turkey). Furthermore, the result of analyses of morphological structures revealed peculiarities of some traits with taxonomic relevance, such as the shape of the fourth leg sensory organs, primary clavae and toe discs, consistent with the three main clusters evidenced by quantitative data.

Conclusions: These results allowed us to distinguish three different species, supporting the hypothesis of a B. pennaki species complex. They are B. pennaki from the Western Atlantic coast and two new species that will be formally described elsewhere: a new Batillipes species from the Mediterranean Sea, and a new species from the Eastern Atlantic coast. The geographic morphometric variability exhibited by some populations within those species constitutes an additional argument in favour of a more diversified B. pennaki species complex. It is expected that, in the near future, a comprehensive evaluation of morphological characters used in the taxonomy of the genus Batillipes and deeper analysis of variability, including molecular data could detect further cryptic species within the B. pennaki complex.

Keywords: biogeography, cryptic species, Heterotardigrada, marine, morphology, taxonomy
Description of a new genus and species
(Eutardigrada, Richtersiidae) from Colombia, with discussion on Richtersiidae

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Background: Macrobiotus crenulatus Richters, 1904 is a species whose definition was very dubious until Binda [1] clarified it and designed new type material. We encountered specimens apparently attributable to M. crenulatus from Sierra Nevada de Santa Marta (Colombia), firstly reported as M. cf. crenulatus [2]. Subsequently, we noticed a peculiar character of the ventral lamina in both the Colombian and the new type material of the species. Observation of claw structure and shape, and reevaluation of other morphological characters, let us realize that the type material of the species and the Colombian specimens shared characters relevant at genus level.

Results: A genus will be described, attributed to the family Richtersiidae and characterized by an additional ventral ridge on the anterior portion of the ventral lamina, modified stylet furcae, claws similar to those of Adorybiotus, Richtersius, Diaforobiotus, and Tenuibiotus, and, in the known species, cuticular pores, two macroplacoids, large well dentate lunules and characteristic eggs. The Colombian specimens and the types of M. crenulatus are indeed distinguishable from each other, so we describe for the Colombian material a new species. In addition, observations on Tenuibiotus allow us to assign also it to the Richtersiidae and to make statements, from the morphological point of view, about possible phylogenetic relationships among the Richtersiidae genera and their position within Macrobiotidea.

Conclusions: In the last decades, many new genera have been described deriving from the historically old eutardigrade genus Macrobiotus. The present presentation gives a contribution to this process. Another important process in act is the distinction of sibling species, which in the past were considered a single, often cosmopolitan and rather variable, species. Future goals include barcoding of new taxa added to Richtersiidae, and clarifying in detail the structure of the ventral lamina in all Macrobiotidea, as started by Guidetti et al. [3].


Keywords: phylogeny, Sierra Nevada de Santa Marta, tardigrades, ventral lamina.
Integrative redescription of Paramacrobiotus (Amicrobiotus) areolatus (Murray, 1907) with notes on the phylogeny of the genus Paramacrobiotus Guidetti et al. (2009)

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Background: One of the most noticeable obstacles in modern taxonomy are outdated and incomplete descriptions of nominal taxa. They are often the main cause behind misidentifications and classifications of multiple morphologically similar taxa as a single species. This, in turn, may lead to underestimation of overall species diversity as well as to overestimation of geographic ranges of poorly described nominal species (such species are very often considered cosmopolitan). This process occurred also within a macrobiotid genus Paramacrobiotus, where two subgenera are recognised: Paramacrobiotus and Amicrobiotus. The nominal taxa for both subgenera, P. (P.) richtersi and P. (A.) areolatus, respectively, were allegedly recorded from all continents except the Antarctic. Here, we attempt to remove the key taxonomic obstacle within Amicrobiotus by integratively redescribing the nominal species for the subgenus. Moreover, we delineate a new Amicrobiotus species collected in Europe, which would be classified as P. (A.) areolatus without the redescription of the nominal species. Finally, we utilise new molecular data for both subgenera to test whether they indeed represent independent evolutionary lineages within the genus Paramacrobiotus.

Results: Integrative taxonomy approach allowed us to present a redescription of P. (A.) areolatus and a description of a related species from Europe. Both, the redescription and the description, are based on detailed morphological and morphometric data obtained with phase contrast light microscope and scanning electron microscope as well as on DNA sequences of three nuclear markers (18S rRNA, 28S rRNA, ITS-2) and a mitochondrial gene (COI). New DNA sequences, added to earlier Paramacrobiotus sequences, enabled a reconstruction of an updated molecular phylogeny of the genus.

Conclusions: By providing an integrative redescription of P. (A.) areolatus, we remove the taxonomic impediment and, by this, we open the door for confident delineation of Amicrobiotus species that are morphologically similar to the nominal taxon. Our results constitute a foundation for further systematic and phylogenetic research on the genus Paramacrobiotus, that will be able to develop fully when the redescription of P. (P.) richtersi, the nominal species for the other subgenus, is also available.

Key words: biodiversity, integrative taxonomy, molecular markers, phylogeny, species delineation
Is it time to change how we present morphology-based taxonomic information to make it more accessible?

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**Background:** Traditionally trained morphological taxonomists and systematists use dichotomous identification keys to both identify specimens and clarify different diagnoses. Experienced researchers can often jump to the most relevant key, or part of the key, when diagnosing an uncertain specimen. We also have the years of knowledge of what a particular character should look like, including its approximate size, shape, colour and texture for a specimen with certain size ranges. Whereas for someone new to either the taxonomic group, the locality, or the habitat type (marine vs freshwater vs terrestrial), the traditional dichotomous key can represent a number of challenges. For example, a specimen’s orientation on the microscope slide might obscure a specific characteristic, such as the apophysis for the insertion of the stylet muscle. Thus, forcing the inexperienced key user into making subjective guesses to try out various possible scenarios in an attempt to find a “reasonable” diagnosis. Can make the same information more accessible by switching from using dichotomous keys, to using data matrices?

**Results:** Working at different taxonomic levels across the phylum, I illustrate a range of data matrices, focusing on different groups of morphological characters, for example the characteristics of the buccal apparatus, claw-types, and cuticular appendices. Using some examples to highlight specific taxonomic issues.

**Conclusions:** The potential to construct and use these morphological character-sets goes beyond the simple taxonomic questions about what identification is my specimen? These datasets can be translated for combined analysis with molecular data. However, at the moment, whilst we can upload molecular sequences into international repositories like GenBank or BOLD; where and how should we code tardigrade morphological datasets? Certainly, one option could be Tardigrada.Net, where data relating to a new individual species can be upload. But what about larger datasets covering all the taxa of a genus, family or superfamily?

**Keywords:** molecular datasets, morphological datasets, systematic, Tardigrada
Tardigrades (Arthrotardigrada) from the manganese nodule area of the Eastern Equatorial Pacific

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**Background:** The first known deep-sea tardigrades were collected in the Indian Ocean by Thiel in 1966 and later described by Renaud-Mornant in 1974 as new family, Coronarctidae. Since then, less than 30 species have been described from abyssal and bathyal depths and the data about the worldwide distribution of deep-sea tardigrades are still very scarce. While the majority of deep-sea species have been found in mud, 3 species are associated with manganese nodules. Polymetallic/manganese nodules occur in high abundances on the sediment-covered abyssal plains of all oceans, where sedimentation rates are low (<10 mm per 1000 years). They are formed by the precipitation of mainly manganese oxides and iron oxihydroxides metals and grow at rates of 10-20 mm per million years. With a size range of 2-8 cm (max. 15 cm), the nodules are embedded in the sediment surface and provide a sub-seafloor habitat of hard substratum on abyssal plains otherwise dominated by soft sediment. Nodule surfaces are often covered with sessile organisms, but crevices are also inhabited by meiofauna. Several studies in the Clarion-Clipperton Fracture Zone have shown that tardigrades make up 1-2% of the nodule associated meiofauna. Only a single study of Bussau in 1992 has investigated the actual species composition of tardigrades, revealing one endemic genus *Moebjergarctus* and three endemic species (*Moebjergarctus manganis, Angursa capsula* and *Angursa lingua*) on cauliflower manganese nodules of the SE Pacific (DISCOL-project).

**Results:** In 2010, samples were collected from the German license area (the Peru Basin, SE Pacific) at 4000 – 4400 m depths revealing many tardigrade specimens. For a first insight, 22 specimens have been mounted on permanent microslides. So far, all the species are new to science and belong to the subfamily Euclavarctinae. To facilitate future investigations of the tardigrade fauna, further expeditions in 2012, 2013, 2014, 2015 and 2016 have collected manganese nodules in the eastern part of the German license area.

**Conclusions:** Manganese nodule crevices provide a sub-seafloor habitat on abyssal plains, which potentially host a unique faunal composition of tardigrades.

**Keywords:** abyssal and bathyal depths, cauliflower manganese nodules, Clarion-Clipperton Fracture Zone, endemic species of arthrotardigrades
Tethys elements among Japanese marine tardigrades

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**Background:** In the Mediterranean Sea, several marine caves are inhabited by very rare arthrotardigrades, such as *Actinarctus neretinus* Grimaldi de Zio et al., 1982; *Pseudostygarctus mirabilis* de Zio Grimaldi et al., 1998; and *Parastygarctus mediterranicus* Gallo D’Addabbo et al., 2001. These tardigrades should have survived the Messinian Salinity Crisis with the isolated marine caves serving as refugia. The report of *Actinarctus neretinus* from Australian marine caves, very distant from the Mediterranean Sea, suggested the presence of a relict Tethys fauna. Several specimens very similar to the above mentioned arthrotardigrades were also found in Shimabara Bay (Japan), which has the greatest tidal range in Japan. Here I will show three species of such tardigrades.

**Results:** Subtidal sediment samples consisting of shelly gravel were collected with a Smith-McIntire grab at about 15 m depth in Shimabara Bay, Japan. Among the arthrotardigrade specimens from these samples, *Actinarctus cf. neretinus* and *Pseudostygarctus mirabilis* were included. Japanese *Actinarctus* has slightly fewer wing pillars and longer clavae than those of *A. neretinus*. *Parastygarctus cf. mediterranicus*, found from a sandy beach near Aitsu Marine Station, shows strong resemblance with the Mediterranean species in shape of the head segment as well as the body plates with two pairs of lateral processes, but it has a different shape of the lateral process behind cirrus E.

**Conclusions:** At least three Japanese marine tardigrades show strong affinity with Mediterranean troglobionts, which might be Tethys-fauna relicts. Although their habitats in Shimabara Bay are in open water, the bay might represent a unique area that has preserved several old tardigrades from the Far East Tethys Sea.

**Keywords:** Arthrotardigrada, marine cave, Mediterranean, relicts, Shimabara Bay
Marine intertidal tardigrades along the length of the Chilean coast

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Background. Marine tardigrades in Chilean territorial waters have not been studied, and there are currently no published species descriptions from the area. Quantitative sediment samples were collected from intertidal and shallow subtidal sediments at 109 sites along the length of the Chilean coast from Arica in the north (18°S) to Puerto Williams in the south (54°S), including shallow-water samples from Easter Island (27°S).

Results. An analysis of the samples indicated the presence of 11 tardigrade genera: Angursa (1 site), Archechiniscus (1 site), Batillipes (9 sites), Chrysoarctus (4 sites), Dipodarctus (1 site), Echiniscoides (1 site), Mesostygarctus (1 site), Orzelicus (3 sites), Ramajendas (1 site), Stygarctus (3 sites) and Tanarctus (1 site). Of the 109 sites sampled, tardigrades were present at 25 of them. The most widely distributed genus was Batillipes which was found at various sites in both the north and south of Chile, on both exposed and protected shores. Qualitative samples collected at the sites also indicate that the surface quantitative sampling has not captured the true extent of tardigrade diversity and distributions. For example, qualitative samples taken at 50 cm deep in the sediments at Punta Choros revealed the abundant presence of Stygarctus, but quantitative samples taken from the sediment surface of this beach did not contain a single tardigrade. In addition, temporal variability in population sizes may also mask tardigrade diversity. For example monthly sampling at Coihuin in southern Chile has revealed considerable temporal variation in the population of Batillipes, with an average of 1.9 individuals per 50 ml of sediment in March 2014 increasing to 347.4 in September. Thus samples taken in September would have been more likely to capture the presence of tardigrades than samples taken in March.

Conclusions. This initial snapshot of the diversity and distributions of tardigrades in intertidal and shallow subtidal sediments indicates that there is considerable tardigrade diversity along the coast of Chile. However, qualitative observations indicate that the diversity may well be considerably higher than the 11 genera so far identified and both repeated sampling of the sandy beaches and of other intertidal and subtidal habitats will be necessary in order to obtain a true picture of marine tardigrade diversity in Chile.

Keywords: Batillipes, Chile, diversity, Easter Island, Stygarctus
An initial survey of marine tardigrades from Prince Edward Island, Canada

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Background: There are no records of marine tardigrades from the east coast of Canada. Hypothesizing this reflects a lack of sampling rather than a true absence of the phylum, an expedition was arranged in the summer of 2017. Prince Edward Island, Canada is famous for its sandy beaches which are common along three sides of the island making it an ideal location to pilot a systematic survey of marine intertidal tardigrades. Four beaches were selected along the north, east and southern shores of the Island (the west coast is mostly cliffs). On each beach, three transects of four samples were collected for meiofaunal extraction and sediment analysis. Meiofaunal phyla were recorded if present; tardigrades were mounted in PVA on slides for taxonomic identification. Sediment samples were dried. Subsamples were analyzed for organic and carbonate content by loss on ignition analysis. Each sample was also sieved for grain size analysis.

Results: Three species of marine tardigrade were found in the sandy beaches on Prince Edward Island. Batillipes mirus were found on the eastern shore at Basin Head. Batillipes tubernatis and Orzeliscus cf asiaticus were found on the southern shore at Argyle Provincial Park. All species were found in the lower intertidal zones in medium grained, well-sorted, strongly fine-skewed siliceous sand with little organic matter.

Conclusions: These are the first records of tardigrades from the east coast of Canada and Prince Edward Island and are the most northern records of these species in the Americas. Batillipes mirus has been documented throughout the eulittoral sediments and algae of the east coast of the United States suggesting that it is common and widely distributed. Batillipes tubernatis appears to be less common but still widely distributed as it has been recorded in both Massachusetts and Florida. The genus Orzeliscus has only recently been recorded from South Carolina, although it was discovered in Brazil. In 2017, a second species, Orzeliscus asiaticus, in this genus was described from Korea and Japan. The Prince Edward Island specimens most closely resemble the oriental species, despite the geographical separation. The presence of these three species in Prince Edward Island extends the known biogeographic range of each, and confirms that more sampling is needed obtain a meaningful picture of marine tardigrade biogeography.

Keywords: biogeography, ecology, Heterotardigrada, marine, morphology
Tardigrade diversity in shallow subtidal shell gravel sediment off the North West Iberian Peninsula

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Background: In the last years our knowledge about marine tardigrades from the Iberian Peninsula has increased significantly. However, almost all these recent studies were done on intertidal habitats. In order to improve our knowledge about the marine tardigrade biodiversity we have sampled several subtidal areas from the North West Iberian Peninsula. In this study we will analyse the results from three sites sampled at Ría de Ferrol.

Results: The sediment of the three study sites consisted of clean shell gravel (F12) or shell gravel with different amounts of silt and clay (sites F3 and F2). We found a total of 103 individuals. These individuals were distributed among 11 different species. Only two species were previously described, Actinarctus doryphorus Schulz, 1935 is reported for the first time in the waters surrounding the Iberian Peninsula and was present at F3 (16 individuals) and F12 (4 individuals) and Halechiniscus greveni Renaud-Mornant & Deroux, 1976 was present at F3 (15 individuals), F12 (2 individuals) and F2 (1 individual). Four new species were also found in significant numbers, Dipodarctus n. sp with 20 individuals in F3, Raiarctus n. sp with 10 individuals in F3, Batillipes n. sp. with 8 individuals in F3 and 1 in F12 and Styraconyx n. sp. with 7 individuals in F2 and 6 individuals in F3. Another 5 new species were represented only in F3 by two or a single animal, Tanarctus sp1 (1 individual) Tanarctus n. sp (2 adults, 7 larvae) Rhomboarctus n. sp (1 individual), Halechiniscus n. sp. (1 individual) and Styraconyxinae indet. (1 individual).

Conclusions: The study of shallow subtidal shell gravel sediment increased in 10 the number of tardigrade species recorded off the Iberian Peninsula, including several new species for science. All the species found were present at F3, 3 were present at F12 and only 2 in F2. These results suggest that shell gravel sediment mixed with silt and clay harbored a high diversity of marine tardigrades, while clean shell gravel (F2) harbored lower tardigrade diversity.

Keywords: Iberian Peninsula, new species, shallow subtidal zone, shell gravel
Do marine tardigrades follow Bergmann’s Rule?

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**Background:** Homeotherms and many poikilotherms display a positive relationship between body size and latitude, but this has rarely been investigated in microscopic animals. Do microscopic animals follow the same ecogeographic rules as macroscopic animals? We analyse a database on marine Tardigrada to address the generality of Bergmann's Rule for microscopic marine invertebrates.

**Results:** No latitudinal pattern could be detected for species richness at any spatial scale. Sampling bias (number of records) was the strongest and most significant correlate of species richness. A hump-shaped increase in mean and median body size with latitude was found at all spatial scales, and the effect was significant for the Northern Hemisphere but not for the Southern. The most significant effect supporting Bergmann's Rule was on minimum (but not maximum) body size, with smaller species disappearing at higher latitudes.

**Conclusions:** Biogeographic signals are difficult to detect in poorly studied groups due to swamping from biased sampling effort. This was true for our analysis of species richness; yet statistically significant latitudinal patterns were observed for body size consistent with Bergmann's Rule. In general, microorganisms are more likely to have cosmopolitan distributions than macroorganisms, so Bergmann's Rule should be uncommon for microscopic animals. This is the first evidence of latitudinal patterns in body size for an interspecific comparison of microinvertebrates. Ecologically relevant Net Primary Productivity (eNPP), suggested as a driver of Bergmann's Rule for other marine organisms, does not correlate with body size in marine tardigrades.

**Keywords:** body size-latitude relationships, ecogeographic rules, Everything is Everywhere, eNPP, Tardigrada
Tardigrades of Victoria Land (Antarctica)

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Background: Our capacity of predictions of future scenarios, in order to foresee the effects of human-mediated environmental change on Antarctic biodiversity and for successful conservation management, is limited by the low knowledge to the biota. To increase the knowledge of tardigrade community in one of the more affected Antarctic areas by human activities, we performed a latitudinal gradient survey (from 72° to 76° parallel) collecting different substrate types along the coastal, ice-free areas of Victoria Land.

Results: From the analyses of more than 120 substrates, tardigrades were absent from only 24% of samples (mainly lichens), testifying to the abundance of the phylum in Victoria Land. We identified 17 tardigrade species, six of which are new to science, but found no species specific relationship between species and kind of colonized substrates. Of the 24 species recorded for Victoria Land, 16 (66.7%) are endemic to this area. The results showed some species only found at one site, others occurred in more than one site, and a few occurred throughout the transect (e.g. *Acutuncus antarcticus*, *Milnesium antarcticum*, *Minibiotus vinciguerrae*). The geographic distribution of the species was unrelated to the two biogeographic areas identified in Victoria Land. *Diphascon sanae* was present in several areas of Victoria Land. The absence of a drop-like thickening in this species lead us to perform a phylogenetic study to find its place within the Hypsibiidae lineage. The results confirmed its belonging to the *Diphascon* genus.

Conclusions: These data increase our knowledge on the biodiversity in Victoria Land, which must be considered a diversity hotspot for Antarctic tardigrades, and the number of endemic tardigrade species reported from Antarctica which deserve to be protected. The escalating reports of new tardigrade species from Victoria Land and all around Antarctica testifies that this continent does not represent extreme or inhospitable habitats to the tardigrades, but is a well colonized and exploitable environment to which tardigrades are well adapted. Our results finally suggest that the large-scale distribution of terrestrial tardigrades is probably determined more by geo-glaciological events and the presence of past refugia rather than latitudinal variations in current climatic and environmental conditions. This work was supported by the grant PNRA16_00234 to LR.

Keywords: biodiversity, biogeography, endemism, refugia
Identification of tardigrades from Half Moon Island, Antarctic Peninsula

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Background: Tardigrades are 8 legged animals less than 1 mm in size that are capable of tolerating extreme environments. A broad variety of species are distributed worldwide, from deserts to high altitudes, jungles and oceans, and as far as Antarctica. Antarctic tardigrades have been studied since the beginning of the 20th century with increasing intensity since the 90s. In 2005, Convey and McInnes [1] reported tardigrade species on 10 of the 11 major islands of the Antarctic Peninsula, and including previous reports, around 55 species have been reported in the Antarctic region. The Antarctic Peninsula has a higher variety of tardigrade species than continental Antarctica and the peninsula was therefore selected as target for this project. Also, Velasco, et al. 2015 [2] indicated through molecular analysis that there is greater diversity of tardigrade species in Antarctica than was previously thought.

Results: The study of the Antarctic tardigrades started with the 2nd Colombian Antarctic Expedition (summer 2015-2016) collecting samples on Half Moon Island. The analysis of 10 samples of moss distributed along the whole island has allowed the identification of 3 main tardigrade families Hypsibiidae, Macrobiotidae, and Echiniscidae. Among these, we have identified the genera Diphascon, Hypsibius, Macrobiotus, and Echiniscus. Some of the confirmed species have been previously reported on the Antarctic continent, but this is the first report in Half Moon Island for Diphascon victoriae, Hypsibius conwentzii, Hypsibius dujardini, and Diphascon rudnicki. Five other species are in the process of confirmation.

Conclusions: We have found a variety of tardigrade species on the hills of Half Moon Island, but also Antarctic hair grass and climate change is facilitating their expansion [3]. The ecosystem is changing, and more alien species are colonizing Antarctica brought by tourists and scientists visiting every year. For these reasons identification of tardigrade species and their distribution among the islands or the continent is important. There are endemic tardigrade species in this area and these changes might have an effect on their population.


Keywords: Antarctica, Half Moon Island, identification, tardigrade
Tardigrades from moss and lichens in the Great Smoky Mountains National Park (Tennessee and North Carolina, USA)

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Background: A large-scale, multihabitat inventory of tardigrades in the Great Smoky Mountains National Park (GSMNP) was conducted as part of the All Taxa Biodiversity Inventory (ATBI). Our current tardigrade database consists of 780 samples, which yielded 15,618 specimens identified to 81 species, with at least 14 species new to science. In the present study we compared tardigrade species composition in our moss and lichen samples and analysed environmental correlates of tardigrade community structure. The basic dataset for this study consisted of moss and lichen samples from trees at 19 ATBI plots: moss at breast height, moss at ground level, lichen at breast height and lichen at ground level. Supplemental collections from rocks included: six samples of lichens from granite and six from dolomitic limestone; seven samples of mosses from sandstone, nine samples from Anakeesta rock formations, and one from dolomitic limestone rock.

Results: The basic dataset from tree moss and lichens consisted of 302 samples and 9749 individuals identified to 46 species. The supplemental dataset from rock moss and lichens consisted of 29 samples and 811 individuals identified to 41 species. Collectively, there was a total of 57 species present in moss and lichens. Sampling effort was much lower on rocks versus trees and many species are quite rare; however, 11 species were found uniquely on rock habitats. Two-way ANOVAs for species richness, abundance, Shannon’s diversity index, and evenness showed no significant differences between substrate (moss versus lichen) or height (ground level vs breast height); however, there were significant differences between ATBI plots. Partition tree analyses found that temperature range and land cover type explained the differences in tardigrade abundance between sites; canopy cover was the most important variable affecting diversity; and evenness was impacted most by land cover type.

Conclusions: Tardigrade communities were not significantly different between mosses and lichens. However, 17 environmental variables affected tardigrade community structure in the plots, including temperature range, land cover type, and canopy cover.

Keywords: ATBI, partition tree analysis, species diversity, tardigrade community structure
Seasonal variation in the distribution and abundance of Tardigrada on Eymir Lake and Çubuk 2 Dam, Turkey

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**Background:** This study was carried out in order to investigate and compare the seasonal species composition and diversity of the semi-terrestrial Tardigrada in two areas: Eymir Lake and Çubuk 2 Dam. Samples of moss, lichen and leaf litter were taken at four locations in Eymir Lake area and four locations in Çubuk 2 Dam area. In each area, the locations were sampled during four seasons, adding up to a total of 593 samples for Eymir Lake area and 627 samples for Çubuk 2 Dam area.

**Results:** A total of 1220 specimens and 125 eggs were identified to 26 species (20 in Eymir Lake area and 24 in Çubuk 2 Dam area). The species *Ramazzottius anomalus*, *Macrobiotus recens* and *Hypsibius dujardini* are new records for Turkey. The distribution of some cosmopolitan species (*Hypsibius dujardini*, *M. hufelandi hufelandi*, *Hypsibius convergens*, *Ramazzottius oberhaeuseri* and *Echiniscus testudo*) were found to be in agreement with the literature. The qualitative composition of the other species in the two investigated areas remains stable in general, even though fluctuations were observed throughout the entire period. However, differences were observed in the abundance of species obtained from different habitats in the same area.

**Conclusions:** It is thought that the increase or decrease of population density or changes in the presence of particular tardigrade species according to seasons, are due to special ecological requirements. Although the survival requirements of each species remains a mystery for now, these kinds of studies will shed light on the ecology of tardigrades in the future.

**Keywords:** new record, seasonal variation, Tardigrada, taxonomy, Turkey
Checklist of Tardigrada in northern Tunisia

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Background: The current knowledge of the diversity and distribution of tardigrade species in northern Africa is still scarce. In this study we provide an update for faunistic records of tardigrades from various regions of northern Tunisia.

Results: In the present study, 232 specimens belonging to thirteen different species of tardigrades were isolated from samples of mosses and lichens: 202 specimens from Feyja, belonging to the following nine-species: Macrobiotus sp, (hufelandi group), Macrobiotus furcatus Ehrenberg, 1859, Paramacrobiotus sp, (richtersi group), Minibiotus sp, Hypsibius convergens Urbanowicz, 1925, Ramazzottius oberhaeuseri Doyère, 1840, Milnesium sp, Echiniscus blumi Richters, 1903, Isohypsibius prosostomus Thulin, 1928; 20 specimens found in Hawaria representing the following five species: Macrobiotus sp, (hufelandi group), Paramacrobiotus sp, (richtersi group); 12 specimens collected around the Faculty of Science of Tunis, belonging to two species: Paramacrobiotus sp, (richtersi group) and Macrobiotus sp, (hufelandi group). In total, two classes (Heterotardigrada and Eutardigrada), three orders (Echiniscoidea, Apochela and Parachela), six families (Echiniscidae, Milnesiidae, Macrobiotidae, Hypsibiidae, Ramazzottiidae and Isohypsibiidae) and eight genera were represented.

Conclusions: The total number of specimens found in the Feyja site, represent 60% of the specimens collected in total in the various sampling sites in northern Tunisia. This result evidences the quality of the moss characterizing this sub-humid zone, evidently a favorable habitat for tardigrades. In total, this study led to the identification of four new species for Tunisian fauna: two are based on molecular and morphological analysis (Marcobiotus sp, hufelandi group, probably a new species - R. oberhaeuseri) and two are based on morphological analysis only (M. furcatus - I. prosostomus).

Keywords: diversity, molecular analysis, morphological analysis, Tardigrada, Tunisian fauna
The structure of tardigrade communities at fine spatial scales in an Andean *Polylepis* forest

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**Background:** The biogeography of tardigrades is poorly understood, but tardigrades do provide a tractable phylum for understanding distribution patterns of micrometazoans at a variety of scales. We are exploring this potential in high-altitude Andean forests, which occur from Venezuela to Argentina. Before making comparisons at landscape, regional and continental scales, it is necessary to examine variation at fine scales. To achieve this, we sampled intensively and with replication in a single forest in Ecuador. Tardigrades were identified to operational taxonomic units and spatial patterns of abundance established at fine scales within and across host bryophytes. The aim was to determine the patterns of spatial variation at this scale, and to inform suitable sampling strategies for comparing tardigrade composition and abundance among a range of sites at coarser spatial scales.

**Results:** Some new species were discovered during this work; one is introduced briefly here. Some species were common, both in abundance and occupancy, but others were rare and restricted to a single sample. Rarefaction curves suggest that samples of at least 50 samples would be needed to characterize tardigrade diversity in this forest. There were differences in composition according to host bryophyte (perhaps related to physical structure and chemistry). Distance between samples did not explain differences in composition.

**Conclusions:** The sparsity of some taxa and the variability in numbers within samples means that large numbers of samples, across a range of host bryophytes, would be needed to make reliable comparisons between different forests. It is not yet clear whether this finding is applicable to a wider range of habitats and ecosystems. Such studies are a vital step before broader geographical comparisons can be made.

**Keywords:** biogeography, bryophyte hosts, diversity, forest
DNA barcoding and tardigrade species diversity

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Background: Even though DNA barcoding is a standard research tool in many animal groups, it is limitedly implemented in tardigrade taxonomy. One of the reasons may be that DNA sequencing is still expensive and obtaining quality genomic DNA from microscopic invertebrates is sometimes challenging. Moreover, the most popular barcode, the mitochondrial cytochrome oxidase subunit I (COI), is very often difficult to amplify and some of its biological properties limit the taxonomic and phylogenetic inference (e.g. uniparental inheritance, lack of recombination, nuclear pseudogenes). Another barcode, the nuclear ribosomal ITS-2 region, is increasingly utilised both in tardigrade species delineation and phylogeny. However, the performance of the two barcodes in tardigrade taxonomy has never been systematically compared.

Results: In this study, we compare classical and molecular estimates of species diversity in three eutardigrade groups: in genera Milnesium and Paramacrobiotus, and in the Macrobiotus hufelandi group. Moreover, we compare success rates of PCR amplification in both COI and ITS-2 as well as the performance of the two markers in phylogeny reconstruction and species delineation.

Conclusions: The percentage of tardigrade species associated with type or neotype DNA sequences is extremely low and multiple COI and/or ITS-2 haplotypes are known only for a handful of species. Thus, in order to facilitate the development and modernisation of tardigrade taxonomy, a much greater effort has to be made to obtain both nuclear and mitochondrial barcodes for as many tardigrade species as possible (with nominal taxa being the priority). A rich barcode library associated with phenotypic data not only will facilitate species identification and discovery, but it will also help to better understand tardigrade phylogeny, distribution and ecology. Moreover, sequences for multiple populations of known species are needed to estimate ranges of intra- and inter-specific variation that are ultimately necessary to estimate genetic distance thresholds for species delineation. In parallel, we should aim at collecting morphometric data based on proper sample sizes as the number of recognised pseudocryptic species, that are identifiable phenotypically only via statistical testing, is likely to increase thanks to DNA sequencing. Last but not least, new tardigrade-specific primers for standard barcodes and the development of primers for new reliable DNA markers are hoped to facilitate the implementation of the integrative taxonomy framework in tardigrade taxonomy.

Keywords: barcode gap, Macrobiotus hufelandi group, Milnesium, Paramacrobiotus, species delineation
Microbiome of tardigrades

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Background: Symbiotic associations of metazoans with bacteria are ubiquitous and virtually no animal is axenic. The bacterial community associated with animals (the microbiome) influences almost every aspect of their host biology. Unveiling the composition of tardigrade microbiomes and characterize potential symbionts could provide new insight into tardigrade biology. Here, we present the characterization of the microbiome from wild and cultured tardigrades from six species along with the molecular characterization of new putative tardigrade symbiotic bacteria.

Results: The microbiomes of six limnoterrestrial tardigrade species (Echiniscus trisetosus, Acutuncus antarcticus, Ramazzottius oberhaeuseri, Richtersius coronifer, Macrobiotus macrocalix, Paramacrobiotus areolatus) in tandem with the microbiomes of their respective substrates through the 16S metabarcoding approach were studied. The experimental design enabled us: to define the microbial community of the same tardigrade species sampled from different environment and the bacterial communities of different tardigrade species from the same environment, and to determine the effects of both the environment and the host (tardigrade) genetic background on the tardigrade microbiome. Our 16S rRNA gene amplicon approach indicated that the tardigrade microbiome is species-specific and well differentiated from the environment. Tardigrade species showed a much lower microbial diversity compared to their substrates, with only one significant exception (Acutuncus antarcticus). Four bacteria taxa representing most probably three new genera belonging to the orders Holosporales and Rickettsiales (that comprise only endosymbiotic bacteria) were identified and characterized. These bacteria are good candidates as tardigrades endosymbionts. The infection prevalence of these bacteria in the examined tardigrade populations was low (from 10% to 40% according to the symbiont and tardigrade species) suggesting that these bacterial symbionts are not essential for tardigrade survival and reproduction, but can anyway be beneficial, commensals or pathogens. Additionally, Fluorescent In Situ Hybridization (FISH) protocol allowed to identify bacteria in the ovary of one of the analyzed tardigrade species (E. trisetosus). Conclusion: The work presented here proved that, as other animal phyla, also tardigrades harbor a defined microbiome community and endosymbiotic bacteria. These bacteria communities are high and diversified with a species-specific association. They could play a major role in tardigrades feeding, reproduction and stress resistance.

Keywords: endosymbiont, Holosporales, metabarcoding, microbiome, Rickettsiales, Tardigrada
Tardigrade dietary preferences and diet effects on tardigrade life history traits

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\textbf{Background:} Despite the ubiquity of tardigrades in various ecosystems throughout the world, the basic knowledge of their biology, such as dietary preferences, is very limited. Surveys on tardigrade diet are scarce and usually focused on one type of food. Here, we show results of two laboratory experiments that investigated relationships between buccal apparatus anatomy and tardigrade dietary preferences. In the first experiment, we tested three eutardigrade species with different types of buccopharyngeal apparatuses for differences in their diet. Tardigrades were offered 18 foods that represent various organisms (cyanobacteria, algae, fungi, plants, animals) and were subsequently observed for ingestion. Moreover, we tested how different foods affect survival and fecundity of the three eutardigrade species. In the second experiment, we tested whether buccopharyngeal apparatus anatomy in nine eutardigrade species predicts preferences for different types of animal prey.

\textbf{Results:} In the first experiment, \textit{Hypsibius exemplaris}, classified as herbivore, fed on cyanobacteria, algae and fungi. Survival and reproductive success of \textit{Hypsibius} did not only differ among cyanobacteria, algae and fungi, but also between different species of algae and fungi. \textit{Milnesium cf. alpigenum}, a presumed carnivore, apart from animal prey, ingested also algal and fungal cells. However, although a purely algal diet extended \textit{Milnesium} lifespan compared to starved control, the species reproduced only on animal prey, which confirmed its carnivorous status. \textit{Paramacrobiotus cf. richtersi}, a supposed omnivore, fed on cyanobacteria, algae, fungi and animal prey, but it achieved highest reproductive rates when it was given access to animal prey. The second experiment indicated that most tardigrade species avoided feeding on tardigrades. However, individuals with longer buccal tubes foraged on tardigrade prey more willingly than those with shorter buccal tubes. Both, traits on buccopharyngeal apparatus and identity of tardigrade species explained tardigrade dietary preferences with similar statistical power.

\textbf{Conclusions:} Our results support earlier observations that buccopharyngeal apparatus anatomy is associated with general dietary preferences (carnivory, herbivory, omnivory) as well as preferences between different types of animal prey. But, in contrast to previous studies, our experiment showed that all tardigrade species have similar preferences that differ only in relative amount of eaten prey.

\textbf{Keywords:} diet, feeding traits, LHT, survival, Tardigrada
Tardigrade community response to nitrogen and phosphorous addition

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**Background:** Tardigrades prey on microbial communities that are likely limited by nutrient availability. However, no studies to date have examined the effect of nutrient manipulations on tardigrade communities. Here, we assess the response of tardigrade communities to fertilization with nitrogen and phosphorus across nine northern hardwood forest stands of three age classes: young, mid-aged, and mature. In August 2017, we collected 108 samples of moss from rock surfaces in the Bartlett Experimental Forest in the White Mountains of New Hampshire, USA, where research plots had been fertilized annually since 2011 with 30 kg N/ha/yr as NH₄NO₃, 10 kg P/ha/yr as NaH₂PO₄, a combined N+P treatment at the same rates, and a control with no fertilizer added.

**Results:** Tardigrade abundance increased in both N and NP treated plots ($p = 0.05$) and had marginally different community composition ($p = 0.07$). Additionally, moss in these N-treated plots had 28% more tardigrades compared to plots not amended with N.

**Conclusions:** Tardigrade communities may respond to nutrient additions due to changes in their food source or changes in other factors affecting their population dynamics, such as migration, fecundity, longevity, or mortality. This impact on tardigrade abundance may have cascading effects throughout forest ecosystem food webs.

**Keywords:** element limitation, nitrogen, morphology, phosphorus, tardigrade ecology
Predator and prey interaction in
*Milnesium lagniappe* and *Macrobiotus acadianus*

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**Background:** The behavior of water bears has only rarely been studied; the few investigations that have been conducted have focused primarily on the response of tardigrades to environmental conditions. Predator-prey interactions have received some attention, but not how predators and prey might detect one another. It is possible, but unlikely, that vision is important in hunting prey or avoiding predation; detection of other animals more likely involves physical contact or olfaction. Using an experimental setup inspired by Clark Beasley’s experiments, we investigated whether a predatory tardigrade species is attracted to, and a potential prey tardigrade avoids, areas previously occupied by the other.

**Results:** We used two species of tardigrade commonly found in southwestern Louisiana, USA. *Milnesium lagniappe* is a large tardigrade that preys on nematodes, rotifers, and small tardigrades. Among these potential prey items is the small water bear *Macrobiotus acadianus*. Petri dishes with non-nutrient agar were used as experimental chambers. To test whether the distribution of *M. lagniappe* was independent of the presence of *M. acadianus*, in one treatment we allowed 21 *M. acadianus* to roam over half of the agar for 20 hours, while leaving the other half free of *M. acadianus*. The mean speed at which tardigrades moved over the agar was sufficient for the animals to travel the length of the available agar surface many times in 20 hours. We then removed *M. acadianus*, and added 24 *M. lagniappe*. In the other treatment no *M. acadianus* were added. Petri dishes were kept in the dark. Results indicated that *M. lagniappe* were significantly concentrated in the area previously occupied by *M. acadianus*, while no such concentration was evident when *M. acadianus* had not been present (Fisher’s exact test; *p*=0.0145). A similar protocol was used to test whether *M. acadianus* avoided areas previously occupied by *M. lagniappe*. *Macrobiotus acadianus* were significantly concentrated in the area never occupied by *M. lagniappe*, unlike in the control (Fisher’s exact test, *p*=0.0251).

**Conclusions:** Given that the experiments were conducted in darkness, these results suggest that both species can detect the other without physical contact and react accordingly. Detection is probably olfactory.

**Keywords:** *Macrobiotus acadianus, Milnesium lagniappe*, predator-prey interaction
Comparison of sexual reproductive behaviors in two species of Macrobiotidae

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Background: In tardigrades, several reproductive modes are known, including sexual reproduction. However, mating behaviors remain only scarcely observed in most species, or entirely unknown especially in freely ovipositing tardigrades. In this study, we used two sexually reproducing tardigrade species that lay eggs freely, Paramacrobiotus richtersi TYO strain and Macrobiotus shonaicus to investigate and compare courtship, mating, and chromosomal behaviors.

Results: Courtship behaviors were observed and recorded in both species, during which an attracted male pursued a female. The entire mating sequence, including the courtship behaviors, was observed and can be categorized into six discrete steps common to both species: (1) Tracking: a male tracks and orientates toward a female; (2) Touching: the male touches the cloaca of the female; (3) Entering: the male enters the female’s ventral side; (4) Preparing: the female stops moving to prepare for male ejaculation; (5) Ejaculation: the male curls his caudal part and ejaculates into female cloaca from close range; (6) Squeezing: the female squeezes her ventral side after mating to capture the sperms. Some notable differences, however, were observed in the behaviors between the two species; e.g., during ejaculation, the females of P. richtersi arrested their motion, whereas there was a warping of the caudal part in M. shonaicus females. Ejaculated sperms were deposited in the external environment in close proximity of the cloaca. First ovipositions were observed at 40 min in P. richtersi, and a few days after mating in M. shonaicus, respectively. In both species, oocyte maturation was arrested in the metaphase I until oviposition. Immediately after oviposition, meiosis progressed, and sperm attached to the interior of the chorions.

Conclusions: We recorded distinct courtship and mating behaviors in two tardigrade species that lay eggs freely, i.e. P. richtersi TYO strain and M. shonaicus with noticeable differences in observed behaviors. Chromosomal behaviors suggest that oocytes, which had been arrested in the metaphase I restarted meiosis after sperm attachment.

Keywords: chromosomal analyses, Macrobiotus shonaicus, mating behavior, Paramacrobiotus richtersi, sexual reproduction
Phototactic behaviour in the eutardigrade *Hypsibius exemplaris*

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**Background:** An insufficiently investigated aspect of tardigrade vision is their light-induced behaviour, including phototaxis. Moreover the few available studies come to partly opposing conclusions, for example positively and negatively phototactic behaviour or even no reaction to light at all, depending on the investigated species. The light-induced behaviour of tardigrades is even more complex, as developmental aspects seem to influence their phototactic behaviour as well. Regarding these contradictory results it is possible that those reactions could be partly a result of the respective experimental design and artificial conditions, especially concerning the light source and presentation. Our aims were to design an appropriate experimental setup to test tardigrades on their light-induced behaviour and to get the first results on the phototactic behaviour of adult specimens of the eutardigrade *Hypsibius dujardini*.

**Results:** We were able to build an experimental setup that is suitable for observing the locomotion of individual tardigrade specimens in a rectangular arena (15 × 15 mm). It was possible to illuminate defined areas of the arena by an LED that emits white light and has an adjustable emission intensity. The analysis of the resulting videos allowed for a semiautomatic tracking of individual animals. For the investigation of phototactic behaviour in *H. dujardini*, one half of the arena was illuminated at different light intensities while the other half was left unilluminated. Statistical analysis of respective experiments with *H. dujardini* indicates that phototactic behaviour is positive at low light intensities, less significant at increasing light intensities, and absent at high light intensities.

**Conclusions:** Since *H. dujardini* lives in freshwater habitats and feeds on algae, the positive phototaxis could be linked to the higher chance of finding algae in areas exposed to the sun. In contrast, extreme sun exposure could involve the risk of desiccation. These results supplement the existent data on light-induced behaviour in tardigrades, which will shed light on the general situation in tardigrades. The behavioural aspect of photoreception will complement molecular and anatomical findings on vision in tardigrades.

**Keywords:** behaviour, phototaxis, vision
Launching out into structural genomics on anhydrobiotic tardigrades

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**Background:** Several research groups have recently started genomics and molecular biological studies to unveil the molecular basis for anhydrobiosis of tardigrades. Some proteins discovered in these studies are thought to be keys to eccentricity of tardigrades since they show low or no identity to known proteins found in phyla other than Tardigrada. Because their functions are yet to be investigated in detail, we have performed structural analysis on such proteins unique to tardigrades.

**Results:** We have tried crystallization of SAHS proteins from *Ramazzottius varieornatus* (*Rv*SAHS1, *Rv*SAHS4, and *Rv*SAHS13) and determined high resolution crystal structures of *Rv*SAHS1 and *Rv*SAHS4. Crystal structures revealed that SAHS proteins have a β-barrel structure resembling fatty acid binding proteins and two putative ligand binding sites (LBS1 and LBS2). Comparing *Rv*SAHS1 and *Rv*SAHS4 suggests that they are optimized for different functions. While *Rv*SAHS1 seemed to use both LBS1 and LBS2 to capture compounds having carboxyl groups such as fatty acid and heme, *Rv*SAHS4 appeared to mainly use LBS2 to interact with neutral molecules such as alcohol and aldehyde. As the next target, we focused on heme proteins that have essential roles in almost all living systems. We found a structural gene of a myoglobin-like protein in the genome of *R. varieornatus* and designated it as Kumamushi globin (Kgb). Spectroscopic and crystallographic analysis on Kgb showed that it is a hexacoordinated globin protein with an unprecedented coordination structure. We hypothesized that Kgb is an electron transferring protein or an enzyme involved in generation of reactive oxygen species rather than an oxygen transporting/storing protein. We also determined structures of an iron-containing protein, superoxide dismutase, and other several proteins. We will provide their structures and would like to discuss their functions in the symposium.

**Conclusions:** We have achieved structural determination of tardigrade proteins. Our results indicate that X-ray crystallography is a powerful technique to study tardigrade at molecular levels and tardigrades are good targets for structural genomics.

**Keywords:** crystal structure, metalloprotein, *Ramazzottius varieornatus*, structural genomics, SAHS protein
Comparative genomics of the tardigrades *Hypsibius dujardini* and *Ramazzottius varieornatus*

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**Background:** Tardigrada, a phylum of meiofaunal organisms, have been at the center of discussions of the evolution of Metazoa, the biology of survival in extreme environments, and the role of horizontal gene transfer in animal evolution. Tardigrada are placed as sisters to Arthropoda and Onychophora (velvet worms) in the superphylum Panarthropoda by morphological analyses, but many molecular phylogenies fail to recover this relationship. This tension between molecular and morphological understanding may be very revealing of the mode and patterns of evolution of major groups. Limnoterrestrial tardigrades display extreme cryptobiotic abilities, including anhydrobiosis and cryobiosis, as do bdelloid rotifers, nematodes, and other animals of the water film. These extremophile behaviors challenge understanding of normal, aqueous physiology: how does a multicellular organism avoid lethal cellular collapse in the absence of liquid water? Meiofaunal species have been reported to have elevated levels of horizontal gene transfer (HGT) events, but how important this is in evolution, and particularly in the evolution of extremophile physiology, is unclear. To address these questions, we resequenced and reassembled the genome of *H. dujardini*, a limno terrestrial tardigrade that can undergo anhydrobiosis only after extensive pre-exposure to drying conditions, and compared it to the genome of *R. varieornatus*, a related species with tolerance to rapid desiccation. **Results:** The two species had contrasting gene expression responses to anhydrobiosis, with major transcriptional change in *H. dujardini* but limited regulation in *R. varieornatus*. We identified few horizontally transferred genes, but some of these were shown to be involved in entry into anhydrobiosis. Whole-genome molecular phylogenies supported a Tardigrada+Nematoda relationship over Tardigrada+Arthropoda, but rare genomic changes tended to support Tardigrada+Arthropoda. **Conclusions:** These findings will serve to be a platform for future analysis on anhydrobiosis and various aspects of molecular tardigradology.

**Keywords:** anhydrobiosis, comparative genomics, horizontal gene transfer tardigrade, transcriptomic analysis
Tardigrades differ from other panarthropods in their antibacterial gene repertoire

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Background: Tardigrades are exposed to multiple biotic stresses, such as fungal and ciliate parasites, and internal microbiomes. However, the mechanisms on how tardigrades fight these stresses are unknown. Determining the tardigrade immune system and how it works could shed light in understanding tardigrade biotic stress responses. The Toll pathway is the best-studied and most common antibacterial pathway used by \textit{Drosophila melanogaster} and humans. Most of the \textit{D. melanogaster} Toll pathway genes are conserved in arthropods. The Imd and Jnk pathways are also involved in \textit{D. melanogaster} antibacterial response. \textit{Caenorhabditis elegans}, on the other hand, uses a completely different antibacterial pathway from arthropods. Given the close relationship of tardigrades to these two model organisms, it is not intuitive whether tardigrades possess an immune system similar to arthropods or nematodes. In this study, comparative genomic analysis was done to mine homologs of canonical \textit{D. melanogaster} and \textit{C. elegans} immune genes from six tardigrades (\textit{Echiniscus testudo}, \textit{Milnesium tardigradum}, \textit{Hypsibius dujardini}, \textit{Ramazzottius varieornatus}, \textit{Paramacrobiotus richtersi}, \textit{Mesobiotus philippinicus}), and three non-arthropod ecdysozoans (Onycophora: \textit{Epiperipatus} sp., Nematomorpha: \textit{Paragordius varius}, Priapulida: \textit{Priapulus caudatus}) to give insights on the tardigrade antibacterial system. Results: Analyses showed that homologs of all the intracellular components of the Toll pathway were not detected in any of the six tardigrades while \textit{Epiperipatus} has homologs of most of the Toll pathway genes. Interestingly, \textit{Paragordius varius} and \textit{Priapulus caudatus} have homologs of some intracellular Toll pathway components. Homologs of most of the Imd pathway genes were not detected in tardigrades, nor in the other ecdysozoans. The Jnk pathway, on the other hand, is conserved in all ecdysozoans. Analyses also showed that homologs of the \textit{C. elegans} antibacterial pathway were not only present in tardigrades but also in the other ecdysozoans. However, as in \textit{C. elegans}, only the tardigrades showed undetectable homologs of the dimerizing transcription factor NFκB, which is the major activator of antimicrobial response gene expression. Conclusion: Overall results showed that tardigrades have a different immune gene repertoire from other panarthropods indicating a possible presence of a unique tardigrade antibacterial response. Results also provide insights on how immune pathways could vary and evolve between different ecdysozoan groups.

Keywords: biotic stress response, ecdysozoan immunity, tardigrade immunity
Transcriptomes from *Echiniscoides sigismundi* and *Richtersius coronifer* provide new insights into lineage specific gene content

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**Background:** Tardigrades (water bears) are microscopic aquatic animals found worldwide in a range of habitats. They are renowned for their cryptobiotic abilities, which include tolerance towards desiccation, freezing, severe osmotic stress and possibly environmental toxicants. Cryptobiosis, defined by a reversible shut-down of metabolism, is found among a range of life-forms. Within Animalia, species within Nematoda, Rotifera, Tardigrada and Arthropoda, exhibit cryptobiotic capabilities. **Results:** We provide two new tardigrade transcriptomes, i.e. the first transcriptome from the marine tidal heterotardigrade *Echiniscoides sigismundi*, which holds a unique evolutionary position within the phylum Tardigrada, as well as a transcriptome from *Richtersius coronifer* representing the first transcriptome within the eutardigrade family Richtersiidae. We compare the two new transcriptomes with available eutardigrade genomic data (from *Hypsibius dujardini* and *Ramazzottius varieornatus*) and also with data from six model organisms spanning a wide spectrum of evolutionary lineages (*Drosophila melanogaster*, *Caenorhabditis elegans*, *Xenopus tropicalis*, *Danio rerio*, *Homo sapiens* and *Saccharomyces cerevisiae*). The overall comparison between the tardigrades and the listed model organisms reveal that tardigrades encode more genes in several COG based functional categories (e.g. post-translational modification, protein turnover and chaperones, defense mechanisms, translation, ribosomal structure and biogenesis, intracellular trafficking, secretion and vesicular transport, energy production and conversion, inorganic ion transport and metabolism, secondary metabolites biosynthesis, transport and catabolism). Investigating 107 gene families, our study further provides a thorough analysis of tardigrade gene content with focus on stress tolerance. Our results reveal both gene expansions and losses that to some extent are lineage specific within the phylum Tardigrada. **Conclusions:** Our results show common gene losses and expansions within stress related gene pathways in tardigrades, but also indicate that different evolutionary lineages have a high degree of divergence involving unique molecular adaptations and possible unknown functional homologues. The study was supported by The Independent Research Fund Denmark (grant-ID: DFF–4090-00145) and research grant (17522) from VILLUM FONDEN. MK is a Marie Curie fellow funded from the European Union’s Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 747087.

**Keywords:** comparative transcriptomics, Heterotardigrada, model organisms, stress genes
Differential gene expression in the heterotardigrade *Echiniscus testudo* during desiccation

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**Background:** Knowledge of the molecular mechanisms of stress tolerance in tardigrades is growing every year, but virtually all studies have been conducted on eutardigrades. The heterotardigrade *Echiniscus testudo* exhibits high levels of stress tolerance, especially with regards to desiccation. In this study, differential gene expression analysis through RNA-seq was explored on single individuals of *E. testudo* in four different conditions: pre-tun, desiccated, rehydrating and a control group of active animals. **Results:** Transcriptomes were sequenced for all four conditions and a reference transcriptome was constructed de novo using Trinity. Differential gene expression data was mined by doing pairwise comparisons between all four conditions. We found a large number of transcripts to be up-regulated in the stressed conditions compared to the control group. Many of these transcripts were identified as enzymes such as kinases, ligases, dehydrogenases and phosphatases. A number of transcripts were identified as proteins involved in DNA replication and repair as well as several transcripts with homology to homeobox-containing genes. We were unable to identify any transcripts with homology to Dsup or the heat soluble proteins, CAHS, SAHS and MAHS found in eutardigrades and putatively related to resistance to desiccation. **Conclusions:** Our data suggests many similarities between heterotardigrades and eutardigrades in the molecular mechanisms of desiccation tolerance, but also some differences. The lack of homologs to Dsup, CAHS, SAHS and MAHS is surprising; however, more data is needed, in order to verify whether or not these discrepancies are a result of divergently evolved stress pathways or due to the very small amount of input mRNA. Efficient DNA repair is undoubtedly central for surviving extreme desiccation, but homeobox genes also seem to play a role. This is the first differential gene expression analysis of desiccation tolerance in a heterotardigrade, which consequently will shed more light on the molecular mechanisms involved in this type of stress tolerance, particularly on possible differences between eutardigrades and heterotardigrades.

**Keywords:** Gene expression, Heterotardigrada, RNA-seq, stress tolerance
Investigation of regulatory mechanisms of anhydrobiosis in *Hypsibius dujardini* by phosphoproteomics

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**Background:** An anhydrobiotic tardigrade, *Hypsibius dujardini* requires preconditioning at high relative humidity (RH) environment to acquire tolerance against severe loss of body water contents caused under low RH condition. This suggests that *H. dujardini* senses the sign of desiccation of surrounding environments and prepare required machinery during a preconditioning period to tolerate upcoming dehydration. However, such molecular regulatory mechanisms remain largely unknown. We have suggested so far that *H. dujardini* requires *de novo* gene expression and the activity of protein phosphatase (PP) 1 and PP2A (PP1/PP2A) for successful anhydrobiosis, by chemical genetic approach. PP1/PP2A are multifunctional phosphatases and have been reported to interact with hundreds of substrates. Therefore, we performed phosphoproteomics to investigate the signaling pathways involved in desiccation response in *H. dujardini*. **Results:** Phosphoproteome of animals preconditioned at 97% RH for 0, 15, 60 and 180 minutes were compared, and 68 peptides were found to be significantly changed in phosphorylation levels during preconditioning. In order to characterize these peptides i.e. original proteins, further analysis with String 10 was performed, and enrichment of RNA transport and AMP-activated protein kinase (AMPK) signaling were observed. Catalytic subunit of AMPK was dephosphorylated during preconditioning. Intriguingly, AMPK is reported as the substrate of PP2A in mammals, suggesting that dephosphorylation of AMPK by PP2A may play a role in desiccation stress response in *H. dujardini*. However, it should be noted that the phosphorylated site found in this study was not identical to that reported in mammals. In addition, to investigate the transcriptional responses, we explored transcription-related factors and found three proteins. One of them is transcriptional coactivator, Endothelial Differentiation-related Factor 1 (EDF-1). In mammals and flies, EDF-1 regulates the activity of various transcription factors including AP-1 and ATF-2 which are known to be involved in oxidative stress response. Desiccation stress could cause oxidative stress as well, suggesting that these transcription factors may be involved in anhydrobiosis of tardigrades. **Conclusions:** Comparative phosphoproteomics partially revealed the initial molecular responses to desiccation stress in *H. dujardini*. Dephosphorylation of AMPK could play a role in this response. Furthermore, there might be common transcriptional mechanisms with known oxidative stress response. To confirm these possibilities, further studies are in progress.

**Keywords:** anhydrobiosis, AMPK, EDF-1, phosphoproteomics, transcriptional response
Antioxidant defense activity under anhydrobiosis in two desiccation tolerant eutardigrades

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Background: Besides aerobic metabolism, several forms of stress can produce Reactive Oxygen Species (ROS). Under desiccation stress, the production of ROS seems to increase as cellular water content decreases. Here we investigate the activity of ROS scavenging enzymes and the content of other molecules capable to counteract oxidative stress during anhydrobiosis in two desiccation tolerant eutardigrades inhabiting different environments: Acutuncus antarcticus (freshwater sediments) and Paramacrobiotus richtersi (leaf litter).

Results: In A. antarcticus, no significant differences were seen in total protein content between desiccated specimens, specimens rehydrated for 1h and 24h, and hydrated specimens used as controls. Significant differences were found in the activity of superoxide dismutase (SOD) among the experimental groups. The activity of SOD was lower in desiccated animals as compared to controls. No significant differences were evidenced in the activities of catalase (CAT), glutathione reductase, and glutathione peroxidase (GPX), comparing the four experimental groups. The content of glutathione was lower in desiccated specimens than in specimens rehydrated for 24h, whereas no significant differences were seen between the other experimental groups. In P. richtersi, the total amount of proteins detected in desiccated specimens was significantly lower with respect to controls and the tardigrades rehydrated for 1h and 24h. As regards scavenging enzymes, significant differences were recorded in the activity of CAT. Higher CAT activity was recorded in desiccated specimens as compared to controls and rehydrated animals for 24h, and in specimens rehydrated for 1h as compared to those rehydrated for 24h. No significant differences in the activity of SOD, glutathione reductase and glutathione peroxidase and in the content of glutathione were detected among the four experimental groups.

Conclusions: These data suggest that the activity of the antioxidant defense could be strictly related to the anhydrobiosis, even though the involvement of specific antioxidant molecules is species-specific. SOD, CAT and GPX are the most active enzymes in P. richtersi, while in A. antarcticus the main compounds are SOD and glutathione. Work supported by PdR 2013/AZ1.13 to L.R. and PdR 2013 B1/01 to R.G: both granted by PNRA-MIUR.

Keywords: anhydrobiosis, desiccation stress, glutathione, ROS, scavenging enzymes
Functional roles of bioprotectants during dehydration and rehydration in *Paramacrobiotus richtersi* revealed by RNA interference

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**Background:** To test if antioxidant enzymes, aquaporin proteins and trehalose have a role in the desiccation process of tardigrades, the RNAi technique was performed to disrupt the function of target genes by the injections of a specific double stranded RNA (dsRNA) in *Paramacrobiotus richtersi*. Then, the survival of injected and control animals immediately (t₀), and 1 (t₁), 24 (t₂₄), and 48 (t₄₈) hours after the end of rehydration was evaluated and compared. Locomotion was the criterion used to check animal survival.

**Results:** Control tardigrades were always alive. The survival of injected animals never reached 100%, with significant differences among experimental groups (p<0.01). Tardigrades injected with dsRNA for genes of catalase (*cat*), glutathione reductase (*gr*) and superoxide dismutase (*sod*) showed significantly lower motilities, recorded at t₀ with respect to controls (*cat* and *sod*: p<0.05; *gr*: p<0.01). The silencing of glutathione peroxidase (*gpx*) gene caused a low post-desiccation motility at every phase of rehydration process (t₀: p<0.001; t₁, t₂₄, t₄₈: p<0.01). At t₀, the motility of animals injected with dsRNA of aquaporin 3 (*aqp3*) and 9 (*aqp9*) genes were significantly lower than controls (*aqp 3*: p<0.05; *aqp 9*: p<0.01), while no differences were evidenced injecting dsRNA of aquaporin 10. The silencing of trehalose-6-phoshate synthase gene (*tps*) showed no difference between injected and control animals. The Reverse Transcriptase PCR, used to check if RNA interference worked in *P. richtersi*, showed a marked decrease in the expression level of each target gene in comparison to *DNA polymerase II* control gene.

**Conclusions:** Present results show that genes encoding antioxidant enzymes (*cat, gr* and *sod*) and aquaporins (*aqp3* and *aqp9*) could play a role during the rehydration phase, the *gpx* gene could be involved in every phase of anhydrobiosis, while trehalose is not the key molecule to preserve desiccated cells. In tardigrades, desiccation tolerance is influenced by the action of different molecules working in a synergic way to allow anhydrobiosis.

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**Keywords:** anhydrobiosis, antioxidant enzymes, aquaporin proteins, RNA interference, tps enzyme
Differences in tolerance to anhydrobiotic conditions among tardigrade species

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Background: Tardigrada are known for their cryptobiotic abilities. Many studies have shown that in this state tardigrades can survive a wide range of unfavourable environmental conditions, even for many years. A specific type of cryptobiosis is anhydrobiosis, which is a response to the lack of liquid water in the environment. Successful anhydrobiosis includes entering, permanent and leaving stages corresponding to the initial dehydration, forming of “tun” and rehydration stages, respectively. This ability is especially important in temporary environments, where lack of water appears periodically. Available data confirm that different species have different anhydrobiotic capabilities. However, detailed and comparative studies between different taxa living in diverse microenvironments have never been performed.

Results: In the present study we applied six taxa to estimate the impact of the duration of the “tun” stage on the capability to recover to full activity. In total, 490 specimens of each species were used. Specimens were dried for three days and stored in small, plastic Petri dishes on filter paper under controlled conditions in an environmental chamber. To test the anhydrobiotic capabilities, specimens of each species were rehydrated after seven different periods spent in anhydrobiosis (0-, 7-, 14-, 30-, 60-, 120-, 240-days). During rehydration, tardigrades were observed in order to detect whether correct tun formation occurred, and for each dish the time of first signs of movement and returning to full activity were monitored. Statistical analyses were performed using standard methods. All tested species displayed the ability to form a correct tun and return to active life. However, differences in the execution of anhydrobiosis were noted, not only between different taxa inhabiting different habitats, but also between different specimens of the same species. The strongest anhydrobiotic capabilities were observed for species of the genera Echiniscus, Milnesium and Ramazzottius.

Conclusions: Our study reveals different anhydrobiotic capabilities in different tardigrade species. The work was supported by the research grant of National Science Centre, Poland, NCN 2016/21/B/NZ4/00131.

Keywords: anhydrobiosis, tolerance, comparison, survivability
Non-electrolyte solutions induce tun-formation and osmobiotic survival in *Echiniscus testudo* and *Hypsibius dujardini*

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**Background:** *Echiniscus testudo* has an upper lethal limit of around 200 mOsm·kg\(^{-1}\) to NaCl solution shock exposures [1]. *E. testudo* (1910 specimens including controls) and for comparison *Hypsibius dujardini* (304 specimens including controls) were gradually transferred from medium hard reconstituted water (MHRW, 4 mOsm·kg\(^{-1}\)) into NaCl or sucrose solutions of increasing osmolality. Activity and morphological response was monitored during exposures and at 2-5 time points from 2 hours up to 6 days following direct or gradual retransfer to MHRW.

**Results:** For *E. testudo*, 58±2% (mean±SEM) retained activity following gradual transfers into 278±1 mOsm·kg\(^{-1}\) NaCl solutions. Activity increased to 99±1% two hours after retransfer to MHRW. At osmolalities >300 mOsm·kg\(^{-1}\) all specimens became inactive, but partially recovered upon retransfers in a time and concentration dependent manner. As an example, activity was 1±1% two hours after retransfer from exposure to 873±5 mOsm kg\(^{-1}\), but increased to 73±6% after 48 hours, whereas only 6±6% and 18±5%, respectively, were active 48 hours and 6 days following recovery from exposure to 1865±6 mOsm·kg\(^{-1}\). *E. testudo* did not undergo tun formation in NaCl solutions, but either retained activity or became passive and bloated. Conversely, exposure to sucrose solutions induced tun formation at ≥343±2 mOsm·kg\(^{-1}\) and *E. testudo* readily regained activity following gradual transfer into and out of 1847±3 mOsm·kg\(^{-1}\) solutions (92±2% activity, 24 hours after commence of retransfer). For *H. dujardini*, tun formation was also induced by high [sucrose] solutions with 49±8% activity 24 hours after commence of retransfer from 1847±3 mOsm·kg\(^{-1}\).

**Conclusions:** As compared to shock exposures, *E. testudo*\(^{'s}\) tolerance towards high [NaCl] solutions is markedly increased when provided time to acclimate to increasing salt loads. The tardigrade, however, fails to produce tuns. We hypothesize that ion diffusion across the integument shifts electrochemical potentials within this limno-terrestrial tardigrade with consequences for excitable tissues, rendering animals immobile and incapable of contraction and tun-formation. High concentration non-electrolyte (sucrose) solutions, on the other hand, readily induce tun formation in both *E. testudo* and *H. dujardini*, accompanied by an increase in tolerance towards high external osmotic pressure. [1] Heidemann et al. 2016. *Zool J Linn Soc.* 178: 912-918. The study was supported by The Independent Research Fund Denmark (grant-ID: DFF–4090-00145).

**Keywords:** electrolytes, limno-terrestrial, osmobiosis, osmotic stress tolerance, tun
Tun formation in the marine arthrotardigrade *Archechiniscus* (Archechiniscidae) from the tidal zone of Kitahama beach, Seto, Japan

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**Background:** Tardigrades are known to exhibit various adaptations to life in extreme, fluctuating environments. While cryptobiotic abilities characterize semi-terrestrial eutardigrades and echiniscoideans, cryptobiosis has only been experimentally evidenced in a single arthrotardigrade species, *Styraconyx haploceros* (Styraconyxidae) by Jørgensen and Møbjerg in 2015 [1]. This species lives on lichens in the upper tidal zone, experiencing high fluctuations in environmental parameters (salinity, temperature, oxygen tension, etc.). Although many tidal arthrotardigrade species have been described, the knowledge of the ability of arthrotardigrades to withstand the environmental extremes of the tidal zone remains limited. In the present study we tested the tolerance of osmotic stress and desiccation in an undescribed species of *Archechiniscus* living on gooseneck barnacles (Pedunculata) attached to rocks in the upper tidal zone of Kitahama beach, Seto, Japan. As the rocks of the study area are fully exposed during low tides, any associated epifauna must cope with regular short-term desiccation between tides, as well as occasionally heavy rain during low tides resulting in fluctuations of salinity.

**Results:** Upon exposure to salinities of more than 65 ‰, the *Archechiniscus* species enters a tun state, in which it also survives desiccation from seawater. When exposed to 15 ‰, leg movement is temporarily reduced. However, normal activity is resumed after a short exposure time (10 min). The species further survives exposure to low salinity seawater (1.8 ‰) assuming a turgid body form.

**Conclusion:** The observed cryptobiotic responses of this second species confirm the presence of these capabilities among arthrotardigrade taxa that are phylogenetically distinct, yet ecologically somewhat similar in terms of habitat niche. The occurrence of cryptobiosis among other arthrotardigrade species is hypothesized; however the relationship between the degree of fluctuation of the natural environment conditions and the frequency of occurrence among taxa has yet to be determined. [1] Jørgensen & Møbjerg. 2015. *Mar. Biol. Res.*, 11(2): 214-217.

**Keywords:** *Archechiniscus*, arthrotardigrades, cryptobiosis, extreme environments, tun formation.
New insights into cryobiosis and storage cell dynamics in *Ramazzottius oberhauseri*

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**Background:** *Ramazzottius* contains strong cryptobionts with well-documented tolerance towards desiccation, high-dose irradiation and osmotic stress. Here, we investigate survival, morphological response, and DNA damage in *R. oberhauseri* following exposure to sub-zero temperatures. Tardigrades (880 specimens) were transferred to 1.5 ml of ultrapure water and exposed to -20°C, -80°C and liquid nitrogen (gradual transfer) for 4 time intervals spanning from 1 week to 5 months. Activity was monitored 1, 2, 4, and 24 hours post-thaw. Morphological response and storage cell DNA damage was investigated in tardigrades frozen at -20°C and -80°C.

**Results:** *R. oberhauseri* endures freezing at all temperatures and time periods with 24-hour activity levels of 80-100%, with exception of -20°C for 5 month (69% activity). For specimens frozen at -80°C and in liquid nitrogen activity at 1 hour was lower than 24 hours post-thaw. Control specimens (300 total) placed with habitat sediment, filter paper, or without substrate showed 100% activity after 24 hours. After ≥5 weeks, post-thaw activity of tardigrades exposed to freezing surpassed those of controls regardless of substrate conditions. Specimens exposed to -20°C and -80°C contracted to a mean±SEM to 72±1% and 66±2% of pre-frozen body length, respectively. Storage cells (~300 cells total) isolated post-thaw from tardigrades frozen at -20°C and -80°C for 24 hours were examined by comet assay immediately after thawing with 7.1±2.0% and 6.6±2.0% DNA in tail. Storage cells analysed 24 hours post-thaw, contained lower levels of DNA damage (above negative control) with 3.4±1.4% (-20°C) and 1.9±0.6% (-80°C) DNA in tail. We found a clear correlation between storage cell quantity and tardigrade length with an increase of +0.85 cell/µm and a size dependent number of 50 to 350 cells.

**Conclusions:** *R. oberhauseri* is a strong cryobiote surviving exposure to sub-zero temperatures including liquid nitrogen (approx. −196°C) for extended periods of time. Freezing is accompanied by DNA damage that is repaired post-thaw. Our data indicate that DNA repair is faster in tardigrades exposed to -80°C as compared to -20°C. Furthermore, tardigrades exposed to -20°C for longer periods have lower recovery. We hypothesize that tardigrades kept at -20°C are not fully ametabolic with implications for post freezing energy levels and survival. The study was supported by The Independent Research Fund Denmark (grant-ID: DFF–4090-00145).

**Keywords:** Cryobiosis, storage cells, comet assay, survival.
Respiration and metabolic rate of
*Richtersius coronifer* determined using O₂-microsensor technology

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**Background:** We present data from O₂-microsensors used to measure the O₂-consumption of individual tardigrades at various temperatures, as well as microscopic analysis to determine their size and thus calculate the metabolic rate. This method is highly reliable and relatively inexpensive. In addition, it can be repurposed for a wide range of experimental set-ups – opening up a rich field of inquiry into the metabolism and energetics of tardigrade biology. The purpose of the study was to provide insight into the metabolic activity of tardigrades and to demonstrate the ability of the method to further elucidate the energetics involved in the cryptobiotic and extremophilic behavior of tardigrades.

**Results:** The metabolic rate of *Richtersius coronifer* at 22 °C was 10.8 ± 1.8 nmol O₂ · mg⁻¹ · hour⁻¹. This is lower than that of the smaller more mobile species *Macrobiotus macrocalix* – which was 13.1 ± 2.3 nmol O₂ · mg⁻¹ · hour⁻¹. Q₁₀-values for the metabolic rate of *R. coronifer* were ~ 1.5 in the temperature ranges 2-11, 16-22 and 22-33 °C, but ~ 5.5 in the range of 11-16 °C. From the Arrhenius equation, an activation energy of the rate-limiting step in the metabolic pathway was calculated to be 50.8 kJ/mole O₂.

**Conclusions:** The microsensor method allows precise determination of the O₂-consumption of individual tardigrades under a vast array of conditions in an accessible and inexpensive manner. In this study we have determined the respiration and metabolic rate of *R. coronifer* in distilled water at various temperatures. We found an instability in the metabolism in the range of 11-16 °C, although it remains stable at both higher and lower temperatures. Finally, we determined the activation energy of its metabolism. This should provide insight into the chemical nature of its metabolic pathway and thus help inform the design and interpretation of future energetics-related studies of tardigrades.

**Keywords:** energetics, Eutardigrada, metabolic rate, O₂-microsensor technology
Analyses of nervous system patterning genes in the tardigrade *Hypsibius dujardini* support a unipartite tardigrade brain

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**Background:** Both euarthropods and vertebrates have tripartite brains. In vertebrates, the brain parts are referred to as the forebrain, midbrain, and hindbrain. In euarthropods, the brain parts are referred to as the protocerebrum, deutocerebrum, and tritocerebrum. Intriguingly, a suite of genes is expressed in similar regionalized patterns during brain development in both vertebrates and euarthropods. These similarities have been used to support direct homology of the tripartite brains of vertebrates and euarthropods. However, this hypothesis must be tested in other animals. Tardigrades are an interesting lineage on which to test this hypothesis because they are relatively closely related to euarthropods, and whether they have a tripartite brain or unipartite brain has recently been a focus of debate.

**Results:** We tested this hypothesis by analyzing the expression patterns of *six3*, *orthodenticle*, *pax6*, *unplugged*, and *pax2/5/8* during brain development in the tardigrade *Hypsibius dujardini*. These genes were expressed in a staggered anteroposterior order in *H. dujardini*, similar to what has been reported for mice and flies. However, only *six3*, *orthodenticle*, and *pax6* were expressed in the developing brain. *Unplugged* and *pax2/5/8* were expressed in the developing trunk.

**Conclusions:** These results buttress the conclusion of our previous study of Hox genes—that the brain of tardigrades is only homologous to the protocerebrum of euarthropods. This result supports the model based on fossil evidence that suggests that the last common ancestor of tardigrades and euarthropods exhibited a unipartite brain. Taken together, these results challenge the hypothesis that the tripartite brain of euarthropods is directly homologous to the tripartite brain of vertebrates.

**Keywords:** brain, development, evolution
Ciliary structures revealed by cryopreparation and electron microscopy of *Hypsibius dujardini*

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**Background:** Cilia and flagella are cellular extensions that can either be motile, like sperm tails, or act as antennae and sense the extracellular milieu. Inside a flagellum is a complex molecular machine based around a specialized arrangement of microtubules and lots of associated proteins – the axoneme. The structure of the axoneme is relatively conserved in eukaryotes, with nine doublet microtubules in sensory cilia (9+0), and in motile cilia, two central singlet microtubules (9+2). The flagellum originates inside the cell at the basal body which is a centriole-like structure. In the nematode *Caenorhabditis elegans*, only sensory cilia are found and they are unusually structured with a tapered basal body and branched structures. Little is known about the tardigrade ciliary structure to date.

**Results:** We have high pressure frozen whole animals of *Hypsibius dujardini* and prepared them for thin section electron microscopy. Inside these animals we found ciliated cells in two locations: in cells attached to the brain extending into the cuticle around the mouth and the previously shown light sensitive cilium inside the eye. The cilia found around the mouth showed tapered basal body morphology and branched structures, similar to those seen in *C. elegans*. A surprising finding was that the cilium structure diverged from the canonical 9+0 sensory cilium arrangement and showed 15 doublet microtubules close to the basal body. We also did a rudimentary 3D reconstruction of the eye, showing two cells extending arborous cilia into a lumen surrounded by a cell with microvilli and a pigmented cell.

**Conclusions:** This work shows that it is important to study ciliary structure in more organisms beyond the common model organisms to fully evaluate the structural evolution of this cellular component. It also shows that the ciliary structure of *H. dujardini* resembles, but are not identical, to that of *C. elegans*. We are now starting investigations in what sort of signals these cilia are receiving and the molecular mechanisms behind the ciliary structure we discovered.

**Keywords:** anatomy, cell biology, cilia, electron microscopy, structure
Germ cell cluster organisation and fate during oogenesis in Parachela

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Background: Two types of gonadal oogenesis can be distinguished in animals: panoistic and meroistic. In panoistic oogenesis all the germ cells have the potential to become oocytes while in meroistic oogenesis a part of the germ cells develop into oocytes, whereas the remaining cells differentiate into trophocytes. The characteristic feature of meroistic oogenesis is the formation of germ cell clusters. Three major types of germ cell clusters can be distinguished in animal oogenesis: linear germ cell clusters, clusters in the shape of a rosette (branched), and clusters with a central mass of cytoplasm.

Results: In Parachela, meroistic oogenesis occurs during which the germ cell clusters are formed as a result of incomplete cytokinesis of the germ cells. The germ cells in each cluster are interconnected by cytoplasmic bridges that allow the directional transport of macromolecules (rRNAs), organelles and yolk material between the cytoplasm of adjacent cells. The number of germ cells in the clusters can vary in different species. In Dactylobiotus parthenogeneticus, the germ cell cluster consists of 8 cells, while in Thulinius ruffoi the cluster is huge and contains more than thirty germ cells. In all analysed species (Dactylobiotus parthenogeneticus, Dactylobiotus dispar, Thulinius ruffoi, Isohypsibius granulifer granulifer, Hypsibius dujardini, Macrobiotus polonicus) germ cells are branched and their fate during oogenesis is similar. Only one cell in the cluster differentiates into an oocyte, while the remaining cells become trophocytes that support the oocyte. In our studies, we used various methods, including: light microscopy, transmission electron microscopy, confocal microscopy, serial block-face scanning electron microscopy and open-source software for creating 3D reconstructions.

Conclusions: In Parachela, meroistic oogenesis takes place. The germ cell clusters are branched. One cell in each cluster differentiates into an oocyte, while the remaining cells become trophocytes. Part of this work was supported by research grant UMO-2014/15/N/NZ4/04350 from the National Science Centre.

Keywords: cytoplasmic bridge, gametogenesis, oocyte, tardigrades, trophocyte
Reproductive performance of individuals of an Antarctic tardigrade revived after being frozen for over 30 years, and of their offspring

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Background: Long-term survival has been one of the most studied of the extraordinary physiological characteristics of cryptobiosis in micrometazoans such as nematodes, tardigrades and rotifers. In previous studies of long-term survival of micrometazoans, instances of survival have been the primary observation, but subsequent reproduction of the revived individuals and of their offspring are generally not reported. We revived several individual tardigrades, Acutuncus antarcticus, from a frozen moss sample collected in Antarctica in 1983 and stored at -20°C for 30.5 years. Then, we recorded the reproduction of the resuscitated individuals, and subsequently of the hatchlings.

Results: One resuscitated individual, Sleeping Beauty (SB) -1, deposited 19 eggs in total, 14 of which hatched. These 14 hatchlings produced an average of 19.5 eggs in total throughout their lifetimes, 86.5% of which successfully hatched. Another resuscitated individual, SB-3, deposited 15 eggs, with seven successfully hatching. These seven hatchlings produced an average of 28.3 eggs, with a hatching success of 61.3%. The days to the first oviposition of SB-3 (14 days) was longer than the average period to first oviposition of the offspring of both SB-1 and SB-3 (9.7 and 9.0 days respectively). However, other reproductive parameters including clutch size, oviposition interval, total numbers of oviposition events and eggs produced per individual, and hatching success did not differ between the revived individuals and their first generation offspring.

Conclusions: The current study demonstrated successful reproduction in individuals of the Antarctic tardigrade, A. antarcticus, after over 30 years in a cryptobiocytic frozen state, and in their first generation offspring. This demonstration of reproductive viability in both the frozen individuals and their offspring confirms their potential to re-establish populations after long-term cryptobiosis.

Keywords: cryobiosis, cryptobiosis, freezing, long-term survival, reproduction
Snow White water bears – quantum-like decreased embryogenesis time for tardigrades reared with increased cold exposure time

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Background: Three models have been proposed to explain lifespan extension resulting from exposure to extreme desiccation in microscopic animals: individuals (1) stop ageing (becoming ‘Sleeping Beauties’); (2) continue ageing (becoming ‘Rip van Winkles’); or (3) age but at a diminished rate. Whether cold temperatures could produce similar effects at earlier life history stages is unknown. We used the eutardigrade species Hypsibius exemplaris to test this, exposing eggs to 0 °C for different time periods.

Results: Embryonic cell divisions were retarded when eggs were reared initially at 0 °C. Compared to control specimens reared at 22 °C, juveniles that hatched from eggs exposed to 0 °C for 4 days and returned to 22 °C experienced a three-day lag, indicating that their biological age was less than their chronological age. As cold exposure duration increased (days = 10, 20, 40), subsequent incubation period at 22 °C decreased incrementally (days = 3, 2, 1), suggesting that H. exemplaris involves a threshold-determined, quantum-like, energetic-based system for controlling embryogenesis.

Conclusions: We found support for model (3); specimens in H. exemplaris age but at a diminished rate when they are reared initially at 0 °C and returned to 22 °C. To distinguish the observed cryobiotic responses from anhydrobiotic responses (and cryobiotic responses to extreme-cold exposures) in adults reported previously, we propose the ‘Snow White’ model: development is retarded in chilled eggs as a consequence from a quantized decrease in average cell division rate. We hypothesise, on this basis, that embryos in the eutardigrade species H. exemplaris age partially when they are reared at cold temperatures and returned to ambient temperatures (they are neither Sleeping Beauties nor Rip van Winkles). We cannot falsify the hypothesis that specimens entered suspended animation within each (i.e., 0-9, 10-19, 20-39 and ≥40 days) threshold exposure duration, possibly demonstrating a Sleeping-Beauty-esque embryonic response similar to Dauer larvae in nematodes. We also cannot falsify the hypothesis that embryogenesis rate increased after return to ambient temperature following cold exposure. All three non-mutually-exclusive hypotheses remain for future, definitive testing.

Keywords: ageing rate, cold tolerance, development, Eutardigrada
Modelling reproductive phenology of tardigrades: a new approach

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Background: Phenology—the study of recurrent biological events and their causes—has been used to study flowering, budburst, migration, hibernation, emergence and breeding. A conceptual and mathematical model for examining the time distribution of biological phenomena has recently been developed that offers biologically meaningful parameters for interpretation. This approach could be used with tardigrades to explore the timing of events associated with cryptobiosis and reproduction, as well as the impacts of environmental conditions on these processes. An explanation of the model and its parameters will be given. As an example, the phenology of egg laying and hatching in the Antarctic tardigrade (*Acutuncus antarcticus*) across a range of controlled temperatures will be explored.

Results: The model fitted the data well, and allowed clear comparisons of different aspects of the egg laying and hatching processes. An analysis was also carried out to model reproductive performance for individual clutches of eggs. Temperature clearly affected reproductive performance, and important variation was found between individuals.

Conclusions: Detailed monitoring of cryptobiosis and reproduction in controlled conditions can provide important insights into the ecology of tardigrades, and their potential response to environmental changes, such as warming temperatures. Phenological models can be useful ways to analyze and interpret these processes, both to extract biologically meaningful patterns from complex datasets, and to simplify the communication of key patterns to others.

Keywords: phenology, modelling, reproduction, cryptobiosis
X-ray vision: 3D imaging of whole tardigrades using nanocomputed tomography

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Background: X-ray computed tomography (=CT) has long been used for non-invasive imaging of biological samples, for example in the field of human medicine. Ongoing advances in CT devices and reconstruction algorithms have been constantly pushing the resolution limits of these methods to the point that CT imaging has not only become invaluable for studying fossils, but is now also routinely used to complement traditional morphological studies. However, conventional CT systems rarely achieve resolutions below the micrometer range, limiting their utility for studying microscopic samples. Optics-based X-ray microscopy is able to achieve nanometer-range resolutions but the samples are limited to only several micrometers in size. Unfortunately, many microinvertebrates, such as tardigrades, fall within the size-range gap between these two methods and are therefore unsuitable for either type of device. Recently, a new tabletop nanoCT setup was introduced that is able to bridge this gap. We demonstrate its potential for studying microinvertebrates by imaging a whole tardigrade and reconstructing its internal anatomy.

Results: The new CT device combines a prototype nanofocus X-ray source with a single-photon counting detector, and operates on the principle of geometric magnification, achieving high magnifications as well as resolutions of 100 nm under ideal conditions. A full-body scan of an adult specimen of Hypsibius exemplaris revealed all major organs and structures at a voxel size of ~270 nm. An additional detailed scan of the head region achieved a 200 nm voxel size. Manual segmentation of each structure allowed us to present the whole body in 3D and analyze the spatial relationships of the major internal organs without introducing sectioning artifacts. Furthermore, the isometric voxel sizes make it possible to measure the volumes of the segmented structures. To take full advantage of this feature, we segmented each storage cell individually and calculated their size and volume distributions.

Conclusions: This imaging technique allowed us to visualize a whole tardigrade in full 3D, demonstrating that recent advances in CT technology may finally make this technique applicable for imaging microinvertebrates. Finally, the relatively straightforward sample preparation procedure facilitates the use of this technique not only for research (for example for morphological analysis), but also as a potentially useful tool for generating educational models.

Keywords: 3D reconstruction, nanoCT, volume rendering
Tardigrade rearing methods: a review

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Background: For a wide range of studies on the biology of tardigrades such as biochemistry, genomic, proteomic, life history and adaptive strategies, it is helpful or even mandatory to access laboratory cultures, strains that are reliably delivering large quantities of specimens reared under defined conditions of temperature, photoperiod, humidity, and with an axenic food source. The number of methods to successfully culture tardigrades has substantially increased during the last decades, but they mostly concern freshwater tardigrades, which commonly are easier to rear compared to terrestrial ones. The present paper gives an overview on the existing culturing methods for tardigrades, with a focus on true terrestrial species.

Results: Difficulties culturing terrestrial species often lie in their sensibility to hypoxia: a small film of water is decisive for their active life, but larger amounts of water may easily lead to oxygen deficiency. Another challenge in starting a successful tardigrade culture is to find out about their species-specific requirements. It begins with the provision of an adequate diet, which often means trying a number of different food sources, since for the majority of terrestrial species even the general food category (“algae”, “microorganisms”, “protists” or “metazoans”) are not yet known and may be rather specific. Culturing success may even involve the necessity of a 3-dimensional matrix by simulating the population’s natural habitat, in order to warrant tardigrade mobility. To summarize our present knowledge as well as current knowledge gaps, we further discuss the rearing attempts of some “difficult” tardigrade species (e.g. Ramazzottius oberhaeuseri, Richtersius coronifer, Apodibius confusus) together with their presumed shortcomings. In addition, the different objectives of massive and single cultures, other than their related problems, will be presented.

Conclusions: The presented overview on culturing methods of tardigrade species, and in particular terrestrial ones – successful attempts as well as some failures – shall provide an annotated catalogue of the presently available information and may be helpful to increase the number of cultures of “difficult” terrestrial tardigrade species.

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Keywords: Culturing methods, laboratory strains, terrestrial tardigrades.
Microfluidic separation of tardigrade

*Hypsibius dujardini* from raw culture for on-demand sample preparation

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**Background:** Increasing research trends in the field of Tardigrada demand advancements of supportive technologies for the ease of experimentation and development of standard protocols. Growth cultures of tardigrades contain different age groups of tardigrades such as adults, newborns, juveniles of variable sizes, their eggs, empty cuticles, and their algal food. For tardigrade experimentation, pure sample preparation is a highly demanding and laborious work, which is usually done manually by using a loop, tweezer or needle. In our step-by-step approach to the tardigrade separation and on-demand sample preparation, we propose a simple, disposable, low cost and rapid microfluidic filtration device.

**Results:** For the microfluidic separation of tardigrade *Hypsibius dujardini*, polydimethylsiloxane (PDMS)-based filtration devices were designed using computer-aided design software and fabricated by soft lithography techniques. The cultures of the tardigrade and *Chlorococcum* algae (tardigrade’s food) were ordered from Sciento (UK) and grown in Chalkley’s medium and Bold’s Basal medium, respectively. The size of most of the tardigrades *Hypsibius dujardini* ranged between 100–300 μm. The eggs were 35–70 μm, while the *Chlorococcum* algae were 5–16 μm. A semi-circular array of six pillars, with five equidistant passways in between, form a single trap inside the PDMS microchannel to capture the tardigrade and eggs and let the smaller algal food particles to pass through the trap (see Schematic). The total number of traps designed inside a single PDMS device was 1229 with a minimum passage dimension of 20 μm, which was slightly bigger than the size of *Chlorococcum* algae. As the raw sample is pumped through the filtration device using a syringe, it acts as a sieve that let the smaller algal particles to pass through the PDMS traps and capture the larger eggs, cuticles and tardigrades. The captured sample is collected from the device using a backward flow.

**Conclusions:** The proposed microfluidic chip can be used to capture tardigrades based on their sizes and to prepare a pure sample devoid of any algal food for subsequent usage in other experiments e.g. tardigrade genomic and proteomic studies.

**Keywords:** Microfluidics, Tardigrade separation, Culture samples, *Hypsibius dujardini*
Tardigrade space research — past, present & future

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Background: Tardigrades have been used as a model organism in space research with good perspective for future space research [1,2]. In this regard, we would like to present our own experiences and ongoing efforts in Korea on tardigrade space research.

Results: We became interested in tardigrades after having a meeting in which we shared our research interest on space biology at the Korea Polar Research Institute (KoPRI). Subsequently, we have cultured Hypsibius dujardini in order to utilize this tardigrade as a science-mission payload for a KRIIBB (Korea Research Institute of Bioscience and Biotechnology) Space-Biology Research Initiative. In 2013 and 2015, we applied the ‘CubeSat Contest in Korea’ [3] as a KRIIBB-campus team named ‘Bio-Astronaut’. Although our applications failed to get awarded, they resulted in our support for the newly awarded CubeSat team ‘KMSL’ (Korea Microgravity Science Lab) [4]. The 3U-CubeSat KMSL mission includes 1U-payload engineering mission and two 1U-payload science missions, one of which is the tardigrade culture mission [5]. In addition, we have collaborated with a KAIST group and our effort in this regard will be presented at the current symposium [6]. Together with our support of the KMSL team, we aim to further develop tardigrade research initiatives in Korea with KoPRI and KAIST groups. Through our experiences, we find that tardigrades are not only very robust at extreme conditions in their tun form, but also very vulnerable to death in their normal active form. Hence tardigrades can be utilized not only as a surviving model organism but also as a dying model organism under various stress conditions.

Conclusions: We think that tardigrades represent a good organism for space research as well as a platform for future biotechnology. We would like to have discussions at this conference regarding research directions for biotechnology development in future, sharing our biotechnology vision for space colonization.


Keywords: Biotechnology, CubeSat, Extremophile, Space Research, Tardigrade
POSTER PRESENTATIONS
A new species of *Dactylobiotus* (Parachela, Eutardigrada) from King George Island, Antarctica

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**Background:** Limno-terrestrial tardigrades form one of the most dominant groups in the scanty terrestrial freshwater ecosystem of Antarctica. However, due to their limited key morphological characters and restricted access to the habitats, taxonomic study on Antarctic tardigrades largely remains to be challenged. KOPRI (Korea Polar Research Institute) ecology team collected several species of tardigrades near the King Sejong Station, King George Island, Antarctica during 2014-2015 seasons.

**Results:** Among the collected freshwater tardigrades, one group shows a buccal-pharyngeal apparatus with ten peribuccal lamellae, and a cuticular connection which joins two claws in each limb. These characters warrant a generic assignment to *Dactylobiotus*. The new species is quite large in size (600-700 μm) with prominent eyespots and smooth cuticle. The egg has circular or slightly hexagonal cone-type processes with a tip. Compared to *D. ambiguus* and *D. caldarellai* which have a rather similar morphology, the new species shows claws with longer primary branch at all limbs. The 18S, 28S rDNA and cytochrome c oxidase subunit 1 (CO1) sequences do not correspond to any previously-reported sequence, although only limited molecular data of tardigrades have been reported so far.

**Conclusions:** Based on the *pt*-ratio (the ratio of the length of a given structure to the length of the buccal tube) of the buccal-pharyngeal apparatus and claws, the morphology of eggs, and the DNA sequences of three partial genes, this species is considered as a new species of *Dactylobiotus*.

**Keywords:** Antarctica, *Dactylobiotus*, Taxonomy
New tardigrade species from India and the problems with the taxonomic status of *Macrobiotus occidentalis* Murray, 1910

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**Background:** Tardigrades of India are very poorly known with only ca. 40 species reported. We examined four moss samples from Himalaya in which two tardigrade species were found. *Macrobiotus occidentalis* Murray, 1911 was described from British Columbia (Canada) and later reported from many localities throughout the world, probably also including many different taxa attributed to this taxon. As a result, the diagnosis of this species is unclear (uncertainty about presence of lamellae around moth opening, pores in cuticle, microplacoid and teeth on lunules). In modern taxonomy all these characters are species specific being quite uniform within a species, with very low intraspecific variability. Moreover, adults and eggs of *M. occidentalis* are similar to *Diaforobiotus islandicus* (Richters, 1904) (former *Macrobiotus*). Also, subspecies were described for these two species: *M. o. striatus* Dastych, 1974 and *D. i. nicaraguensis* (Séméria, 1985) complicating the taxonomic status of *M. occidentalis* even more.

**Results:** We found a species very similar to *M. occidentalis* but it is probably new for science. We compared our specimens with the original description of *M. occidentalis* and description of the genus *Diaforobiotus* Guidetti et al. 2016. The adults of Indian taxon agree with characters proposed for the genus *Diaforobiotus*, however the eggs are more similar to *M. occidentalis*. Therefore, we attribute our specimens to the genus *Diaforobiotus* and hypothesize that *M. occidentalis* should be also considered as a member of this genus. We also found a new species belonging to the *Echiniscus arctomys* group which differs from other species by the details of dorsal sculpture.

**Conclusions:** At present, it is not possible to definitively resolve the taxonomic problem in the *islandicus-occidentalis* group and redescriptions or reevaluation of *D. i. islandicus*, *D. i. nicaraguensis*, *M. o. occidentalis* and *M. o. striatus* are necessary. It is highly probable that the subspecies should be elevated to species level and that *M. o. occidentalis* and, eventually *M. o. striatus* should be transferred to the genus *Diaforobiotus*.

**Keywords:** *Diaforobiotus*, *islandicus-occidentalis* group, *Macrobiotus*, new species
A possible new genus of Heterotardigrada

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**Background:** Recently we examined three *Echiniscus* species, *E. danieli* Meyer et al., 2017, *E. maesi* Séméria, 1985 and *E. perarmatus* Murray, 1907, all sharing the presence of additional short appendages (spines) on legs II and III, which in other congeners are present only on legs I (spine) and IV (papilla). Another feature characteristic of these species is a dorsal sculpture, which is also characteristic of species of the *bigranulatus* group.

**Results:** Based on morphological similarities of the examined taxa we hypothesized that all three species may belong to a new genus. We then analysed 18S, 28S, and COI sequences of *E. danieli* from two localities in North and South Carolina (USA). We confirmed that both populations belong to the same species. The 18S and 28S sequences were analysed along with other sequences from GenBank belonging to *Echiniscus* taxa. Analysis of 18S sequences suggests that *E. danielli* and *E. bigranulatus* form a monophyletic group that is the sister group to a monophyletic group of all other *Echiniscus* species. However, the 28S phylogeny does not support a sister group relationship between *E. danielli* and *E. bigranulatus*. In the 28S phylogeny, both of these lineages are nested within *Echiniscus* and they are both recovered as more closely related to other *Echiniscus* species than they are to each other.

**Conclusions:** At present we have two possible explanations of our ambiguous results. First, *E. danielli* (and probably other species with *bigranulatus* type sculpture) and *E. bigranulatus* are a lineage that is separate from other *Echiniscus* species. Second, *E. danielli* and *E. bigranulatus* are clearly nested within *Echiniscus* and are both more closely related to other *Echiniscus* species than they are to each other. It also needs to be stated that spines on legs II and III are not present in other members of the *bigranulatus* group and the taxonomic value, on the generic level, of the presence of these unique spines still needs to be confirmed.

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**Key words:** 18S, 28S, *bigranulatus* group, *Echiniscus*, USA
Two new *Echiniscus* species or only one?

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**Background:** The majority of species in the genus *Echiniscus* are described based on differences in chaetotaxy (number, arrangements and shape of dorsal and lateral appendages) or dorsal sculpture. The dorsal sculpture is in general considered as species specific and not very variable. However, it relies heavily on the interpretation of microscope images and quality of optical equipment. Opposite to dorsal sculpture, chaetotaxy is much easier to interpret (presence or absence of the appendages), even using microscopes of not very good quality, but it is not a very conservative approach. Such situation creates a taxonomic mess not only in the genus *Echiniscus* but in tardigrade taxonomy in general. A good example is the *Echiniscus blumi-canadensis* complex, with few morphospecies erected, having the same sculpture type but variable chaetotaxy. It also needs to be stated that in many species chaetotaxy is different depending on life stage. In the present study we examined *Echiniscus* specimens with two different types of chaetotaxy found in the same sample collected at Ivohibory Cristal Mountain Forest (Republic of Madagascar).

**Results:** The analysed specimens are characterized in general by two types of chaetotaxy i.e. A-C-D⁴-E and A-D⁴-E, and minor differences in dorsal sculpture. We also found a wide range of variation in shape and length of appendages (from very thick, short spines to short filaments) and observed some minor differences in dorsal sculpture i.e. specific pattern on terminal plate, number and diameter of pores. Observed differences were not correlated to body length, which can negate our hypothesis about correlation with life stages. However, this aspect as well as sex-depended differences will be analysed in the near future. We also started molecular analyses of CO1, 18S and 28S sequences of both morphospecies but up to now without positive results.

**Conclusions:** We can assume that two morphospecies are present in the same environment, differing in chaetotaxy and some minor characters of dorsal sculpture. Future molecular analysis will hopefully answer the question whether these two forms are two separate species or one species with high variability in chaetotaxy.

**Key words:** chaetotaxy, Madagascar, morphospecies, taxonomy
The *Macrobiotus polonicus-persimilis* group (Eutardigrada, Macrobiotidae), another example of problematic species identifications in tardigrades

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**Background:** For many years, *Macrobiotus hufelandi* was considered to be only one species, with large morphological variability. Based on new characters and in-depth evaluations of old characters currently about 50 species are known (*M. hufelandi* group). Among them, some species share a similar egg type, i.e. *persimilis* morphotype. To better define this group, we carried out morphological and molecular analyses (*cox1* and 18SRNA genes) on populations from Poland (*locus typicus* of *Macrobiotus polonicus*), Italy and France.

**Results:** Our results support the existence of a monophyletic group that includes all specimens with a *persimilis* egg morphotype. The population from France has a little genetic distance from that of the *locus typicus* (p-distance 1.7%), but differs by having longer claws and placoids. The other populations differ from that of the type locality for at least 16% *cox1* p-distance. Bayesian and Maximum likelihood analyses reveal the presence of at least four more lineages. In Enna (Sicily) two lineages are found: morphology indicates that one of them could be *M. persimilis*. Southern (Lecce) and Central Italy (Osimo) populations share another lineage containing similar *cox1* sequences (p-distance: 2.9-3.9%). In Lecce there is only one animal and egg morphotype, both are also present in Osimo, together with another type of animal and egg. The Novellara (Northern Italy) population shows one molecular lineage possibly related to one of the lineages from Enna, but with animals showing the longest placoids and claws of all. In all samples, males (with two lateral gibbosities on the hind legs) and females (with seminal receptacle) were present.

**Conclusions:** Within the *M. hufelandi* group exists a monophyletic *polonicus-persimilis* group that includes *M. polonicus, M. persimilis* and probably at least other four species. Molecular data suggest that the populations from Poland and France belong to the same species. If so, the morphological variability should be considered intraspecific. Alternatively, differences not supported by molecular data could reflect lack of finding all haplotypes.

**Keywords:** integrative taxonomy, *Macrobiotus polonicus-persimilis* group, Tardigrada
Challenging taxonomy of the *Pseudechiniscus suillus* complex

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**Background:** The genus *Pseudechiniscus* was emended by Kristensen in 1987 [1]. It is a homogenous taxon and most species are similar to each other. The morphological characters, which are most often used in species discrimination, include: dorsal sculpture, shape and number of dorsal plates and structure of dorsal appendages. Also geometric morphometrics were proposed as a useful tool in the taxonomy of *Pseudechiniscus*. The *Pseudechiniscus suillus* group is one of the most challenging groups of tardigrades. All the species are similar, without any dorsal and lateral appendages (except for cephalic appendages and cirri A). Moreover, not only the nominal species, but also other members of the *suillus* group were insufficiently described. As a result, authors have used inadequate characters to describe new taxa and to identify specimens as *P. suillus*. At present it is hard to decide which characters are species specific and useful in species identification. In our study we examined populations/species from Africa, Antarctica, Arctic, Asia, Europe, South and Central America, which would traditionally be attributed to *P. suillus*. The populations/species were analysed by classical morphology and morphometry using phase contrast light microscopy and scanning electron microscopy as well as by molecular data.

**Results:** In addition to differences in dorsal sculpture, we found some important differences in ventral sculpture of different taxa within the *suillus* group. We also found clear differences in morphometry between *suillus* taxa. Our results suggest that at least some of the examined populations need to be considered as separate taxa. Some of them should probably be described as species new to science, but without a redescription of the nominal *P. suillus* and some of the other members of the complex this is not possible.

**Conclusions:** Summarizing, we can conclude that minor differences in dorsal and ventral sculpture are species specific. Nominal *P. suillus* is probably not a cosmopolitan taxon and *P. suillus* specimens reported from different regions belong to totally different species.


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**Key words:** barcoding, Heterotardigrada, taxonomy, *suillus* group, ventral sculpture
Ferdinand Richters (1849–1914)  
– a major “tardigradologist” at the beginning of the 20th century

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**Background:** In modern tardigradology, former tardigradologists play only a rather minor role, if at all. It is worthwhile, however, to remember some, to realize that also ‘tardigradology’ has a history. If, in addition, parts of their collections still exist, it may be possible to use them or at least to understand how their gatherers were working. The German Ferdinand Richters is one of the early tardigradologists, who has created such a specimen collection, from which some important preparations are preserved. We trace some stages of Richters’ life and scientific work presenting some of his published drawings and micrographs and some micrographs from specimens of his collection recently made.

**Results:** Richters was a specialist in crustaceans, before he began working with tardigrades. As from about 1900 he collected and described tardigrades from his near environment, later he received samples from all over the world. He published more than 30 articles, including popular ones. The first publications prove that he was a good observer and draftsman. Nevertheless, he later (apparently for the first time) used photomicrographs to illustrate his work. Through the years he described more than forty species, of which more than the half is still valid, although most have been renamed at the generic level. The most spectacular new species were Batillipes mirus (still valid) and Halechiniscus guiteli (dubious species). Occasionally he also discussed phylogenetic relationships favouring relations to annelids (Polychaeta). Posthumously, a review was published in the "Handbook of Zoology" together with Thilo Krumbach (1874-1949) and assisted by Richter's son. Richters built a sizeable collection of slides with specimens probably fixed and embedded in formol (at least in part). This collection was mostly destroyed during the war chaos. However, approximately 58 slides containing a single specimen or several specimens and their eggs "survived".

**Conclusions:** Ferdinand Richters was one of the most productive alpha-taxonomist at the beginning of the 20th century, and probably one of the first to produce a usable collection of specimens. Although available only in fragments, some preparations of this collection are in such a good condition that they have been used and are currently used for taxonomic revisions.

**Keywords:** ancient collection, alpha taxonomy, history, Tardigrada
Phylogeny of Itaquasconinae in the light of new integrative analyses and the discovery of a new genus from Borneo

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Background: Over 30 years passed since the last and only revision of the Itaquasconinae, a hypsibiid subfamily, which was originally monotypic. The 1987 revision established three new genera and in the following decades four more new itaquasconin genera were erected. Nevertheless, phylogenetic relationships between the majority of genera within the subfamily remain unknown. This may be so because of notorious problems with obtaining sufficient numbers of individuals required for integrative analyses – not only are itaquasconin species typically rare but, if found, they usually occur in low densities. Here, we present an attempt to solve some of phyletic affinities within the subfamily, using three molecular markers (18S rRNA, 28S rRNA, COI) and fine morphology of the bucco-pharyngeal apparatus (especially the annulation of the pharyngeal tube analysed in Scanning Electron Microscopy).

Results: We collected molecular and morphological data for fourteen species representing five of eight itaquasconin genera: Adropion, Astatumen, Itaquascon, Mesocrista and Platicrista. The type of the pharyngeal tube annulation holds a reliable phylogenetic signal at the genus level. However, it is homoplasious at higher taxonomic ranks, which makes it unsuitable for the classification of genera. Two general types of annulation can be distinguished: (1) simple, composed of typical annuli, being plesiomorphic to the whole family Hypsibiidae; and (2) complex, net-like annulation, present only in some itaquasconin genera. Moreover, Adropion is shown to be polyphyletic, as one of the morphological groups classified within this genus is the sister group to all other analysed Itaquasconinae. Furthermore, our analyses suggest that the reduction of placoids in the pharynx evolved convergently within the subfamily. Finally, a new itaquasconin genus from Western Borneo (Sarawak) possesses a mixture of traits of two genera. Similarly to Astatumen, it has no stylet supports, but – alike some Itaquascon species – it exhibits a narrow and elongated pharyngeal tube finished with a circular pharynx with no placoids. This finding suggests that even profound morphological changes, such as the loss of stylet supports or the presence of concave claw bases, may not allow for correct inference on affinities between eutardigrade genera.

Conclusions: The presented phylogeny shows explicitly that, at least in some tardigrade groups, accurate taxonomic classification at levels higher than generic requires sound molecular data.

Keywords: biodiversity, bucco-pharyngeal apparatus, Hypsibiidae, morphology, phylogeny
Deceptive conservatism of claws: distinct phyletic lineages hidden within the Isohypsibioida (Eutardigrada: Parachela)

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Background: Among the four superfamilies of Parachela, Isohypsibioida appear the least modified relative to the hypothetical common ancestor of this tardigrade lineage, thus presumably exhibit conserved plesiomorphic traits of all limno-terrestrial parachelans. Unfortunately, in contrast to other superfamilies, the relationships within this clade remain mostly unsolved, preventing the formulation of hypotheses on the evolution of taxonomically important traits, such as claw or apophyses for the insertion of the stylet supports morphology, within Eutardigrada. This is primarily because of the lack of molecular data and the classification of almost all isoypsibioid taxa based on the Isohypsibius type claws. However, Isohypsibioida are a very diverse group, both in terms of morphology (various levels of claw reduction or elongation, evolution of the ventral lamina), and inhabited environment (mostly mosses or lichens, but also soil, intertidal, and marine habitats, which are rare in the remaining three eutardigrade superfamilies).

Results: DNA sequencing and Scanning Electron Microscopy analyses of over 25 isoypsibioid species reveal major phyletic clades of Isohypsibioida. These groups can be characterised as follows: (a) limno-terrestrial Isohypsibiidae sensu stricto, comprising many Isohypsibius sensu lato taxa + Eremobiotus + Fractonotus; (b) marine Halobiotus, distantly related to all other isoypsibioids; (c) soil Hexapodibiidae; and sister (d) aquatic Isohypsibius spp. + Pseudobiotus + Thulinius + Doryphoribius + Apodibius. Based on these novel findings, a hypothesis describing the evolution of claw morphology in Eutardigrada is proposed.

Conclusions: In addition to the existing Isohypsibiidae and Hexapodibiidae, at least two new families should be erected within the Isohypsibioida, although Isohypsibius and Doryphoribius still remain largely undersampled and polyphyletic. Interestingly, the majority of species representing closely related genera inhabit similar environments (i.e. either aquatic or limno-terrestrial). Last but not least, our study shows that morphology alone cannot resolve deep phyletic eutardigrade affinities and some of the morphological differences between the lineages may become apparent only post hoc molecular analyses.

Keywords: claws, convergence, homoplasy, morphology, phylogeny
Apodibius confusus in Fish Creek Provincial Park, Alberta, Canada

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Background: In 2011, two specimens of a tardigrade species were found in Fish Creek Provincial Park (FCPP). These specimens completely lacked claws which is a rare phenotype amongst tardigrades. There are three known clawless eutardigrade species that are all classified under the Apodibius genus. No Apodibius species has been recorded in North America, but one Apodibius specimen has recently been found in South America by Dr. Hieronymus Dastych (2017). The identity of the specimen from South America was not confirmed but it is in the phyletic line of A. nuntius. To confirm identification of the FCPP specimens, a search was conducted in Spring/Summer 2017 for additional specimens in the same location.

Results: There were 26 total Apodibius found in four different samples of soil and ground moss. Of the 26 specimens, one specimen was lost, four specimens are preserved in alcohol and 21 were mounted on slides in polyvinyl alcohol. By measurement these 21 specimens and the original two specimens were determined to be A. confusus, later confirmed by Dr. Dastych. Mean values of specimen measurements are within one standard deviation of previous measurements of A. confusus except for the first macroplacoid percent ratio of the buccal tube.

Conclusions: This is the first recording of an Apodibius species in North America. Our data contribute morphological and geographical information on the known species, A. confusus. The current research contributes information about Alberta’s tardigrades, specifically in FCPP.

Keywords: Apodibius, Eutardigrada, North America
Morphological and phylogenetic analyses of *Milnesium cf. tardigradum* found in Japan

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**Background:** Tardigrades belonging to genus *Milnesium* have ordinarily two or three points on each secondary branch of the double claws. *Milnesium tardigradum*, the type species of *Milnesium*, has two and three points ([2-3] type). However, *Milnesium* species found in Japan have mostly three points on each secondary branch ([3-3] type). *M. cf. tardigradum* specimens having the characteristic claw arrangement of the type species have previously only been from Hokkaido Island (unpublished). Here we will discuss the morphology and molecular phylogeny of a newly discovered *M. cf. tardigradum* from Nagano and Iwate prefectures on Honshu Island, and the distantly located islands of Okinawa, and Ogasawara.

**Results:** We examined $P_t$ values of the buccal tube width and stylet support length, and gene sequences such as cytochrome c oxidase subunit I (COI) of *M. cf. tardigradum* from four localities. Although these individuals showed similar $P_t$ values to *M. tardigradum*, the molecular analysis was able to distinguish two groups among them. The specimens of the Nagano-Iwate group and the Okinawa-Ogasawara group formed different clades in the phylogenetic tree based on the COI sequences. The Okinawa-Ogasawara group branched early from the other *Milnesium* species and formed an independent clade. Interestingly, they showed the developmental changes of the opposite group e.g., juveniles of the Okinawa-Ogasawara group with [2-2] type claws increased the points during development to result in an adult [2-3] type. In contrast, the Iwate group juveniles with [3-3] claws decreased the points to [2-3]. They had modifications of the secondary branches in ontogeny.

**Conclusions:** There are several groups of *Milnesium cf. tardigradum* with [2-3] type claws in Japan. Considering their developmental traits, as well as the molecular analysis, Okinawa-Ogasawara and Iwate may be different species.

**Key words:** COI, Milnesiidae., ontogeny, secondary branch, , taxonomy
An integrative description of *Richtersius coronifer* (Richters, 1903) from the original *locus typicus* in Spitsbergen

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**Background:** *Richtersius coronifer* was described in 1903 from Spitsbergen and it is the only recognised species in the genus. This species is an example of common problems in modern tardigrade taxonomy, where old and incomplete descriptions of taxa hinder species delineation which translates to difficulties in biodiversity assessment. Although over the decades many researchers recorded *R. coronifer* throughout the world, recent research indicates that these records likely represent a species complex rather than a single cosmopolitan species. However, in order to recognise and name the species diversity within the complex, first, an integrative redescription of the nominal species is needed. Although Maucci & Ramazzotti (1981) established the neotype for *R. coronifer*, they redescribed the species using specimens from continental Norway. Thus, there is a considerable risk that the neotype represents a different species than *R. coronifer* sensu stricto. Therefore, the neotype needs to be compared both with specimens from Spitsbergen (type locality) and continental Norway (neotype locality) to establish whether they represent a single or two species. Here, we provide an integrative description of a *R. coronifer* population collected from the exact original *locus typicus* and we compare it with specimens from continental Norway and other European countries. We also compare COI sequences from Spitsbergen with sequences available from GenBank to test for hidden species diversity within this group.

**Results:** Integrative taxonomy approach allowed us to describe a topotype population of *R. coronifer* and perform molecular species delimitation within the genus. Our description is based on detailed morphological and morphometric data obtained with phase contrast light and scanning electron microscope as well as on DNA sequences of three nuclear markers (18S rRNA, 28S rRNA, ITS-2) and a mitochondrial gene (COI). The analysis of available COI sequences confirmed the presence of several species within the genus *Richtersius*.

**Conclusion:** This work is an example of integrative taxonomic research which overcomes the impediment caused by an inaccurate original description of the nominal species. By providing an integrative description of a topotype *R. coronifer* population, we mark a starting point for further research on the taxonomy and species diversity within the genus. Moreover, we confirm prospective existence of several distinct species within the genus *Richtersius*.

**Keywords:** biodiversity, integrative taxonomy, species delineation, Svalbard archipelago, taxonomic impediment
Preliminary study of the tardigrades of La Malinche National Park, Tlaxcala, Mexico

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Background: The diversity of the Tardigrada in Mexico is represented by two classes: Heterotardigrada (with two families, four genera and 12 species) and Eutardigrada, (with five families, 11 genera and 32 species). To date, 44 species have been recorded (41 terrestrial and three from freshwater environments). However, there are very few works related to the Mexican tardigrades, some species are doubtful and need confirmation. Of the 32 federal entities, only nine present records, which indicates that the tardigradological fauna of Mexico is still far from being well known. The present study was carried out at La Malinche National Park in central Mexico, with a predominant vegetation of oyamel, oak and pine forest, and where no previous records of tardigrades are known.

Results: Twenty two tardigrade species have been identified from a total of 1850 specimens isolated from mosses samples collected in september 2016 and august 2017. All species represent new records for the state of Tlaxcala, and the species of Echiniscus arctomys, E. merokensis, Milnesium katarzynae, Platicrista sp., Pseudechiniscus spinerecutus, and Ramazzottius sp., represent new records for Mexico. The four species of mosses analyzed (Ceratodon sp., Hypnum amabile, Neckera chlorocaulis, and Thuidium delicatulum), had never previously been reported as tardigrade associated species. The species of mosses H. amabile, N. chlorocaulis and T. delicatulum represent new records for the state of Tlaxcala.

Conclusions: Despite its biological importance and potential in other areas such as Biomedicine and Biotechnology, there have been little advances in the knowledge of the diversity of tardigrades in Mexico. The study of Tardigrada needs to continue in the state of Tlaxcala as well as the analysis of its composition at different altitudes to understand how these organisms differ between the microhabitats.

Keywords: La Malinche, Mexico, Tardigrada, Tlaxcala
A discovery and phylogenetic analysis of a putative novel species of a limnoterrestrial tardigrade from Diliman, Quezon City, Philippines

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Background: There are currently over 1200 known tardigrade species documented worldwide, but the data on the tardigrade fauna in tropical regions are scarce. In the Philippines, only six tardigrade species have been recorded. In this study, a limnoterrestrial tardigrade was isolated from moss on the surface of a small rock and is here preliminarily characterised using both morphological and molecular analyses.

Results: Phase contrast microscopy and scanning electron microscopy of animals and eggs isolated from an isogenic culture revealed that the species represents the Mesobiotus furciger group. This was further supported by phylogenetic analyses of DNA sequences of three nuclear and one mitochondrial markers: the small and large ribosomal subunits (18S rRNA and 28S rRNA, respectively), internal transcribed spacer-2 (ITS-2), and cytochrome oxidase subunit I (COI), which all clustered with other Mesobiotus sequences available from the GenBank. The p-distances between COI sequences of our species and other Mesobiotus spp. ranged from 21.6% to 26.1%, i.e. the minimal distance was well above the 3% threshold typically used for species delineation. Preliminary phenotypic analysis suggests that the strain is the most similar to Mesobiotus creber and Mesobiotus orcadensis, but differs from them by the presence of multiple finger-like projections on the apices of egg processes, larger meshes on the egg surface, and less slender egg processes. Taken together, these results suggest that the strain represents a new species of the genus Mesobiotus.

Conclusions: The results of our analyses suggest that the isogenic strain may represent a new tardigrade species of the furciger group. The species belongs to the same genus as other previously described limnoterrestrial tardigrade species in the Philippines. However, this is the first report of a species of the furciger group from the country. Additional morphological and morphometric data are necessary to confirm the identity of the species.

Keywords: integrative taxonomy, Mesobiotus furciger group, Philippines, Tardigrada
Are one-to-one morphometric comparisons reliable in tardigrade species identification?

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**Background:** Despite advances in integrative taxonomy, tardigrade systematics still relies mostly on phenotypic traits. This is so, because the great majority of the known tardigrade species were described before the DNA sequencing era and only a handful of old taxa have been redescribed by means of integrative taxonomy. However, even nowadays, many species descriptions are based solely on phenotypic traits. Moreover, it is not infrequent that such classical descriptions rely on morphometric measurements of a very few (sometimes even single) individuals. Thus, when faced with scarce morphometric data, taxonomists often compare dimensions of taxonomically important traits in single individuals of similar body size and conclude that individuals represent different species if the dimensions diverge. Although one-to-one comparisons are widely used in differential diagnoses, the accuracy of this method has never been rigorously tested. Here, we test this method by applying computer simulations to a large data set for six species representing four eutardigrade families.

**Results:** Our simulations show that conspecific individuals of similar body lengths (±10 μm) or similar buccal tube lengths (±1 μm) exhibited considerable differences in other measured traits, such as placoid or claw lengths. Whereas body or buccal tube length ranges constituted only a fraction of the population ranges, in the majority of absolute and relative taxonomic traits the values oscillated around 50% and 70% of the population ranges, respectively.

**Conclusions:** With the constantly increasing number of described tardigrade species, and the limited number of morphological and morphometric traits, morphometric comparisons will have to become more accurate in order to remain useful for species delineation. Our study shows explicitly that one-to-one comparisons may be misleading as they often result in false positives, *i.e.* individuals of a single species that are similar in body size may exhibit divergent dimensions of other morphometric traits. This, in turn, may lead to an incorrect assignment of conspecific individuals to different species. Thus, comparisons based on larger sample sizes are strongly recommended as the primary method of morphometric comparisons and phenotypic species delineation, especially that molecularly identified pseudocryptic species may be morphometrically distinguishable only via statistical testing that requires sufficient sample sizes to be reliable.

**Keywords:** morphometry, sample size, species delineation, Tardigrada, taxonomy
A revaluation of *Diphascon pingue brunsvicense*

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**Background:** In 1972, Argue described *Diphascon pingue brunsvicense* as a new sub-species during his survey of New Brunswick tardigrades, thus splitting *D. pingue* into two sub-species. *Diphascon pingue brunsvicense* was defined as distinct from *D. p. pingue* based on a higher mean body size and higher width-to-length ratio. *D. p. brunsvicense* has only been documented in southern New Brunswick during Argue’s survey while *D. p. pingue* has been found throughout the continental United States and Europe. Modern morphometric analysis of Argue’s original type specimens, European *D. pingue* type specimens, 25 *D. pingue* specimens collected in Maine, and 5 new *D. pingue* specimens collected from Argue’s original sampling sites in New Brunswick allowed us to determine whether *D. p. brunsvicense* is a valid sub-species.

**Results:** Buccal tube length (BTL) was used as a metric for body size comparison. Body and buccal tube widths were corrected for body size effects utilizing Thorpe’s normalization. No significant differences were found between any of the populations’ mean BTLs (ANOVA, $p = 0.2377$), normalized widths (ANOVA, $p = 0.1143$), or normalized buccal tube widths (Kruskal-Wallis, $p = 0.1043$).

**Conclusions:** These results suggest that *Diphascon pingue brunsvicense* is a synonym for *Diphascon pingue pingue* and all specimens of this species should be referred to as *Diphascon pingue*. As Argue originally noted, no significant differences exist between the populations’ buccal apparatuses and this study found no significant differences in the populations’ body sizes and shapes.

**Keywords:** *Diphascon pingue*, Eutardigrada, morphometrics, taxonomy
Clarification of taxonomic status and geographic distribution of *Echiniscus merokensis* Richters, 1904 *sensu lato* in the light of integrative taxonomy

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**Background:** *Echiniscus merokensis* was described by Richters in 1904 [1] from samples collected in southern Norway. Seven years later Thulin [2] found specimens very similar to *E. merokensis* (*E. merokensis* var. *suecica*) in Sweden, but noted a different size of the animals and the presence of cirri *B*, which were absent in the nominal taxon described by Richters [1]. Since that time, both forms (with and without cirri *B*) have been reported from many localities in and outside Europe. Therefore, in recent years a careful revision of all records outside the Holarctic has been suggested. In our study we examined representatives of a few populations from Antarctica, Arctic, Asia, Europe and South America morphologically attributed to *E. merokensis*. The specimens were analysed by classical morphology and morphometry based on phase contrast light microscopy (PCM) and scanning electron microscopy (SEM) as well as a molecular approach based on COI (cytochrome c oxidase subunit 1) sequence analyses.

**Results:** The analysis of COI sequences as well as morphology and morphometry of the animals confirmed the wide distribution of *E. merokensis*, which could suggest a cosmopolitan or at least semi-cosmopolitan distribution of this taxon (*p*-distance of specimens from different continents = 0.4-1.0%). Our data also enabled us to prepare a detailed redescription of the species and a clarification of its taxonomic status. We also partially verified the geographic distribution of this taxon.

**Conclusions:** We clarified the taxonomic status of *E. merokensis* and its distribution throughout the world and confirmed the presence of this taxon on at least four continents using different methods (PCM and SEM analyses as well as COI sequence analysis). Our results support the previously negated proposition that at least some tardigrade species have a cosmopolitan distribution.


**Keywords:** barcoding, Heterotardigrada, species complex, taxonomy
Remarks and updated key to the genus *Mesobiotus* Vecchi, Cesari, Bertolani, Jönsson, Rebecchi & Guidetti, 2016 (Eutardigrada, Macrobiotidae) and description of a new species from the Republic of Madagascar

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**Background:** The genus *Mesobiotus* was recently established by Vecchi [1] and comprises traditionally recognized species groups: *Mesobiotus harmsworthi* and *M. furciger*, specifically. Few years earlier, Kaczmarek [2] published a dichotomic key to the species of the *M. harmsworthi* group, whereas the comprehensive key to the *M. furciger* group has never been prepared. In the light of recent taxonomic changes and numerous new species descriptions, an updated and exhaustive examination of the species composition within the *Mesobiotus* genus as well as a key for their identification is extremely necessary.

**Results:** In the present study we analysed all *Mesobiotus* species based on original descriptions and other relevant literature as well as direct examination of taxa. We collected an extensive morphological data set enabling recognition of several different types of egg processes and egg shells. The data was used to prepare a dichotomic key to the genus, based mostly on newly proposed types of egg ornamentation, as well as a discussion on geographical distribution of this taxon. Furthermore, in the mosses and lichens collected in the Republic of Madagascar we found a new *Mesobiotus* species from the *furciger* group, which is described by means of integrative taxonomy. The species description and species delineation involved morphological and morphometric data obtained using phase contrast light and scanning electron microscopy as well as DNA barcoding.

**Conclusions:** Based on egg morphology we recognized more species groups in the genus *Mesobiotus*. Species from this genus are widely distributed throughout the world and most of them have restricted distribution or are known only from type localities. Except of a probably incorrect report of *M. harmsworthi*, this is the first report of *Mesobiotus* species from Madagascar.


**Keywords:** Africa, *furciger* group, *harmsworthi* group, *Mesobiotus fiedleri* sp. nov.
Splitting the *Pseudechiniscus* line

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Background: In 1987, Kristensen [1] divided the Echiniscidae into two major lines of evolution based on the presences or absence of pseudosegmental plates, the *Pseudechiniscus*-line and the *Echiniscus*-line. *Acanthechiniscus, Antechiniscus, Cornechiniscus, Hypechiniscus, Multipseudechiniscus, and Mopsechiniscus* have the classic pseudosegmental plate IV but most have pseudosegmental plates on other segments too. For decades the *Pseudechiniscus*-line has been considered polyphyletic. Most recently, integrative taxonomy was used to move the *P. victor* group to a new genus *Acanthechiniscus* [2]. In their amended descriptions, the presence of additional pseudosegmental plates is mentioned in neither the description of *Acanthechiniscus* nor *Pseudechiniscus*. However, examination of our collections using autofluorescence imagery are showing more and more differences within the dorsal plates of the members of this line.

Results: Specimens of *Pseudechiniscus* from America and Europe (The Azores and Portugal), *Hypechiniscus* and *Multipseudechiniscus* were examined using autofluorescence microscopy in addition to Nomarski interference contrast and phase microscopy. Images of these members of the *Pseudechiniscus*-line show new divisions and pairings in dorsal segmental plates, varying numbers and shapes of median plates and the presence of previously undescribed lateral plates on specimens that under light microscopy were not described. Several of these patterns do not fit existing generic descriptions.

Conclusions: We think it is time to more clearly define the dorsal plate patterns in each of the genera of the *Pseudechiniscus*-line. We believe that clearly stating what plates or pieces should be present or absent within each genera and how they are shaped will produce some clarity within the lineage.


Keywords: dorsal plates *Pseudechiniscus*, pseudoplates.
Classification of instars in the genus *Milnesium* Doyère, 1840 based on morphometric data

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**Background:** Identification of tardigrade species still relies mostly on morphological and morphometric traits. Given that the number of available taxonomically significant traits in the apochelang genus *Milnesium* is exceptionally low, ontogenetic variability may provide additional ways of species delineation. However, establishing whether both immature and mature instars are present in examined populations is notoriously difficult, especially if the number of available individuals is low. The most reliable method of detecting ontogenetic changes is developmental tracking. Unfortunately, however, the method is time-consuming and requires culturing of live animals, which is not always possible. Therefore, the goal of this study was to develop an analytical morphometric method that helps to test whether immature life stages are present in a set of individuals fixed on permanent microscope slides.

**Results:** We analysed morphometric data for hatchlings, juveniles and adults representing eight *Milnesium* species. We found that body length is an inadequate criterion for differentiating between instars, because body size varies significantly between *Milnesium* species and it is prone to deformation. However, we also found that other traits, such as buccal tube or posterior primary claw branch may independently allow to assign individuals to immature and mature instars with reasonable probability. Moreover, given that missing measurements are common in tardigrade taxonomy, various methods of handling missing data were also evaluated in aiding instar classification.

**Conclusions:** Classical taxonomy is becoming insufficient in delineating species and describing the diversity within the genus *Milnesium*. Since the identification of ontogenetic variability may be crucial in species delimitation, our test for the presence of immature and adult instars in *Milnesium* populations fixed on microscope slides could be a useful tool in detecting developmental variability and thus also in species delineation. Given that collecting morphometric data is extensively time-consuming, data sharing via public repositories is hoped to aid and accelerate taxonomic research.

**Keywords:** Apochela, hatchling, juvenile, life stages, morphometry
Morphological and genetic data show that North American *Diploechiniscus* are not *Diploechiniscus oihonnae*

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**Background:** *Diploechiniscus oihonnae*, a terrestrial tardigrade, is the only described species in its genus. Originally published in 1903 from Norwegian material as *Echiniscus oihonnae*, it was redescribed in 2016 from European samples and designated the type species of a new genus. In North America the species has been reported from British Columbia, California and Wisconsin. In 2017 we collected samples from Mountain Lake Biological Station, Virginia of what initially appeared to be *D. oihonnae*. We also examined specimens from the Robinson-Winter Museum of Natural History identified as *D. oihonnae* from Alaska and Georgia. We sequenced the *cox1* region of five Virginia specimens. The purpose of this investigation is to determine whether North American specimens of *Diploechiniscus* are significantly different from *D. oihonnae* sensu stricto, and therefore represent an undescribed species.

**Results:** In most respects the habitus and morphometrics of Virginia specimens closely resemble those of *Diploechiniscus oihonnae*. However, tubercles on the scapular plate of Virginia specimens are wider than those on European *D. oihonnae* (maximum 1.6 μm diameter vs. 1.0 μm). The most significant difference is in lateral and dorsal appendages. In European *D. oihonnae* appendages A, B, C, C\(^d\), D and E are always filaments, while D\(^d\) is a spine. Virginia specimens differ in that appendages B, C\(^d\) and D are always long spines in Virginia. Appendages in Georgia specimens are the same as in Virginia animals. Specimens from Alaska lack appendage B, and have spines at C\(^d\) and D. We also plan to examine other museum specimens identified as *D. oihonnae* that were collected in California. Among Virginia *Diploechiniscus*, *cox1* p-differences ranged from 0.7% to 2.7%; p-distance between Virginia *Diploechiniscus* and European *D. oihonnae* ranged from 11.1% to 12.1%.

**Conclusions:** The combination of morphological differences and genetic distance indicate that the Virginia population of *Diploechiniscus* is not *D. oihonnae* sensu stricto, but rather an undescribed species. Morphological traits suggest that Georgia, and possibly Alaska, *Diploechiniscus* are the same species as in Virginia.

**Keywords:** biodiversity, biogeography, DNA sequence data, *Diploechiniscus* sp., Echiniscidae
A new species of the genus *Milnesium* Doyère, 1840 with an intriguing pattern of ontogenetic variability in claw configuration

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**Background:** It was recently shown that ontogenetic variability in claw morphology (claw configuration change) is present in some *Milnesium* species. So far, the change was shown to occur either between the hatchling (1\textsuperscript{st} instar) and the juvenile (2\textsuperscript{nd} instar) or between the juvenile and the adult (3\textsuperscript{rd} instar). Moreover, the change may result in an increase (positive change) or a decrease (negative change) in the number of points on the secondary branches. How common among *Milnesium* species this phenomenon is, still needs to established, but our preliminary data suggest that it is widespread throughout the mitesiid phylogenetic tree. Here, we describe a new pattern of claw configuration (CC) change in a new species of *Milnesium* from Iceland.

**Results:** The new species exhibits a double change in CC during the ontogeny, which is described here for the first time. The detailed analysis of morphological and morphometric traits was carried out using both light and scanning electron microscopy. A phylogenetic analysis, based on four genetic markers (nuclear 18S rRNA, 28S rRNA, ITS-2 and mitochondrial COI), showed that the new species is closely related to *M. tardigradum*.

**Conclusions:** A double ontogenetic change in CC in the new species highlights the importance of developmental tracking in the taxonomy of *Milnesium*. Moreover, the description of the new species questions the alleged cosmopolitan distribution of the nominal species for the genus and stresses the importance of DNA barcoding in modern *Milnesium* taxonomy.

**Keywords:** barcoding, cryptic species, integrative taxonomy, new species, ontogenetic variability
Morphological and molecular analysis of *Milnesium cf. granulatum* from New Hampshire, USA

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**Background:** Although tardigrade diversity in North America has been well studied in some areas, many areas remain poorly known. One goal of our research is to achieve a better understanding of North American tardigrade distribution. In 2014 we collected tardigrades from moss on trees in New Hampshire (NH), USA. Here we report the results of genetic and morphological analysis of adult and neonate specimens of one NH species.

**Results:** NH specimens most closely resemble *Milnesium granulatum*, a species known from Europe and North Carolina (NC), USA. They have the same adult claw configuration ([3-3]-[3-3]) and bands of granulation on the cuticle. NH a* pt values differ somewhat from NC *M. granulatum*, most notably in that NH animals have a more anterior stylet support insertion point (NC: a* pt 67.1, n=18; NC: 63.6, n=7). However, all morphometric values for NC and NH, including stylet support insertion points, overlap in range. Therefore we refer to NH animals as *Milnesium cf. granulatum*. Unlike other species in the genus, the claw configuration of *Milnesium cf. granulatum* shows no ontogenetic change in claw configuration; i.e., all neonates have [3-3]-[3-3] configuration. Molecular data from the internal transcribed spacer region 2 (ITS2) was collected from 20 adults and from siblings hatched in the laboratory from 8 egg clutches. This region was 96-100% identical among adults. Siblings from the same clutch were 98-100% identical. In one clutch P-distances between siblings ranged from 0.011 to 0.034, indicating sexual recombination. Comparison of the ITS2 region of *Milnesium cf. granulatum* to other *Milnesium* species is as follows: 73% similar to *M. lagniappe* (USA), 78% similar to *M. berladnicorum* (Romania), 80% similar to *M. tardigradum* (Germany), and 79% similar to *M. variefidum* (Scotland).

**Conclusions:** In some *Milnesium* species claw configuration undergoes ontogenetic change; our results show that this is not true in all species in the genus. Genetic data are not available for *M. granulatum* from NC or Europe. Morphometric traits from NH differ in mean from NC, but overlap in range. Therefore, at this time it is premature to conclude that the NC animals are *M. granulatum* sensu stricto.

**Keywords:** biogeography, DNA sequence data, *Milnesium cf. granulatum*
Genetic variability within and among species of *Milnesium*

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**Background:** Classically, tardigrade species have been identified and distinguished using morphological data; recently molecular data have become increasingly important. *Milnesium lagniappe* Meyer, Hinton and Dupré, 2013 was first collected from moss in Calcasieu Parish, southwestern Louisiana, USA, where it is very common. It is characterized by [2-3]-[3-2] claw configuration in adults and the presence of 9 dorsal and lateral sculptured bands of tubercles. The species exhibits ontogenetic change in claw configuration; neonates and juveniles are [2-2]-[2-2]. Based on morphological criteria specimens from Fort Myers, Florida, USA were also identified as *M. lagniappe*. The aim of this study was to collect molecular data on *M. lagniappe* to determine the level of genetic diversity within the species, and compare it to other *Milnesium* species.

**Results:** We examined the internal transcribed spacer region (ITS2), which has a fast rate of evolution and should allow for differentiation between closely related species. DNA isolated from seven Louisiana *M. lagniappe* was analyzed and was between 99.3-100% identical over a 443bp region of ITS2. However, the ITS2 region of *M. lagniappe* is only 76% similar to *M. variefidum* (from Scotland; [2-3]-[2-2], no sculptured bands), 73% similar to *M. berladnicorum* (Romania, [2-3]-[2-2], no sculptured bands), 72% similar to *M. tardigradum* (Germany; [2-3]-[3-2], no sculptured bands), and 73% similar to *Milnesium cf. granulatum* (USA, [3-3]-[3-3], reticular dorsal sculpture). Preliminary results from the ITS2 region of a specimen from Florida suggest that it is 98% identical to Louisiana *M. lagniappe*.

**Conclusions:** Many tardigrade species are known only from their locus typicus or nearby areas. Our preliminary results suggest that the Louisiana and Florida populations, separated by approximately 1,600 km (on land), represent the same species. For the five species compared in this study similarity/differences in claw morphology are not correlated with genetic difference.

**Keywords:** Biogeography, DNA sequence data, *Milnesium lagniappe*
New records and species of Tardigrada from Republic of Madagascar

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**Background:** Madagascar along with neighbouring islands is one of 35 Earth biodiversity hotspots. Madagascar is characterized by a large diversity of ecosystems, ranging from rainforests to hot desert and broad mountain areas. Invertebrate fauna in this region is very rich, however very little is known on tardigrades. To date, only 13 tardigrades species have been reported from the Republic of Madagascar (including four new for science). In 2017, Marta Kepel and Andrzej Kepel collected 122 bryophyte and lichen samples in Ivohibory Cristal Mountain Forest. All of them were dried in paper envelopes and transported to Poland where they were examined for tardigrades with standard methods.

**Results:** During our investigation 122 samples were examined. In 70 positive samples (57.38%), ca. 2015 tardigrades and ca. 221 eggs were found. The species were preliminary identified and attributed to ca. 26 taxa, including at least 7 new for science. The new species belong to the genera **Bryodelphax** Thulin, 1928, **Doryphoribius** Pilato, 1969, **Echiniscus** C.A.S. Schultze, 1840, **Macrobiotus** C.A.S. Schultze, 1840, **Mesobiotus** Vecchi, Cesari, et al., 2016, **Milnesium** Doyère, 1840 and **Minibiotus** R.O. Schuster, 1980. A few species are also new records for Madagascar, most of them were earlier known only from Southern Africa or the archipelagos of the Indian Ocean. Considering that the samples were collected from a rather small area, the diversity of tardigrades was very high.

**Conclusions:** Our results show that the tardigrade fauna of Madagascar is species rich and extremely poorly known. It is also obvious that it is unique, but it is closely related to the South African tardigrade fauna.

**Keywords:** Africa, Eutardigrada, fauna, Heterotardigrada, taxonomy
A survey of the Belgian tardigrade fauna

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Background: The Belgian tardigrade fauna is relatively poorly known, with only four publications mentioning multiple species. As of 2018, 46 species are mentioned for the Belgian fauna. As the most recent publication dates back from 1998, and advances in systematics have led to more detailed species delineation, many of these records (e.g. *Macrobiotus hufelandi*, *Milnesium tardigradum*) require a re-evaluation.

Results: We took limnoterrestrial samples from all over Belgium, finding several species new for the Belgian fauna. These species were investigated under light microscopy, SEM and morphometrically analyzed. Poorly described species were thoroughly described in our study. Additionally, old records were re-evaluated and an updated checklist was composed. As of March 2018, 7 new records for the Belgian fauna have been found: *Milnesium* cf. *alpigenum* Ehrenberg, 1853; *Isohypsibius dastychi* Pilato, Bertolani & Binda, 1982; *Hypsibius microps* Thulin, 1928; *Mesobiotus montanus* (Murray, 1910); *Macrobiotus persimilis* Binda & Pilato, 1972; *M. polonicus* Pilato, Kaczmarek, Michalczyk & Lisi, 2003 and *Xerobiotus pseudohufelandi* (Iharos, 1966). Several morphospecies of the *Echiniscus blumi-canadensis* complex were found, but as morphology is not a sufficient criterium for species delineation in this species complex, we provisionally refrain from adding them to the checklist. Furthermore, specimens suspected to belong to an undescribed species of to the *Macrobiotus hufelandi*-group were found. The species is characterized by the presence of eyes, granulation on all legs, crenate lunules on legs IV, buccal armature of the *maculatus* type, pt-ratio of the insertion of the stylet supports and eggs of the *hufelandi*-type with slightly jagged edges.

Conclusion: The current study is a work in progress and the number of species known from Belgium will undoubtedly increase in the further run of this project. Over the next years, we will take limnoterrestrial, limnic and marine samples in the various ecoregions of Belgium to get a more complete image of the Belgian tardigrade fauna.

Keywords: Belgium, Biodiversity survey, Integrative taxonomy, *Macrobiotus hufelandi* group
The tardigrade diversity of an oak forest in Southern Norway

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**Background:** The Tardigrade diversity of Norway is assumed to be high, but many ecosystems and geographical regions have received little attention. In literature, investigations are skewed towards Svalbard, and northern and eastern parts of mainland Norway. This study aims to investigate the unexplored tardigrade diversity of an oak forest in southern Norway by traditional methods and DNA barcoding.

**Preliminary results:** Our findings suggest an overall diverse composition of tardigrade species from bryophyte, lichen and leaf litter samples with notable differences between samples. In general, bryophyte samples had high abundances and many species, lichens had high abundances and few species, while leaf litter samples contained high diversity, but few individuals. Corrected for biomass, the bryophyte-, lichen- and leaf litter samples contained an average of 261, 164 and 63 individuals, respectively, while, for the same samples, the average numbers of species found were 13, 7 and 11. A total number of 36 species have so far been reported, with five new recordings for Norway: *Mesobiotus coronatus*, *Macrobiotus furcatus*, *Itaquascon placophorum*, *Astatumen trinacriae* and *Echiniscus* cf. *crassispinosus*.

**Concluding remarks:** Although only half of the samples have been investigated so far, a variety of species from several genera have been found. Several specimens have not yet a clear taxonomic identification and should be further investigated for possible new species to science. We intend to develop a DNA barcode library of the contained species to facilitate a planned metabarcoding survey, evaluating a more efficient method of identifying and exploring tardigrade diversity.

**Keywords:** Ecology, DNA barcoding, Metabarcoding, Southern Norway, Tardigrade diversity
An integrated study of the biodiversity within *Pseudechiniscus* (Heterotardigrada, Echiniscidae)

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**Background:** *Pseudechiniscus* is the second most species-rich genus in Heterotardigrada and in the family Echiniscidae, second only to the genus *Echiniscus*. However, previous studies have pointed out polyphyly and heterogeneity in this taxon. The recent erection of the genus *Acanthechiniscus* has restored monophyly to *Pseudechiniscus* but not homogeneity. The present investigation aimed at clarifying biodiversity and taxonomy of *Pseudechiniscus* taxa, with a special focus on species pertaining to the so-called “*suillus-facettalis* group”, by using an integrated approach of morphological (light and scanning electron microscopy) and molecular (18S, 28S and *cox1* genes) investigations.

**Results:** The analysis of sequences from specimens sampled in Europe and Asia and those available in public databases confirmed the monophyly of the genus *Pseudechiniscus*. Inside the genus, two main evolutionary lineages were recognizable: the *P. novaezeelandiae* lineage and the ‘*P. suillus-facettalis* group’ lineage. Their divergence, defined by the different shape of their cephalic papilla, being elongated in the former and dome-like in the latter, was also confirmed by molecular data. Inside the ‘*P. suillus-facettalis* group’, it was possible to describe new species through an integrated approach, but *cox1* molecular data also pointed out a very high variability (up to 26.6% p-distance) among specimens sampled in the same locality, even though not supported by morphological data.

**Conclusions:** The integrated approach to the study of *Pseudechiniscus* allowed to confirm and clarify its taxonomic position and the relationships within the taxon. It also pointed out that specimens sampled in European, Asian, Arctic and Antarctic regions all belong to the *Pseudechiniscus* genus, thus highlighting its global wide distribution. Moreover, the present study reported for the first time the presence of cryptic species in the genus *Pseudechiniscus*.

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**Keywords:** DNA sequence data, Heterotardigrada, integrative taxonomy, *Pseudechiniscus*
Tardigrades in Norwegian forests

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Background: The knowledge of Norwegian tardigrades is poor and their diversity, distribution and
ecology in Norwegian forests is unknown. This project aims to investigate tardigrade diversity
associated with different types of substrates in forests in Norway, evaluate the impact of forestry
management practices on tardigrade biodiversity for future conservation policies, and expand the
DNA barcode library of Norwegian tardigrades. It will also use environmental barcoding of
substrates to test the effectiveness of this method in documenting tardigrade diversity and
distribution.

Results: We collected three hundred bryophyte-, lichen- and leaf litter samples from various
protected deciduous and coniferous forests in Norway in 2017. The vegetation in each sample was
identified, mostly to species-level. Tardigrades were extracted from most bryophyte- and lichen
samples, and some litter samples. Preliminary analyses show that there are differences in abundance
and community composition between both forest- and substrate types. Litter samples show lower
abundances than bryophyte and lichen samples, but a higher diversity than expected.

Conclusions: Remaining samples still need to be processed, but our preliminary conclusion is that
different substrates and forest types host different tardigrade communities. DNA-barcoding will be
performed on single specimens of as many of the sampled species as possible and added to the
Barcode of Life Data Systems database (BOLD). We expect that DNA metabarcoding of
environmental samples from selected localities will record the same diversity as traditional
extraction of specimens, but also add information on the presence of species that were undetected.

Keywords: DNA barcodes, forest, lichens, litter, metabarcoding, moss
What’s in a size?
Phenotypic variation across *Acutuncus antarcticus* populations

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**Background:** Tardigrades, are one of the most diverse and common groups of animals found throughout continental Antarctica and play an important role in biological processes in its challenging terrestrial ecosystems. *Acutuncus antarcticus* is currently thought of as a truly pan-Antarctic species which can survive in most terrestrial habitats in Antarctica, although it is most commonly found within algal mats. Previous studies into phenotypic variation between continental populations of this species showed little difference. Here we build on these studies by measuring phenotypic variation over a geographical area expanded to include the sub- and maritime Antarctic from six populations. These populations were analysed for patterns of variance using principal component analysis for a range of differing morphological characters.

**Results:** Obtained data indicate that variations exist between populations of *A. antarcticus* in Antarctica forming into three groups. Group one included Alexander, Deception and Signy Islands, group two included Livingston Island and Davis Valley and group three included James Ross Island. Each of these groups are defined by a different morphological character, either claw morphology (group one), buccal apparatus morphology (group two), or body size (group 3). These groups however are not defined by geographical area.

**Conclusions:** These differences in morphology could suggest that speciation is occurring in response to differences in local environmental conditions. Further molecular work and the inclusion of more geographical areas would be needed to confirm or deny this.

**Keywords:** *Acutuncus antarcticus*, Antarctica, phenotypic variation, principal component analysis, speciation.
Indian scenario of marine tardigrade research

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Background: Marine tardigrades have been a recent object of increased ecological and systematic investigations. They are part of the meiofauna found in intertidal and sub-tidal areas down to the abyss. Most species are interstitial, but some are algal associates and others are associated with barnacles and other invertebrates. Research on marine tardigrades is limited [1], even though they are a phylogenetically important group [2, 3]. In order to facilitate future studies on Indian marine tardigrades, we here provide an overview of the records of marine tardigrades from Indian waters.

Results: Several species of marine interstitial tardigrades have been described in recent years from different parts of the world, but records of tardigrades from Indian waters are limited with only six studies primarily from the eastern part of the Indian Ocean. The sampled species include *Batillipes carnonensis* (Andhra Pradesh and Odisha), *Stygarctus bradypus* (Odisha), *Parastygarctus higginsi* and *Stygarctus bradypus* (Andaman and Nicobar islands) collected from sediments such as medium sand, organic detritus, medium sand with rich organic debris, medium and fine sand with detritus and medium sand with little detritus [4-7]. Research on marine tardigrades is challenged by rarity of specimens and by the lack of information on species distribution. The tropics encourage the growth of tardigrade competing microbes such as bacteria and fungi, thus making a competitive habitat for tardigrades, which may be an additional reason.

Conclusions: More effort should be placed on marine tardigrade research from tropic regions, which would contribute to the understanding of how tardigrades differ between different environments, in terms of biology and tolerance spectra. Integrative studies, which combine molecular and morphological data to describe species, together with studies of stress tolerance, would be highly beneficial in this regard.


Keywords: *Batillipes carnonensis*, India, marine tardigrades, *Parastygarctus higginsi*, *Stygarctus bradypus*. 
Effects of urbanization on the tardigrade communities in the Argentine Republic

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Background: Communities of urban tardigrades are little studied worldwide. For the Argentine Republic, a single study evaluated the diversity of tardigrades in an urban-rural gradient in the city of Salta. In the present work, we continue this line of research, deepening on the effects that vehicular traffic of the city (classified in High, Medium and Low) may have on tardigrade diversity in these different urban environments. Specifically, we want to determine if a process of biotic homogenization occurs in the area under study. Sites between 1100-1200 masl in the city of Salta were selected, and lichens and mosses that grew on the trunks of trees were sampled. The samples were treated according to the usual methodology and the tardigrade and their eggs were mounted in polyvinyl-lactophenol. Different variables of the micro-habitats were analysed and considered in the evaluations. The data analyses were carried out through PAST, PC-Ord and R programs.

Results: Three thousand forty-nine specimens representing 16 species of heterotardigrades (Echiniscidae) and eutardigrades (species belonging to Milnesidae, Macrobiotidae, Hypsibidae) were collected. The environments with Medium vehicular traffic was 1.12 and 1.23 times more diverse than those with Low and High traffic, respectively. The environments with High traffic showed the lowest diversity of tardigrades. The ordination analyses explained more than 60% of the total variance in two axes, showing a nested pattern in the three environments studied. The indicator value analysis (IndVal) showed that one Milnesium species (Milnesium sp7) (46.6% p = 0.03) is an indicator for Medium vehicular traffic environments. The partition of the $\beta$ diversity in its different components (replacement and loss of species) showed that the replacement of species was higher among the communities with High and Medium traffic, while a loss of species was evidenced between the communities of High and Medium transit.

Conclusions: The Salta urban tardigrade fauna shows a nested pattern between different environments, evidencing a homogenization process of fauna in this area. This pattern implies a significant loss of tardigrade species from High traffic to Medium transit environments.

Keywords: anthropogenic disturbance, city, diversity, Neotropic
Tardigrade inventory in the Black Forest National Park

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**Background:** The Black Forest National Park was established in 2004 between Baden-Baden and Freudenstadt in the Southwest of Germany. The National Park covers an area of around 10,000 hectares with the aim of restoring its wild, natural state. As many protected areas, the Black Forest National Park lacks comprehensive and accurate data about much of its biodiversity, especially for small organisms. The goal of the tardigrade survey was to identify as much tardigrade species as possible within this National Park which is renowned as a highly biodiverse and temperate forest, mainly consisting of conifers, like pine trees, or firs.

**Results:** We studied 325 moss samples and found more than 36 tardigrade species belonging to 17 genera of both classes. Some species were recorded for the first time in Germany (e.g. *Itaquascon placophorum* Maucci, 1973, *Murrayon hibernicus* Murray, 1911) and at least 4 species are new to science (*Diaforobiotus* sp. nov., *Ramazzottius* sp. nov., *Mesocrista* sp. nov. and *Pilatobius* sp. nov.).

**Conclusion:** These first results of the Tardigrada inventory in the Black Forest National Park reflects the high biodiversity of this area. This study did not produce only a mere list of species but it is also become a way to increase the awareness of the local authorities and the general public about the existing biodiversity and the need of its preservation. This inventory will allow to clearly define the actual situation, to compare it with future situations, so as to assess changes in biodiversity in dependence of time and restore activities.

**Key words:** biodiversity, Black Forest, inventory, national park, taxonomy
The first record of marine tardigrades from New Jersey, USA

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Background: There is a paucity of Records of Occurrence of marine tardigrades from the Cold Temperate Northwest Atlantic Marine Province. Intertidal species are of particular interest in that they may clarify the transition of tardigrades from marine to terrestrial environments. Here, we report tardigrades recovered from intertidal barnacles at Barnegat Lighthouse, New Jersey, USA.

Results: Thirty-eight specimens were permanently mounted on glass slides and examined with light and phase-contrast microscopy. An additional five were examined with electron microscopy. Those specimens whose full complement of claws could be resolved average 7.84, 7.84, 7.63, and 7.00 claws on legs I – IV, respectively. Body lengths average 226 µm. The average body length-to-width ratio is 2.52, which falls within the range expected for medium-size species. Appendages appear on all legs; those on I and II are dome-shaped. Black eyespots are present, as are internal and external cirri, cirri A and E, primary clava, and cephalic papillae (secondary clavae). The dorsal cuticle is warty without a substructure of smaller points. Stylet bases rest on the posterior part of the pharyngeal bulb.

Conclusions: We provisionally consider these specimens to be Echiniscoides cf. pollocki. Within the genus, only E. pollocki and E. horningi have both a warty cuticle and an 8,8,8,7 claw configuration. E. horningi has a pair of tertiary clava, however, which is lacking in our specimens and in E. pollocki. More extensive morphometrics and further examination of the anus and gonopore are necessary to identify our species. As the holotype of E. pollocki was found in sediment only 300 km Northeast of Barnegat Lighthouse, similar substrate should also be collected at our site for comparison.

Keywords: barnacle, Heterotardigrada, intertidal, marine, Record of Occurrence
Tardigrades in the canopy: are water bears at the top of the giant Sequoia?

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Background: Giant Sequoias (Sequoiadendron giganteum) are the largest trees in the world, reaching more than 90 meters tall, eight meters wide, and weighing over 1.2 million kilograms, they can be more than 3,000 years old. Sequoias can be found in small groves along the western slope of the Sierra Nevada Mountains in central California, USA. Recent reports of tardigrades in the canopies of younger and smaller trees motivate the question: do tardigrades exist in the canopy of some of the largest, tallest, and oldest trees? During the summer of 2016, 27 sequoia trees in Sequoia National Park were climbed in coordination with U.S. National Park Service. Seventy-one samples of lichen or moss tardigrade habitat were collected from seed cones and live or dead wood substrates from the base, middle and top of the giant trees.

Results: Two hundred twenty-nine tardigrades were found on 23 of the trees, at all heights. Twelve species of water bears were detected; both Heterotardigrades and Eutardigrades are represented in the collection, including the elusive Multipseudechiniscus raneyi and a new species. In general, the species found here were small. Lichen was significantly more common on the trees but tardigrades were significantly more diverse and dense in moss than lichen habitat. More tardigrades were found in the canopy than at the base of the trees. Significantly more tardigrades were found in the habitats growing on the dead wood and cone substrates than that growing on the live substrate. There was no difference in tardigrade abundance between the orientation of the sample; vertical or horizontal.

Conclusions: For 300 years, tardigrade research has been limited to ground level but now we report that several species of tardigrades occupy habitats at all levels throughout the canopy of giant Sequoia. Thus, speculation has been eliminated about tardigrades ability to live in the canopy of the forests of the world. This discovery has raised questions of diversity, distribution, dispersal and habitat suitability in this third dimension of tardigrade ecology.

Keywords: canopy, dispersal, diversity, epiphytes, Giant Sequoia
**Echiniscus testudo** from New Zealand: human-aided dispersal or evidence for the "Everything is Everywhere" hypothesis?

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**Background:** The “Everything is everywhere, but the environment selects” ("EiE") hypothesis postulates that microorganisms (including tardigrades) are free to disperse globally (e.g. with air currents) and thrive wherever the ecological conditions are favourable. The hypothesis quickly dominated the scientific discourse on biogeography of microscopic life. However, so far, there is no convincing evidence that tardigrade species can be cosmopolitan. Although many tardigrade species were recorded globally, cosmopolitan records usually concern nominal taxa with poor original descriptions that are currently recognised as species complexes. Moreover, sound evidence for a cosmopolitan distribution requires DNA sequences of variable molecular markers both for the original account of a species and for the records from other zoogeographic realms, and such data are not available for tardigrades. Here, we describe the first record of a Palaearctic species, *Echiniscus testudo*, from the southern hemisphere. Thanks to a recent integrative redescription of this species, we were able to verify the identity of a population collected in Auckland.

**Results:** Both morphological and molecular (phylogenetic) analyses confirmed undoubtedly that the Auckland population represents *E. testudo sensu stricto*. Thus, the species is now known from two isolated zoogeographic realms: Holarctic and Australasia. The position of the Auckland population among Palaearctic populations on the phylogenetic tree suggests a recent arrival to New Zealand.

**Conclusions:** The discovery of *Echiniscus testudo* in New Zealand shows that tardigrade species may be cosmopolitan. However, although New Zealand has been geographically isolated for over 80 My, the arrival of Europeans dramatically affected native ecosystems, also by introducing new species. Thus, a single Australasian record of *E. testudo* from an urban park in a cosmopolitan city with a history of intense human migration, tourism and trade links with other parts of the world, including Palaearctic, does not allow to discriminate between the “EiE” hypothesis and the endemic origin of the species (introduction by humans). The recent arrival to New Zealand, deduced from phylogeny, excludes low-rate natural dispersal but is expected both in the case of high-rate (ongoing) natural and human-aided dispersal. Thus, testing the “EiE” in tardigrades will require molecular analyses of multiple populations, preferably from primeval habitats in remote localities.

**Keywords:** biogeography, cosmopolitism, dispersal, microinvertebrates, New Zealand
A new species of *Macrobiotus* from rocky shore supralittoral level of North Portugal

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**Background:** Previous studies showed that the diversity of tardigrades living on supralittoral lichens is low, but most of the species recorded were undescribed ones. The objective of this study is to improve our knowledge about the tardigrade fauna inhabiting this particular habitat.

**Results:** A new species of *Macrobiotus* (Eutardigrada) was found in supralittoral lichens of the species *Xanthoria parietina*, growing on rocks at Salgueiros beach (North Portugal). This new species, with two macroplacoids and a microplacoid in the pharynx, and eggs with conical processes, belongs to the *Macrobiotus hufelandi* group. The new species is similar to *Macrobiotus recens*. The new species differs from *M. recens* by some morphometric characters (claws and buccal apparatus) and by the number, dimension and shape of the egg processes. In the new species the stylet insertion on the buccal tube is smaller but consistently more posterior when compared to *M. recens* and the external diameter of the buccal tube is much smaller than in *M. recens*, while all claws were longer in the new species. The eggs of the new species have more processes, longer but with smaller basal diameter than *M. recens*.

**Conclusions:** In conclusion supralittoral lichens seems to harbour a low diversity tardigrade assemblages. However, its tardigrade fauna is poorly studied and harbour many undescribed species.

**Keywords:** *Macrobiotus*, new species, North Portugal, supralittoral
Unfolding the mystery behind *Echiniscus cf. africanus* in Hawaii

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**Background:** *Echiniscus africanus* has been reported from South Africa (type location), Lesotho, Angola, Tanzania and Vietnam. Schuster collected *Echiniscus* specimens in Hawaii, but died before completing his study. We obtained 28 slides of his labeled ‘Ech afr’ from the Bohart Museum. We examined them to determine whether they could be considered *E. africanus* sensu stricto.

**Results:** Cirrus B is missing in the majority of Hawaiian specimens, but if present appears as a pair on both sides. Murray illustrated and described the dorsal cirri at positions C and D as overlapping. However, in the Hawaiian animals these spines, when present, are sometimes overlapping, but more often parallel. Another feature with high variability is the dentate collar: specimens vary in the number of teeth, which also often varies between the right and left legs of the same animal. Several specimens exhibit abnormal variation in traits, perhaps representing developmental abnormalities. For example, Cirrus A is present in most *Echiniscus* specimens; four Hawaiian specimens are missing this cirrus, which is replaced by an extra clava.

**Conclusions:** In order to describe or re-describe a species, it is useful to have the original specimens that were utilized for the characterization of the species. In the case of *E. africanus*, Murray’s original specimens have been lost, as have those from Lesotho, Angola and Vietnam. None of the papers reporting the presence of *E. africanus* included adequate measurement or images of important body structures, such as the spines on the dorsal plates, dentate collar on the fourth pair of legs, or the spines on the posterior margin of the scapular plate. Since we have no type specimens to compare to the Hawaiian animals, we can only compare them to Murray’s written description and illustration. Animals matching Murray’s description would fit within the range of observed variation in Hawaiian animals. Therefore we cannot conclude definitively whether the Hawaiian specimens are *E. africanus* or an undescribed species. Hawaiian specimens vary considerably in their morphology, even within a population. This research underscores that when describing a new species, it is important to use a sufficient number of specimens to account for variability and abnormalities.

**Keywords:** biogeography, *Echiniscus africanus*, Echiniscidae, morphological variability
A new species of the *Echiniscus viridis* group from the Hawaiian Islands

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**Background:** In 1985 R.O. Schuster and colleagues collected *Echiniscus* specimens from moss and lichen in Haleakala National Park, Maui Island, Hawaii, but Schuster died before completing his study. We obtained 17 slides of his labeled ‘Ech arct’ from the Bohart Museum. Morphological examination made it evident that these specimens were not *Echiniscus arctomys* but were an undescribed species. Twelve specimens were suitable for morphometric analysis using phase contrast microscopy.

**Results:** Although the sex of most of the specimens of the new species cannot be discerned, both males and females are present. No specimens show any evidence of pigmentation, even though two slides of Hawaiian *E. viridis* mounted by Schuster at the same time are still dark green. Despite the absence of discernible pigmentation, morphologically the species belongs to the “*viridis* group” (*E. viridis, E. perviridis, E. rufoviridis, E. viridianus* and *E. viridissimus*). It lacks dorsal or lateral trunk appendages and has well-developed claws. Dorsal cuticular ornamentation consists of large, round, slightly-raised tubercles; this character is distinctly different from *E. rufoviridis* and *E. viridissimus*. It differs from the other three species in having much wider dorsal tubercles (maximum 7.3 μm in the new species; maximum 2.4-3.6 μm in the others) and large, robust basal spurs on its claws. It also differs from *E. viridis* in having longer cirrus A (49-75 μm vs. 36-44 μm) and smaller claws (claw IV sc 26-34 vs. 35-38); from *E. perviridis* in having shorter cirrus A (49-75 μm vs. 114-170 μm) and smaller claws (claw IV sc 26-34 vs. 50-53), and from *E. viridianus* in having a longer cirrus A (49-75 μm vs. 29 μm).

**Conclusions:** The new species probably differs from *E. perviridis* and *E. viridianus* in lacking pigmentation. The following combination of independent morphological characters is unique in the “*viridis* group”: maximum tubercle diameter at least twice as wide as other species, cirrus A of intermediate length, and large, robust basal spurs. *Echiniscus viridis* is endemic to Oahu Island, Hawaii. The description of the new species from the Schuster collection will add a second species of the “*viridis* group” to the Hawaiian tardigrade fauna.

**Keywords:** Echiniscidae, *Echiniscus* sp. nov., Hawaiian tardigrades, Heterotardigrada
First 12 years of tardigrade succession in the young soils of a quickly evolving ecosystem

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Background: Great parts of the landscapes in Lusatia (Eastern Germany), as well as in other European regions, underwent massive changes during opencast coal-mining, where the original soil strata are lost due to excavation and dumping practices. For ecologists post-mining sites give an excellent opportunity to investigate primary succession of flora and fauna. Especially during the first years, organisms inhabiting such mining substrates are exposed to extreme environmental conditions, such as unbuffered changes in soil temperature, rapid succession of dry and wet cycles and lack of organic matter. The six hectare study area “Chicken Creek” is located close to Spremberg, Germany, and has been studied since its construction in 2005, point zero of ecosystem development, in terms of abiotic environment and soil fauna development.

Results: In twelve years altogether 13 tardigrade species were determined from the Chicken Creek soils, with species richness increasing to its present maximum of eight in 2017. After the first years with very low tardigrade abundance, we registered a mass occurrence of Apodibius confusus in 2007, which started to decline in October 2009, when overall tardigrade species diversity rose. The dominating species of the subsequent years were Hexapodibius micronyx, Isohypsibius tuberculatus group and Eremobiotus alicatai, followed by Diphascon nobilei that first appeared in 2015 and dominated in 2017. Paralleling ecosystem development abiotic parameters, such as soil pH, moisture and texture, carbon and nitrogen content as well as vegetation cover, have undergone great changes. The main factor impacting tardigrade community was the vegetation cover, while other factors had less influence as demonstrated by Redundancy Analysis (RDA). The increase of shading due to the development of a plant cover over years clearly paralleled the numerical decline of algal feeding Apodibius confusus, possibly as a result of a lower availability of photosynthetic green algae.

Conclusion: The results indicate that tardigrades are introduced by random effects, such as wind and passing birds or small mammals carrying plant parts. The population development of single species might be favored by certain environmental factors and food availability, but the overall influence of the environment seems to be small. This once again underlines that terrestrial tardigrades can withstand harsh conditions, a skill that makes them especially capable of inhabiting new environments and young soils.

Keywords: community ecology, post-mining landscape, primary succession, Tardigrada
Resistance of tardigrades to cyanotoxins

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Background: Tardigrades are microscopic animals capable to survive environmental stresses, such as desiccation, irradiation or vacuum. They can be found in all terrestrial and aquatic ecosystems and their cohabitation with cyanobacteria has been reported in Antarctica [1]. Most cyanobacteria represent a source of natural products with applications in the pharmaceutical, food, cosmetic, agriculture and energy areas. Nevertheless, several cyanobacteria species are also well known to produce cyanotoxins. It has been reported that health of a variety of animals from wild to livestocks and domestic ones can be affected by exposure to cyanotoxins.

Results: In that context, our research focuses on the interaction between tardigrades and cyanobacteria, with the goal to characterize the possible resistance of tardigrades to various cyanotoxins. We are presently selecting cyanobacteria strains that are suitable for our studies from the 800 strains which constitute the live collection of freshwater cyanobacteria of the French National Museum of Natural History. Cosmopolitan cyanobacteria belonging to the gender of Phormidium, Synechococcus, Leptolyngbya, Nostoc, Pseudanabaena, Microcystis and Microcoleus have already been included in our screen. These cyanobacteria differ by their shape, size and capability to produce different cyanotoxins. We are presently monitoring the survival and adaptation of Hypsibius dujardini, our model tardigrade, when fed with these different cyanobacteria. The epigenetic modifications associated with the resistance to cyanotoxins will next be investigated in a genome-wide manner.

Conclusion: The use of tardigrades to investigate the mechanism of action of cyanotoxins represents a novel and original strategy and will shed new light in the field of cyanobacteria and the toxicity of cyanotoxins.


Keywords: cyanobacteria, cyanotoxins, epigenetics, survival, adaptation, tardigrades
Acutuncus antarcticus: an emerging model animal to predict the effect of global warming on sensitive ecosystems

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Background: The eutardigrade Acutuncus antarcticus is one of the most widespread meiofaunal species in Antarctica. The knowledge about its responses and possible adaptations to climate changes could shed light on ecosystems preservation in sensitive cold regions. Here, we review the biological properties of A. antarcticus.

Results: Acutuncus antarcticus is endemic in Antarctica where it colonizes sediments of temporary freshwater ponds, mosses, algal and bacterial mats.

Molecular approaches (18S and 28S genes) reveal that the genus Acutuncus represents a well-supported evolutionary lineage within Hypsibiidae. Acutuncus antarcticus may be considered a pan-Antarctic species because very few phenotypic and genetic differences are found among analyzed populations. Nevertheless, possible cryptic species are present and the potential for further speciation events is suggested by the intraspecific high genetic diversity reported using cox1 gene within Victoria Land populations in Antarctica. A. antarcticus is spread all over Antarctica, probably due to its capacity to reproduce by thelytokous meiotic parthenogenesis and its ability to undergo passive dispersal when in a desiccated state.

The species can be cultured with algae (Chlorella sp. or Chlorococcum sp.) as food either in plastic dishes with a layer of bacto-agar or in flasks with a layer of algae. The life cycle of A. antarcticus is short, as well as the hatching time and the interval of time between two successive ovipositions. It also shows an early age at first oviposition, with an overall high hatching success. A. antarcticus tolerates stress events, such as freezing, desiccation, thermal stress and high ultraviolet (UV) radiation, but survivorship is strongly affected by the synergic action of high temperature and UV radiation.

Conclusions: Acutuncus antarcticus is a good model for predicting adaptive responses to climate changes. Next steps will be to obtain data from genomics, transcriptomics, and epigenetics to define practical and efficient management options to direct conservation planning.

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Keywords: Acutuncus antarcticus, global warming, model species, stress resistance
Encystment in *Thulinius ruffoi* (Parachela: Isohypsibiidae)

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**Background:** In tardigrades, two forms of dormancy have been described - quiescence (cryptobiosis) and diapause. Cryptobiotic abilities are known from many species, and especially from terrestrial species that often are exposed to unfavorable conditions. Diapause is a less studied dormancy state, represented by encystment and resting eggs. Diapause is viewed as a process that depends on external as well as internal stimuli. Encystment is known in only a few terrestrial, limnic and marine species. In this process, an animal undergoes physiological and morphological changes that result in immobilization, encapsulation and in reduction of the metabolic rate of the individual.

**Results:** Here we investigate encystment in the freshwater eutardigrade, *Thulinius ruffoi* employing light microscopy, as well as transmission and scanning electron microscopy. During the encystment process, animals start to synthesize multiple cuticle layers. Serial synthesis of cuticles, together with a contraction of the body and internal organs, leads to tardigrades that are enclosed in cuticular capsules. The individuals are not able to move, eat or reproduce. The contraction of the body results in a compression of the internal organs and reduction of their lumen. However, internal organs and their cells are still recognizable at the ultrastructural level. Reserve materials were observed in the storage cells during encystment. Observed cysts were of various sizes representing encystment of individuals of different sizes. All analyzed cysts had a light or dark brown color and an ovoid shape.

**Conclusions:** *Thulinius ruffoi* is a freshwater species with the ability to encyst. Visible morphological changes of this tardigrade during encystment include contraction of the body together with a compression of the internal organs, and serial synthesis of cuticles by the epithelial cells of the body wall.. This process can be a strategy to cope with unfavourable environmental conditions.

**Keywords:** Diapause, Encystment, Parachela, Tardigrada
Live tardigrades recovered from faeces of White-bellied Seedsnipe (*Attagis malouinus*)

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Background: Tardigrades have been found in bird nests and hence birds are hypothesized to act as potential dispersal vectors. Birds may disperse microscopic organisms or diaspores either ecto- or endogenously. To assess the role of Upland Goose (*Chloephaga picta*) and White-bellied Seedsnipe (*Attagis malouinus*) for the endogenous dispersal of bryophytes in alpine habitats of Navarino Island, southern Chile, we screened 27 Seedsnipe and 18 Goose faeces. Upon the surprising discovery of tardigrades in the faeces, we speculated reasons for their presence.

Results: We discovered 9 tardigrades, 8 of which were living, representing three species. All tardigrades were recovered from the interior of two White-bellied Seedsnipe faeces. Both faeces were collected from a single alpine location (579m asl) in the Dientes de Navarino Mountains. We confirmed two specimens as members of the genus *Diphascon*, one specimen belongs to *Adropion* (Hypsibiidae), and one specimen to *Macrobiotus hufelandi*-group (Macrobiotidae). All specimens are being examined for further identification.

Conclusion: The Tardigrada of Navarino Island have not yet been surveyed extensively. This study will contribute to the knowledge of the range of known species or aid the discovery of new species. We suggest two possible reasons for the presence of tardigrades in White-bellied Seedsnipe faeces. The tardigrades could have been ingested with mosses, which are fed on by the birds, and passed through the birds’ digestive tract. Or, the tardigrades burrowed into the faeces post-defecation. The presence of protozoa and micro-invertebrates in the faeces suggests that the faeces could have served as a rich food source. Given that the recovered tardigrades regained vigour upon hydration, we speculate that they could have been dormant in cryptobiosis as the faeces dried. Tardigrades have been documented occupying the faeces of land snails, to our knowledge, this is the first discovery of tardigrades using bird faeces as a microhabitat.

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Keywords: Bird faeces, Cape Horn Region, *Diphascon*, microhabitat, White-bellied Seedsnipe
Life-history trait of a bisexual tardigrade species
*Mesobiotus* sp. nov. (Eutardigrada; Macrobiotidae) from Elba Island, Italy

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**Background:** Elba is the largest Italian island of the Tuscan Archipelago in the Mediterranean. It is located 10 km from the coastal town of Piombino (province of Livorno), and about 50 km east of the French island of Corsica. Little is known about the tardigrade species which inhabit Elba. A new tardigrade species, *Mesobiotus* sp. nov. is described from moss samples collected in the Barbarossa and Reale Bay at the south-eastern side of the island.

**Results:** Other than morphological studies (LM, SEM), the life cycle of the species, reared under laboratory conditions (at 21°C), was investigated. The new species differs from other species within the genus by the presence of granules on the posterior dorsal cuticle and by the shape of the egg processes. It is further characterised by sex-specific differences in size: the mean length was 301 \(\mu\)m for females and 240 \(\mu\)m for males, each in the same reproductive age. The mean examined life span of the specimens was 86 days, with a maximum of 150 days. Sexual maturity was examined based on the first appearance of laid eggs and a mean time of around 20 days was determined. The mean time interval between egg laying and hatching was 15 days (hatching time).

**Conclusion:** With the current study on the life cycle of a bisexual tardigrade, *Mesobiotus* sp. nov., we gain further information on the ecology and adaptive strategies of the life history of tardigrades, allowing us to have a more complete pattern not only based on the life cycle data of the more analysed parthenogenetic species.

**Keywords:** bisexual, Elba island, life history, life span, *Mesobiotus*
Recovery of a moss dwelling eutardigrade (*Macrobiotus cf. hufelandi*) dried for over 15 years

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**Background:** The phylum Tardigrada consists of ca. 1,300 species that inhabit both aquatic and terrestrial environments throughout the world. Tardigrades have an extraordinary ability to survive extreme environmental conditions, like very low and high temperatures, lack of water, high doses of radiation or high and low pressure, due to their ability to enter into different types of cryptobiosis. Previous reports include observations of terrestrial tardigrades that survive even up to 20 years in a dry state [1]. Such an ability can be very important from ecological point of view, because in anhydrobiotic state tardigrades may survive in unstable and unpredictable environments. Recently, after examination the sample from frozen Antarctic sediments, it was confirmed that in cryobiosis, tardigrades can survive even longer - up to 30 years. Taking into consideration that the lifespan of fully active specimens is up to a few months, such a long time spent in inactive state can have great evolutionary consequences, e.g. reducing the rate of evolution. In the present study we analysed a 15 year old moss sample, collected in a very dry ecosystem in the Kyrgyz Republic, from which we extracted several dozen eutardigrades.

**Results:** Most of the extracted tardigrades were dead (they were fully stretched and almost transparent), but two specimens showed some signs of life (they were milky-white and "bent in shape of a croissant"). We placed these two specimens in clean water and after a few minutes first movements were observed. We subsequently transferred the specimens to a culture in a Petri dish using green algae and rotifers as potential food. The tardigrades returned to normal activity, however both died after two weeks, probably due to lack of food intake (we did not observe hunting on rotifers nor green algae in the intestine). Finally both specimens were placed on permanent microscope slides and were identified as *Macrobiotus cf. hufelandi*.

**Conclusions:** Our observations confirmed the time spent by tardigrades in anhydrobiosis with the successful recovery of up to 15 years in the current study. However, our specimens did not start eating and it is unknown whether the food was inadequate or the eutardigrades failed to return to a normal metabolic activity.


**Keywords:** anhydrobiosis, extremophiles, Kyrgyz Republic, Macrobiontidae
Seasonal abundance of two marine tardigrades, 
*Echiniscoides sigismundi* and *Styraconyx haploceros*, inhabiting the barnacle 
*Fistulobalanus albicostatus* at an intertidal zone, western coast of Korea

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**Background:** Much research on tardigrades has been done in terrestrial ecosystems rather than in marine ecosystems. In the present study, barnacles were sampled monthly from the lower parts of the intertidal rock surface during the period between July 2016 and March 2017, on the western coast of Korea. Tardigrades were collected on the outside and inside of the barnacle shells. The purpose of this study is to contribute to knowledge on the seasonal abundance of the marine tardigrades inhabiting the barnacles in an intertidal coastal zone of the temperate region.

**Results:** Two species of tardigrades, *Echiniscoides sigismundi* and *Styraconyx haploceros*, appeared in all sampling months. *E. sigismundi* was found in 0~30% of the barnacles (mean: 0~4.3±4.04 inds/barnacle) at the upper part of the intertidal zone, and in 100% of the barnacles (mean: 7.9±3.41~12.2±14.97 inds/barnacle) at the lower part. Even though *S. haploceros* was not found at the upper part, it was present in 80~100% of the barnacles (mean: 2.3±1.22~5.5±6.59 inds/barnacle) at the lower part. In addition, in the lower part of the intertidal zone, monthly mean abundances of *E. sigismundi* were 2.5±2~15.8±16.51 inds/barnacle, while monthly mean abundances of *S. haploceros* were 3.3±3.28~72.9±81.47 inds/barnacle from September 2016 to August 2017. In the lower part of the intertidal zone, the monthly abundances of *E. sigismundi* were higher on the outside of the barnacle shell than on the inside of the shell, including mantle cavity and viscera, except in September 2016 and February 2017. Through the whole study period, the monthly abundances of *S. haploceros* were higher on the outside of the barnacle shells than on the inside. Also, both of the two tardigrades were more abundant in the larger barnacles (>14mm) than in the smaller (<9mm) or dead barnacles (>13mm).

**Conclusions:** Both species of tardigrades in the intertidal zone are more abundant from October to December than in other months, prefer the lower part of the zone to the upper one, and live mainly outside of the barnacle shell. The abundance of tardigrades on the barnacles appears to be adversely correlated with exposure to drying and precipitation.

**Keywords:** abundance, barnacle, marine tardigrades
Tolerance to gamma radiation in the desiccation-sensitive tardigrade *Isohypsibius myrops* (Parachela, Eutardigrada)

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**Background:** Some tardigrade species can tolerate high-dose irradiation. It has been hypothesized that their extraordinarily high radiation tolerances are the byproducts of adaptation to desiccation. Radiation tolerance in tardigrades has been examined mainly on desiccation-tolerant species and scarce information is available on desiccation-sensitive species. Therefore, we analyzed the radiotolerance in a highly desiccation-sensitive tardigrade, *Isohypsibius myrops*, which cannot withstand exposure to 98% relative humidity and the isogenic strain was established and is reared in laboratory.

**Results:** To evaluate radiation tolerance, adults of *I. myrops* were irradiated with various doses of gamma radiation ranging from 0 to 4000 Gy, and then, the survival, egg laying and hatchability of laid eggs were continuously examined. As control, the effects of 0-7000 Gy of gamma radiation were examined in highly desiccation-tolerant and radiotolerant *Ramazzottius varieornatus*. The median lethal dose (LD₅₀) in 48 hours post-irradiation was calculated as 2240 Gy in *I. myrops* and 5970 Gy in *R. varieornatus*. In *I. myrops*, the life span was significantly shortened by radiation even with 500 Gy, but, in contrast, *R. varieornatus* exhibited virtually unaffected life span even after 5000 Gy irradiation. The egg layings were also significantly affected by radiation in both species in a dose-dependent manner: During the examined period, the non-irradiated individuals of *I. myrops* laid 45.3 eggs on average; the number of laid eggs decreased to 0.7, 0.3, 0 in the 500, 1000, 2000 Gy irradiated groups, respectively. In *R. varieornatus*, the number of laid eggs were 3.9 eggs in non-irradiated condition and decreased to 0.9, 0 in the 3000 Gy, 5000 Gy irradiated groups. All eggs laid by the irradiated adults failed to hatch in both species.

**Conclusions:** The highly desiccation-sensitive tardigrade, *I. myrops* exhibited much weaker radiation tolerance than those of desiccation-tolerant species having been reported in Eutardigrada. The data supported the correlation between desiccation tolerance and radiotolerance, and provide insights on evolutionary and mechanistic origins of tolerability against different environmental stresses in tardigrades.

**Keywords:** correlation, desiccation-sensitive, radiation tolerance
Mitochondrial alternative oxidase contribution to successful tardigrade anhydrobiosis

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Background: Many tardigrades have the ability to withstand almost complete drying (dehydration) without sustaining damages. In such conditions they form a cyst or tun. The process is known as anhydrobiosis and enables tardigrades (and other invertebrates such as some rotifers and nematodes) to withstand the periodic drying that occurs naturally in their microenvironments. At present, the recovery from the tun stage to the active stage is the only attainable evidence of successful anhydrobiosis. Nevertheless, the available data suggest mitochondria participation in the tun survival, but their role, in successful anhydrobiosis of tardigrades, still remains unexplained. Accordingly it is known that tardigrades in tun stage may show tolerances to abnormally high concentrations of potassium cyanide that imposes the role of mitochondrial alternative oxidase (AOX). Although AOX is an ubiquitous enzyme in plants, it is also found in other eukaryotes including fungi and protists, and only few animal species. Interestingly, analysis of Milnesium tardigradum, Hypsibius dujardini and Ramazzottius varieornatus genomes has indicated the presence of the functional AOX encoding gene. This makes AOX a promising candidate for a mitochondrial marker of successful anhydrobiosis.

Results: The results concern two tardigrade species differing in anhydrobiosis capability; i.e. M. tardigradum and H. dujardini. Under the applied conditions of dehydration and the applied period of the tun stage duration the percentage of recovery from the tun to active stage was ca. 90% and ca. 10%, respectively. The AOX inhibitor, benzohydroxamate (BHAM), was added to active individuals not undergoing or undergoing dehydration. Then the impact of BHAM on their survival or the tun formation and recovery to active life were monitored, respectively. At concentrations not toxic for the active and dehydrating animals, BHAM appeared to delay time required to recover from the tun stage to active life.

Conclusions: The obtained results suggest important role of AOX in tardigrade mitochondria and contribution of the mitochondria functioning to successful anhydrobiosis. This in turn makes AOX an interesting candidate in searching of mitochondrial markers of successful anhydrobiosis.

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Keywords: anhydrobiosis, Hypsibius dujardini, Milnesium tardigradum, mitochondrial alternative oxidase
Effects on longevity traits in frozen tardigrades

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**Background:** There are different documented strategies for tardigrades to survive low temperatures. Besides freeze avoidance, where the animal depresses the temperature of spontaneous freezing by using antifreeze proteins or other cryoprotectants, it is also possible to tolerate ice formation of the extracellular body water. The ‘Sleeping Beauty’ model states that there is no aging during the inactive, dried period, and it was proven to be fitting for tardigrades in the anhydrobiotic state. Therefore, it seems to be also the most promising model to describe the effects of cryobiosis on their longevity.

**Results:** The comparison of the temporarily frozen groups with the control groups of *Milnesium cf. alpigenum* shows significant difference if the frozen time is included, as well as if it is excluded from the age of the tardigrades. If the frozen time is included in the tardigrades’ age, the frozen groups show a distinctly longer life than the control group. The oldest animal in the frozen groups was 169 days (frozen time included) respectively 94 days (frozen time excluded), while the oldest animal in the control group lived 93 days.

**Conclusion:** The results show that while in cryobiosis or anhydrobiosis, *Milnesium cf. alpigenum* does not age. Thus, the ‘Sleeping Beauty’-hypothesis is confirmed for cryobiosis as well as it previously was for anhydrobiosis, although freezing and drying are not similar stress vectors. We showed the effect of cryobiosis on the internal clock in this tardigrade species. This knowledge could help to a better understanding of the mechanisms of cryobiosis and its effects on tardigrades.

**Keywords:** cold tolerance, cryobiosis, freezing, Sleeping Beauty, Tardigrada
Untargeted metabolomic profiling in search for metabolic markers of successful tardigrade anhydrobiosis

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Background: Anhydrobiosis is commonly defined as desiccation tolerance that denotes the ability to survive almost complete dehydration without sustaining damages. This phenomenon has been reported for many microorganisms. Responding to dehydration, tardigrades form a so-called ‘tun’ and simultaneously enter the state of anhydrobiosis. The only true evidence of a successful tardigrade anhydrobiosis attainable at present is the observation of a successful recovery from the tun to the active stage. An analysis of available molecular data suggests that successful anhydrobiosis is based on carbohydrate and lipid metabolism. Therefore, we decided to compare metabolic profiles of active and anhydrobiotic individuals of different tardigrade species in order to identify metabolic marker(s) of successful anhydrobiosis. Results: To follow putative alterations of metabolism within the tun stage, we decided to apply a metabolomic approach, i.e. untargeted metabolomic profiling based on gas chromatography-mass spectrometry (GC-MS). This required a determination of extraction methodology and identification of the detected derivatives, a selection of appropriate tardigrade species preferably differing in their anhydrobiosis capability, as well as an estimation of the numbers of studied individuals ensuring proper method sensitivity. The obtained data proved applicability of an extraction method based on application of a mixer mill to grind and homogenize frozen samples, the mixture of methanol and chloromethane as an extraction solvent, and ultrasonic bath to perform the extraction. The dried extracts were resolved in the presence of N,N-Diethyl(trimethylsilyl)carbamate applied as a derivatization reagent and loaded on the GC column. The unscrambled metabolites detected for the optimal amount of active and anhydrobiotic individuals of Echiniscus testudo and Hypsibius dujardini represented mainly amino acids, monosaccharides, carboxylic acids and membrane lipids. Moreover the metabolites are mainly implicated in oxidation processes occurring in mitochondria. Conclusions: The chosen methodology leads to detection of different metabolites and consequently allow for determination of metabolic marker(s) enabling discrimination between active and tun stages. This in turn could provide cellular markers allowing discrimination between tardigrades differing in their anhydrobiosis capability, with a potential importance for the explanation of life without water. The work was supported by the research grant of National Science Centre, Poland, NCN 2016/21/B/NZ4/00131.

Keywords: anhydrobiosis, Echiniscus testudo, Hypsibius dujardini, metabolic marker(s), untargeted metabolomic profiling
Osmotic stress tolerance by the tidal tardigrade *Echiniscoides* spp.

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**Background:** Tardigrades can withstand extreme environments including desiccation, variable temperature, and variable osmotic pressure. These environmental tolerances were studied mainly with limno-terrestrial tardigrades. Reports on the tolerance of marine tardigrades to extreme environments, however, still remain relatively unexplored. Therefore, we evaluated the osmotic pressure tolerance of the tidal tardigrade *Echiniscoides*, which is widely distributed around the world.

**Results:** Live *Echiniscoides* spp. individuals were collected from living barnacles taken from the intertidal zone of Yokohama, Tokyo Bay. In order to observe the tardigrades’ response to osmotic stress, four salinity conditions (3, 50, 70, and 90) and reference condition (30) were prepared with artificial seawater. Experimental individuals (n=5) were exposed to each condition for 48 hours. The behavior of individuals was documented under microscope at 7 moments during the exposure (30 sec - 48hrs). We documented the morphological change of the individuals and the number of movements of the forefoot per minute. As a result, the individuals formed tun at salinity of 50, 70, and 90. On the other hand, individuals became swelled at salinity 3. After this experimental period, all population was transferred to reference environment of salinity 30, all individuals returned to their normal appearance and active state after 2 hours. Additionally, in order to investigate the survival limit of osmotic stress, individuals of *Echiniscoides* spp. (n=10) were submerged in saturated artificial seawater and milliQ. Activity of population in saturated artificial seawater was immediately halted after transferring, and the body became flattened like a sheet of film, but 84% of individuals recovered activity after 24 hours upon reimmersion in reference water. On the other hand, none of the individuals in milliQ were recovered.

**Conclusions:** The *Echiniscoides* spp. used in this study was able to withstand sudden osmotic stresses. Because *Echiniscoides* spp. used in this study inhabits in the intertidal zone, they are exposed to drying and osmotic stress every 6 hours by tidal cyclicity. In order to adapt to such environment, *Echiniscoides* spp. develops genes and structural resistance mechanisms to dehydration, and therefore it is considered to be able to withstand sudden osmotic stress.

**Keywords:** *Echiniscoides*, Heterotardigrada, marine, osmotic stress
Ice-binding proteins as a potential mechanism to survive freezing: ice-affinity purification and characterization of IBP homologs in *Hypsibius dujardini*

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**Background:** Although tardigrades are widely known for their ability to survive a range of environmental and thermal extremes, little is known about the specific molecular mechanisms that underlie survival against freezing. One strategy to prevent the uncontrolled formation of ice crystals at subzero temperatures is to produce antifreeze or ice-binding proteins (IBPs). Since their initial discovery, a diversity of structurally and functionally distinct IBPs have been described across various taxa. Previous work has not established whether tardigrades produce IBPs, despite their ability to remain active in winter conditions and viable in extreme subzero temperatures. Here, we utilize lab strains of *Hypsibius dujardini* and publicly available proteomic data to uncover whether tardigrades may produce ice-binding proteins (IBPs) to help survive freezing.

**Results:** Elution fractions of ice-affinity purification from *H. dujardini* whole-animal lysate (~20,000 individuals) generate bands of ~10, 25, and 70 kDa faintly visible by silver stain—which are identified via mass spectrometry. Mass-spec identifies ~225 proteins that are uniquely expressed under freezing conditions, as well as ~15 proteins that are found in both ice-affinity purification elution fractions and under freezing conditions. In a parallel approach to determine IBP candidates, 2749 protein sequences from a wide diversity of organisms annotated as “ice-binding” or “antifreeze” (RCSB Protein Data Bank and UniProt) were compared for homology against publicly available *H. dujardini* proteomic data using Protein BLAST. This analysis identified 261 primarily unannotated protein sequences in *H. dujardini*, which share notable homology (E-value 1e-3 or less) with 90 known IBPs spanning numerous classes.

**Conclusions:** Ice-affinity purification, mass-spec, and shared homology with known IBPs in other taxa identify numerous protein candidates in *H. dujardini* that may function as ice-binding proteins. Functional verification of candidate IBPs is needed to determine if *H. dujardini* utilize IBPs as part of their cold tolerance strategy. Future work will explore the ice-binding properties of candidate IBPs *in vitro* as well as the ability of these proteins to confer increased survival in subzero temperatures *in vivo*. This pipeline for IBP discovery and verification will be employed in additional Eutardigrade species—currently cultured in lab—to gain greater insight into the evolutionary history and mechanism of cold tolerance in this unique and understudied phylum.

**Keywords:** antifreeze proteins, cold tolerance, Eutardigrada, *Hypsibius dujardini*, ice-binding proteins
Level of desiccation under different relative humidities in the tardigrade *Richtersius coronifer*

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**Background:** The invertebrate phylum Tardigrada contains a large number of species with an ability to tolerate complete desiccation in all developmental stages. Severe water loss in tardigrades leads to contraction of the body into a so-called tun, and metabolism is then arrested resulting in a state of anhydrobiosis. For successful induction of anhydrobiosis tardigrade samples are often dehydrated slowly under high humidity conditions. The level of desiccation under different humidity conditions has not been much studied, and in the present study we measured the weight of the eutardigrade *Richtersius coronifer* desiccated for 24h under different relative humidities (95, 38, 0% RH) and after drying at 150°C.

**Results:** The average weight of a desiccated individual tardigrade was about 2 ug, and of a hydrated individual about 14 ug. For samples that were measured repeatedly at different humidities, we observed significant decreases in the weight from desiccation at 95% RH to desiccation at 38% RH, and from desiccation at 38% RH to desiccation at 0% RH. Also the transfer from desiccation at 0% RH to 24h drying in oven resulted in a significant decrease in body weight.

**Conclusions:** The general estimates of weight of desiccated *R. coronifer* is in line with previous published estimates. Our results confirm that desiccation at different relative humidities results in different levels of desiccation, i.e. low humidity conditions remove more water from the body than high humidity conditions. However, although significant the absolute differences in desiccated weight were not very large despite the range in humidity from 0% to 95%, and one conclusion of our study is that even at very high humidity, desiccation for 24h will be enough to put the animal in an anhydrobiotic state. This information is important for studies where induction of anhydrobiosis under high humidity is part of the experimental protocol.

**Keywords:** Anhydrobiosis, dehydration, *Richtersius coronifer*
Forays into generation of transgenic *Hypsibius dujardini*

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**Background:** Tardigrade *Hypsibius dujardini* has a compact genome (approximately 20,000 genes) and many shared characteristics with both *C. elegans* and *D. melanogaster*, two widely studied model organisms. These traits, along with *H. dujardini*'s resilience to wide variety of environmental stressors, make the species an attractive potential research model. However, the lack of established techniques for introducing stable genetic modifications has been a limiting factor in the study of *H. dujardini*.

**Results:** Protocols explored in this project include induced random mutagenesis, injection of functional mRNA, and CRISPR/Cas9 genome editing. We found no evidence of survivable random ethylmethane sulfonate -generated mutagenesis, perhaps due to low fecundity. All genome editing was attempted using of microinjection, as good injection technique is not yet established, we attempted to look at the consistency of localization of injection practices using dyes including fluorescein and phenol red, as well as injecting functional GFP mRNA. Baseline autofluorescence in the gut and the permeability and diffusion of some dyes interfered with our attempts to look for localization and germline uptake of injected material. Genes targeted for editing were chosen for high homology with both *C. elegans* and *D. melanogaster* and were assessed through BLAST and other standard bioinformatics tools. We designed CRISPR guide RNAs targeting several genes including *rab-3*, *protein white*, *unc-75*, *chs-1*, *unc-104*, and *unc-18* to create large deletions. Attempted deletions were screened using a nested PCR strategy. Finally, we explored the *in vivo* accessibility of DNA and neuronal cytoplasm by staining animals with SYBR green (nucleic acid staining dye) and Mag-Fura-2-AM ester (a calcium activity indicator).

**Conclusions:** The availability of tools to genetically manipulate tardigrades is crucial to the viability of tardigrades as a genetic model organism. The time-intensive screening process necessitates a consistent and effective method of gene editing. The ability to generate specific modifications in the genome of this organism will allow for a deeper understanding of the molecular machinery and will be an important step forward in the field of tardigrade biology.

**Keywords:** CRISPR/Cas9, gene editing, genome, *Hypsibius dujardini*, microinjection, transgenics
AFM analysis of DNA binding mode of a novel DNA-protection protein, Dsup, unique to a radiotolerant tardigrade

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Background: Ramazzottius varieornatus can withstand high dose irradiation either in a hydrated state or in a dehydrated state. Recently, we identified a novel DNA protection protein, dubbed Damage suppressor (Dsup) from chromatin fraction of the tardigrade. Dsup protein can directly associate with DNA in vitro. Dsup also significantly suppresses DNA damage caused by irradiation and reactive oxygen species (ROS) in cultured human cells and improves the radiotolerance of the cells. Given that no delay was observed in proliferation of Dsup expressing cells compared to unengineered control cells, Dsup likely protects DNA without interfering most cellular activity. The safe DNA protection mechanism by Dsup, however, remains totally unknown.

Results: Ultracentrifugation analysis revealed that Dsup proteins are present mostly as monomers, when purified in native condition from bacterial lysate. The purified Dsup protein significantly shifted the electromobility of DNA, suggesting formation of huge complex. To visualize the morphology of the complex, the Dsup - linear DNA mixture was examined with atomic force microscopy (AFM). DNA associated with Dsup exhibited various forms, mainly as relaxed linear form, linearly folded form and circular form. Dsup proteins were found to attach intensively to a contiguous region of each DNA molecule, suggesting that DNA-bound Dsup protein may recruit another Dsup protein to the neighboring position. When Dsup was mixed with reconstructed nucleosomes, Dsup induced significant condensation of nucleosomes with much lower protein amount than binding to naked DNA, suggesting that Dsup binds to DNA in cooperative manner with histones.

Conclusions: The novel DNA binding protein Dsup is usually present as a monomer, but binds to contiguous region of DNA and induces condensation of nucleosomes. In this study, we revealed the binding mode of Dsup to DNA and the effect on higher-order structure of DNA and chromatin. Our results provide clues to understand a possible mechanism of Dsup enabling enhanced DNA protection against radiation and ROS.

Keywords: atomic force microscopy (AFM), damage suppressor (Dsup), molecular biology, radiation tolerance, reactive oxygen species (ROS)
Multi-omics study of a heterotardigrade *Echiniscus* cf. *testudo* suggests convergent evolution of anhydrobiosis in Tardigrada

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**Background:** Many limno-terrestrial tardigrades are able to enter an ametabolic state termed anhydrobiosis upon desiccation, in which the animals can withstand extreme environments. Through genomic studies, molecular components of anhydrobiosis are beginning to be unveiled, such as the expansion of oxidative stress response genes, loss of stress signaling pathways, and gain of tardigrade-specific heat-soluble protein families designated CAHS and SAHS. However, studies thus far have been predominantly limited to eutardigrades, and molecular mechanisms in heterotardigrades still remain elusive. To this end, here we report a multi-omics study of a heterotardigrade *Echiniscus* cf. *testudo*, which is one of the most desiccation tolerant species.

**Results:** In order to elucidate the molecular basis of anhydrobiosis in *E. cf. testudo*, we employed a multi-omics strategy encompassing genome sequencing, differential transcriptomics, and proteomics. Using ultra-low input library sequencing protocol from a single individual, its genome was first sequenced and assembled, and was annotated using RNA-Seq data. Surprisingly, none of the tardigrade-specific anhydrobiosis-related genes such as CAHS and SAHS were conserved, while loss and expansion of existing pathways were partly shared. Moreover, differential expression analysis turned out to be negative, just as in *Ramazzottius varieornatus* where desiccation is so rapid that expression changes do not take place. Furthermore, we screened for heat-soluble proteoins, revealing several proteins that are highly heat-soluble and constitutively abundant.

**Conclusions:** Our results suggest partly shared but distinct machinery of anhydrobiosis in heterotardigrades, and we therefore suggest a possible convergent evolution of anhydrobiosis in tardigrades.

**Keywords:** anhydrobiosis, *Echiniscus* cf. *testudo*, heat-soluble protein, Heterotardigrada
A new *in situ* labelling technique uncovers carbonyl accumulation in the tardigrade *Hypsibius dujardini* (Doyère, 1840)

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**Background:** UV irradiations promote DNA damages like thymine dimers and double-strand breaks. UVC irradiation of active *Hypsibius dujardini* has been reported to induce thymine dimers and instant death. Failure of DNA repairing systems has been proposed as an explanation of the lower UVC resistance of *Hypsibius dujardini* compared to other tardigrades. To date, only survival or DNA damage has been assessed in response to UV treatments. Here, we examine if a newly developed technique may reveal *in situ* protein carbonylation induced by UVC treatment in active and anhydrobiotic tardigrades.

**Results:** Irradiation of active tardigrades with UVC doses, ranging from 60 to 180 Kjm⁻², induced a proportional increase of the carbonylation labelling with a high correlation degree. We were able to measure an up to five-fold increase in the carbonyl content with the highest UVC dose assessed. For anhydrobiotic tardigrades irradiations in the same range also revealed high correlation to carbonylation labelling with up to a 2.5 fold increase. As staining was observed in both active and anhydrobiotic UVC irradiated tardigrades, it is likely that dehydration, by itself, does not provoke an immediate rise in carbonyl labelling. Additionally, we were not able to detect a saturation of the carbonyl staining signal during UVC irradiation indicating, at least for the anhydrobiotic tardigrade, that the UVC doses assessed do not allow a complete saturation of the molecular sites likely to be affected by carbonylation which are mainly linked to proteins.

**Conclusions:** *Hypsibius dujardini* is the first tardigrade species in which carbonylation has been detected. It will be interesting to assess if other tardigrade species have a comparable molecular signature upon UV irradiation. Carbonylations represent a well-known molecular signature of encountered oxidative stress by proteins and protein carbonylation is known as an age-associated mark with age accumulation documented in numerous organisms. It is possible that carbonyl accumulation may represent a determinant process in the UV resistance for a given tardigrade species. Deciphering the carbonyl clearance - proteostasis maintenance mechanisms used by tardigrades may be of great importance for a comprehensive understanding of the molecular roots of tardigrade resistance.

**Keywords:** aging mark, carbonylation, *Hypsibius dujardini*, UV radiation
Investigating candidate genes of cryobiosis in the eutardigrade *Ramazzottius oberhaeuseri* through real-time qPCR quantification of differential expression

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**Background:** The eutardigrade *Ramazzottius oberhaeuseri* can survive exposure to extreme cold by reversibly entering a latent state of high tolerance, termed cryobiosis. Here we investigate candidate genes with putative association to mechanisms of cryobiotic protection of biomolecular structures, reduction of the damaging effects of ice formation and freeze-induced cellular dehydration as well as post-cryobiobiotic regeneration. The expression patterns of a collection of these genes are examined in correlation to cryobiosis by employing real-time qPCR differential expression analyses to perform relative quantification of expression of active specimens in comparison to 2-hour post-frozen specimens, having been exposed to -80°C for 10 days. **Results:** In this study, significant correlations have been established between cryobiosis and upregulated expression of Secretory Abundant Heat Soluble Protein 1 (*SAHS1*) (3.7-fold, P<0.0001) and Cytoplasmic Abundant Heat Soluble Protein 3 (*CAHS3*) (3.2-fold, P<0.0001). Less significant identifications of upregulated expression for Aquaporin-10 (*AQP10*) (1.6-fold, P=0.0151) and downregulated expression of a putative glucose transporter (*SLC2A1*) (0.6-fold, P=0.0320) were detected. Several genes did not show any differential expression in the 2-hour post-frozen state, specifically Heat Shock Protein 20 (*HSP20*), Heat Shock Protein 70 (*HSP70*) and Late Embryogenesis Abundant Protein 4 (*LEA4*). A more constitutive expression pattern or post-frozen reestablishment of expression is supported by the obtained results for these genes. **Conclusions:** Our results indicate important functions of the eutardigrade-specific SAHS and CAHS proteins in regard to cryoprotective mechanisms in *Ramazzottius*. Combined with recent findings on the structure and localization of SAHS1 from *Ramazzottius varieornatus*, we hypothesize that SAHS1 may have a potential importance in relation to cryobiobiotic survival, potentially through carboxylic acid binding in the extracellular space. Furthermore, the importance of molecular transport and osmotic processes in cryoprotection is supported by differential expression of *AQP10* and *SLC2A1*. The results do not exclude HSP20, HSP70 and LEA4 as important elements of cryoprotection, but merely indicate constitutive expression or reestablishment 2 hours after cryobiosis. This work was supported by research grant (17522) from VILLUM FONDEN. MK is a Marie Curie fellow funded from the European Union’s Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 747087.

**Keywords:** cryobiosis, differential gene expression, eutardigrade, real-time qPCR
Gene expression patterns during anhydrobiosis in *Hypsibius exemplaris*

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**Background:** Identifying and understanding the role of genes underlying adaptive phenotypes is a major goal of modern evolutionary ecology. Anhydrobiosis is phenotypic adaptation that allows individuals to withstand the temporary desiccation of their otherwise aquatic environment. It is common across microinvertebrate groups, namely tardigrades. Gene expression analyses provide a window to the molecular mechanisms that enable organisms to survive and recover from these events. Proteins of three major groups have been suggested to have a key role in such adaptations: heat-shock proteins (HSPs), aquaporins (AQPs) and late embryogenic abundant proteins (LEAs). However, previous studies have largely focused on only a small subset of these proteins, and/or only compared effects between the end points of the anhydrobiosis process (i.e. dry vs. active individuals). We combine for the first time (i) the simultaneous estimate of expression levels in all three key protein groups, and (ii) a comparison across consecutive anhydrobiosis states (active, transitioning and dry), using the tardigrade *Hypsibius exemplaris*. Moreover, by using digital droplet PCR (ddPCR), a novel approach that is optimized for small sampling units, we could obtain more precise expression measures.

**Results:** Initial results show different patterns of gene expression for each target gene. The expression level of HSP did not change from active to transitional state, but was reduced to one fifth in the dry state. AQP expression levels were three times higher in the active state than in the transitional state, and were further lowered to 60% in the dry state. In contrast, there were no changes in LEA transcripts across the observed anhydrobiosis states. These findings suggest similar roles of different stress proteins forms between *H. exemplaris* (hsp70-like I, Aqp10) and *Milnesium tardigradum* (hsp70 isoform I, MtAqp-1). The LEA gene did not seem to have a relevant function for entering anhydrobiotic state.

**Conclusions:** This study offers the first comprehensive view of the interelationships between all three major proteins groups associated with anhydrobiosis within a single tardigrade species. It illustrates the importance of studying species-specific molecular mechanisms of tardigrade adaptations, as well as providing a benchmark for comparative studies and future experimental research.

**Keywords:** HSPs, aquaporins, LEAs, ddPCR
Application of fluorescent dyes to evaluate mitochondrial energetic status and oxidative stress during tardigrade anhydrobiosis

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Background: Many tardigrade species are able to survive complete dehydration and this phenomenon is called anhydrobiosis. During dehydration tardigrades curl up into a little ball called a tun, which can be regarded as a consequence of cytoprotective mechanisms triggered at cellular and molecular levels. The anhydrobiotic state can therefore be defined as an organized state and as such it requires some form of energy supply that imposes mitochondria involvement. Accordingly, in the presence of a so called “mitochondrial uncoupler” tardigrades are able to survive but lose the ability to form the tun that implies mitochondrial coupling supporting ATP synthesis based on the oxidative phosphorylation. Besides, being the main intracellular location for ATP synthesis, mitochondria are also involved in reactive oxygen species (ROS) production. Importantly, an efficient protection against dehydration stress in anhydrobiotic organisms is thought to require ROS scavenging mechanisms. This adds to the mitochondrial role in maintaining proper course during the dehydration process.

Results: We decided to estimate mitochondrial coupling in active and anhydrobiotic (forming tuns) tardigrades by application of the cell-permeant, cationic, lipophilic fluorophore tetramethylrhodamine methyl ester (TMRM) transported into mitochondria in the presence of the mitochondrial inner membrane potential. We also studied the levels of mitochondrial ROS production by the application of the MitoSOX Red fluorescent dye as a mitochondrial superoxide indicator. The resulting fluorescence was analyzed under fluorescence microscopy. We observed that the presence of functional mitochondria in tuns correlates with successful recovery to the active stage. We were also able to detect differences in ROS levels between the tun and the active stage.

Conclusions: Mitochondria appear to be underestimated in studies on cellular mechanisms of successful anhydrobiosis. Thus, there is a need for addressing their functioning during tun formation, the tun stage and rehydration as well as for monitoring their functioning during different dehydration/rehydration stages. The applied fluorescent dyes seem to be useful tools in this kind of studies. The work was supported by the research grant of National Science Centre, Poland, NCN 2016/21/B/NZ4/00131.

Keywords: anhydrobiosis, mitochondria coupling, reactive oxygen species, tardigrades
Awaiting Mr. Perfect - how female tardigrades attract their males

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Background: The present study gives first results on the partner finding behavior of tardigrades. We investigated whether the approach of a male tardigrade of the species *Isohypsibius dastychi* towards a ready-to-mate female is random or targeted. For this purpose, tardigrade couples were positioned in an arena, at 1, 2, 3, and 4 mm distance from each other.

Results: Ready-to-mate females were in late simplex state and thus immobile. The movement of the male, especially the distance traveled, time invested and its speed were video recorded and evaluated with a tracking-program. The smaller the initial distance between the tardigrade pair, the more targeted the male headed for the female. The distance traveled and the time invested increased exponentially with increasing initial distance between male and female, which means that the male lost orientation of the female the larger the distance towards her.

Conclusions: The present findings hint to a still undetermined female pheromone, which builds a concentration gradient in the water and loses strength quickly. Our assumption of a strong communication agent is further confirmed by the rare observations of sperm release without direct contact between partners that nevertheless resulted in fertile offspring. That the females that were partners in such no-contact mating behavior then deposited their eggs – a behavior that was never observed when males were absent from the chamber - further suggests that they likewise can sense a fresh ejaculate of spermatozoa in their direct vicinity. Pheromone signaling explains why tardigrades can afford having a highly unbalanced sex ratio with sexual populations often holding many females and only very few males.

Keywords: Eutardigrada, *Isohypsibius dastychi*, mating behavior, partner finding, pheromone
Characterization of reproductive mode in the tardigrade *Ramazzottius varieornatus*

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**Background:** More than 1200 species have been described in the phylum Tardigrada since the discovery of this invertebrate group by Goeze in 1773. Life history traits and reproductive modes have been characterized in only a few species in this phylum, mainly because of the difficulty in culturing tardigrades. In this study, we investigated the reproductive mode of the tardigrade *Ramazzottius varieornatus* from Sapporo, Japan, via culture experiments and histological analysis.

**Results:** We started an individual culture for *R. varieornatus* ($n = 20$) at 25ºC after each individual hatched. Among them, 11 individuals produced eggs 1–5 times during their lifetime, whereas nine did not lay eggs. The first oviposition after hatching was observed from day 7 to day 25. In total 55 eggs were laid, and 80% of the eggs hatched with an average developmental time of 5.7 days (range: 4–7 days). We then observed 31 *R. varieornatus* adult specimens stained with acetic-lactic orcein. Ovaries were found in 24 specimens and neither spermatozoa nor other male germinal cells were observed. There were six bivalents in a 4’,6-diamidino-2-phenylindole (DAPI) stained specimen’s cell, suggesting that the population was diploid.

**Conclusions:** We believe that the population of *R. varieornatus* comprises only females and reproduces parthenogenetically. Parthenogenesis in *R. varieornatus* may have developed in relation to its anhydrobiotic capacity, in which an animal can reproduce alone in new habitat following dispersal in the anhydrobiotic state.

**Keywords:** parthenogenesis, *Ramazzottius varieornatus*, reproduction, tardigrades
Fine structure of the midgut epithelium in two species of Macrobiotidae (Eutardigrada, Parachela)

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**Background:** The digestive system of tardigrades belonging to Parachela is composed of a foregut, midgut and hindgut. The foregut is responsible for drawing nourishments into the midgut that fulfills the role of a digestive organ, where processes of secretion, absorption and excretion appear. The midgut opens into a short hindgut that ends in a cloaca with the anus. The midgut epithelium of tardigrades is the first line of defence against stressors such as pathogens, excessive levels of heavy metals and toxic substances.

**Results:** As a material for our study we chose two gonochoric, carnivorous species belonging to the Macrobiotidae family: *Macrobiotus polonicus* and *Macrobiotus pallari*. In both species examined here, two types of cells can be distinguished in the midgut epithelium — digestive and regenerative cells. The digestive cells form a single epithelium that lays on the basal lamina, while the regenerative cells form an “epithelial ring” in the posterior end of the midgut. The regenerative cells play the role of midgut stem cells. Reserve material accumulated in the cytoplasm of the digestive cells in female specimens of both analyzed species is constituted by spheres of different size and electron density. In the cytoplasm of male specimens only small spheres of medium electron density can be observed.

**Conclusions:** The midgut epithelium is formed by digestive cells and one “epithelial ring” containing regenerative cells. More reserve material is accumulated in the cytoplasm of digestive cells in female specimens than in males.

**Keywords:** digestive cells, digestive system, regenerative cells, reserve material, tardigrades
Ultrastructural analysis of the dehydrated tardigrade

*Hypsibius dujardini* (Doyère, 1840) unveil an anhydrobiotic specific architecture

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**Background:** Tardigrades can cope with adverse or alien environmental conditions by turning into anhydrobiotes. A process by which tardigrades lose almost all of their body water. By the onset of this process, the animals contract to form a characteristic "tun" shape. As holds for organ functionality conservation, cell structure and organelle integrity must be protected in order to allow a subsequent rehydration process. However, to date, cell ultrastructure of *Hypsibius dujardini*, a limnoterrestrial species, has not been explored under anhydrobiotic conditions.

**Results:** We performed slow desiccation protocols for the induction of anhydrobiotic specimens that allows full anhydrobiote formation in the *Hypsibius dujardini* species. We subsequently used Transmission Electron Microscopy (TEM) to compare cell shape organisation and ultrastructure between hydrated and anhydrobiotic *Hypsibius dujardini*. While the structure of cell organelles was comparable, we observed a reduction of cells size in dehydrated animals of roughly 50% (+/- 10) and a significant reduction of mitochondria size (25% +/− 5). The nucleus appeared with both eu- and hetero-chromatin figures in hydrated as well as dehydrated tardigrades. In anhydrobiotic specimens, we observed secretory active cells with an abundant rough endoplasmic reticulum. Interestingly, these cells were found to be in close relationship with a specific extracellular structure surrounding the anhydrobiote cells. This 100 nm (+/- 10) thick rampart structure has been explored. It is possible that this rampart-like shaped extracellular structure results from the accumulation of anhydrobiotic specific material.

**Conclusions:** Specific expression of intra- and extra-cellular heat-soluble proteins have been reported and are expected to protect from the dehydration induced aggregation of denatured protein by a vitrification process. Members of this protein family have been shown to be essential for desiccation survival in *Hypsibius dujardini*. It is possible that the *Hypsibius dujardini* cell structure modifications during anhydrobiote formation involve such inducible and specific protection to resist the adverse conditions.

**Keywords:** Anhydrobiosis, *Hypsibius dujardini*, tardigrade, transmission electron microscopy.
Capabilities and limits of micro-CT shown with Tardigrada

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**Background:** The organization of tardigrades has so far been investigated by means of very different methods (various light microscopic techniques including immunostaining especially of whole-mount preparations, SEM, TEM) and is, therefore, well-known. All these methods are invasive and seriously affect the integrity of the specimens. A method of choice could be the non-invasive micro-computed tomography (µCT) currently used to produce 3D images of a variety of organisms among others of those which should remain intact, e.g., rare and precious museum specimens. However, the resolution of the most scans (usually down to a pixel size of ca. 1 µm) is too low to allow the study of minute metazoans such as tardigrades. Here we present 3D images of several tardigrades fixed with boiling 80% ethanol and studied with fast white beam µCT and a pixel size of 1.2 µm, available only at synchrotron radiation facilities. In comparison to laboratory X-ray sources, synchrotrons offer various physical contrast mechanisms and much shorter exposure times.

**Results:** Generally, the obtained 3D images (tomograms) do not reach the quality of images obtained with other methods. This applies to many external (e.g., lunulae on the base of the claws, peribuccal lamellae, details of the cuticle) and internal (macroplacoids) characters important for determination. However, 3D images give an insight in the organization of a tardigrade. Segmentation of those tomograms facilitates highlighting of selected organs and their position in relation to each other. This will be shown with some examples.

**Conclusions:** Currently, high resolution 3D images obtained with this technique do not adequately show most relevant taxonomic characters, but gives an impression of the spatial orientation of organ systems. Application of several staining methods (e.g. iodine) may improve the results and further technological developments (nano-CT, X-ray microscopy) are expected to enhance the resolution substantially.

**Keywords:** Non-invasive µCT, anatomy of tardigrades, synchrotron
Earth-like and tardigrade survey of exoplanets and Mars

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Background: Finding life on other worlds is a fascinating area of astrobiology and planetary sciences. Presently over 3500 exoplanets, representing a very wide range of physical and chemical environments, are known. Scientists are not only looking for traces of life outside Earth, but they are also trying to find out which of Earth’s known organisms (e.g. Tardigrada) would be able to survive on other planets.

Results: In our study, we have established a metric tool for distinguishing the potential survivability of active and cryptobiotic tardigrades on rocky-water and water-gas planets in our solar system and exoplanets, taking into consideration the geometrical means of seven physical parameters such as radius, density, escape velocity, revolution, surface temperature, surface pressure and surface gravity of the considered planets. The Active Tardigrade Index (ATI) and Cryobiotic Tardigrade Index (CTI) are two metric indices with minimum value 0 (= tardigrades cannot survive) and maximum 1 (= tardigrades will survive in their respective state). Values between 0 and 1 indicate a percentage chance of the active or cryptobiotic tardigrades to survive on a given exoplanet. Among known planets (except for Earth), the highest values of the ATI and CTI indices were calculated for Mars and considered exoplanets like: Kepler-100d, Kepler-48d, Kepler-289b, TRAPPIST-1 f and Kepler-106e. The results with Mars as the threshold indicate that Mars would be the most suitable rocky-water planet for tardigrades among all considered planets in our analysis.

Conclusions: At present we can conclude that all discovered planets till now are not very suitable even for such extremotolerant Earth invertebrates’ like Tardigrada.

Keywords: Active Tardigrade Index (ATI), Cryobiotic Tardigrade Index (CTI), exoplanets, habitability
DRYNET project: an international research network
to explore dry storage as an alternative strategy to cryostorage

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Background: Recent progresses in regenerative medicine, drug testing, and disease modelling have resulted in massive increase in stored cell lines under liquid nitrogen (-196 °C). Moreover, the international Convention on Biological Diversity calls all signatory nations to set up cells and gametes repositories of species in their respective territories as an instrument to counteract biodiversity decline worldwide. Although effective, cryostorage presents several problems, including high maintenance costs, need for specialised storage facilities and continuous liquid nitrogen supply, energy-dependency, safety concerns, and risk of pathogen transmission, all serious issues in clinical practice. Besides these intrinsic problems, the industrial production of liquid nitrogen has a serious environmental impact, leaving a massive carbon footprint. Hence, alternative storage options for cells are necessary.

Results: The DRYNET project sets an interdisciplinary/sectorial/international research network to explore dry storage as an alternative strategy for cells/germplasm biobanking. In particular, its aims to develop alternative methods to preserve different cell types of mammals based on water subtraction techniques to induce reversible block of macromolecular interactions, allowing cells storage at non-cryogenic temperatures. This will be achieved by the synergy arising from collaborative efforts of theoretical modellers, engineers, embryologists, cryobiologists, zoologists, molecular biologists and leading biobanking experts cooperating in DRYNET. In the context of DRYNET, the screening for important xero-protectants will be performed in two of the best-characterized anhydrobiotic animals, i.e. midges (\textit{Polypedilum vanderplanki}), and tardigrades.

Conclusions: DRYNET research network objective will allow i. to establish an international and multidisciplinary research team, ii. to prepare a new class of researchers with unique expertise and skills in alternative biobanking solutions, iii. to discover new molecules involved in natural desiccation tolerance, iv. to develop a new method to reversible drying sensitive cells, such as mammalian cells. This project has received funding from the European Union’s Horizon 2020 under the Marie Skłodowska-Curie "Research and Innovation Staff Exchange (RISE)" Grant agreement No 73443 - DRYNET

Keywords: Biobanks, desiccation tolerance, international network, xero-protectans
Developing quantitative, automated methods for assaying tardigrade behaviors

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Background: A fundamental question of neuroscience is how dynamical cellular activity in a brain produces reliable and effective behaviors. Tardigrades such as Hypsibius dujardini are promising model organisms for systems neuroscience because of their complex but tractable nervous system which is compatible with emerging noninvasive cellular imaging techniques. Further, for studying the production of movement and movement disorders, tardigrades may represent more natural organisms than Caenorhabditis elegans owing to the presence of 8 limbs than exhibit both a coordinated locomotory gait as well as independent movement. We sought to develop methods to quantify tardigrade behaviors and factors which affect them.

Results: We designed a free-roaming behavioral arena with an overhead camera and circular LED array for selective illumination of the stage. The LEDs are driven by an Arduino microcontroller, allowing for time-sequenced delivery of directed light of different wavelengths. Second, we developed a suite of open-source software tools to track multiple animals simultaneously and analyze their movement. Using these tools we examined how movement was affected by (a) presence of directed light of different wavelengths, (b) surfaces the animals were walking on, and (c) exposure to pharmacological agents which target neurophysiology. Third, we designed a microfluidic chip to lightly restrain animals during live microscopy, allowing them to move their appendages while keeping body segments such as the head still. Finally, we stained tardigrade neuroanatomy to confirm that our chip positions the animal such that its neurons are accessible for live imaging.

Conclusions: Rigorous behaviors are crucial for assaying the effects of genomic and neural perturbations. Taken together, our experiments represent initial steps toward the development of quantitative assays and apparatus for studying the neural production of behaviors of Hypsibius dujardini. These tools can also be used for comparative study across tardigrade species, for example in differentiating behaviors of eutardigrades and heterotardigrades. In this way, our approach may be useful for clarifying evolutionary relationships across species. Overall these experiments lay the foundation for future work on how neural dynamics give rise to behavior.

Keywords: animal tracking, behavior, Hypsibius dujardini, microfluidics, neurobehavioral assay
e-Tardigrada – a computational model for testing hypotheses about water bear life history and conducting astrobiology research

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Background: Tardigrades are renowned for the ability to tolerate exposure to extreme conditions. Most Earth-based tolerance research involves exposure to one extreme factor at a time. Outer-space-based tolerance research can involve simultaneous exposure to multiple extreme factors but is expensive and involves tardy data return. To enable tolerance research involving simultaneous exposure to multiple extreme factors in an inexpensive, immediate data return manner, we developed e-Tardigrada, a computational model that can be used to emulate life histories for individuals in a tardigrade population. The computational model can receive as input data acquired from real-world, single extreme factor experiments, then utilise those data to simulate populations simultaneously exposed to multiple extreme factors over time.

Results: The computational model was discovered to have been designed fortuitously for mimicking experimental protocols (e.g., egg removal from cultures). It returned survivorship curves that were similar qualitatively and quantitatively to survivorship curves obtained in real-world, single extreme factor experiments when trained on radiation and g-equivalent acceleration tolerance data. The ability to accommodate variety of relations between and among effects was required for the computational model to return results from in-silico, simultaneous multiple extreme factor experiments that were similar quantitatively to results obtained from real-world, outer space experiments.

Conclusions: Because the computational code was developed using data for a single parthenogenetic species subjected to particular extreme factors, modifications to the computational model are required before it can be used generally by researchers. Different species should be represented; different reproductive modes should be implementable; and data for additional extreme factors, such as low humidity (e.g., desiccation), temperature (e.g., freezing), and pH (e.g., acidification), should be included. e-Tardigrada then will constitute a tool useful to ‘aquarsologists’ as well as ‘astrobiologists’.

Keywords: growth, outer space, reproduction, survival
The challenges establishing terrestrial tardigrade cultures, fungal infections

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Background: Tardigrades are extremophiles capable of entering a cryptobiotic state (anhydrobiosis, cryobiosis, anoxybiosis, etc.) when faced with certain adverse environmental conditions. This ability, coupled with their reputation for effective and efficient DNA repair, has increased popularity in these organisms, leading to a recent upsurge in research into these processes for fields such as medicine and astrobiology. In order to undertake laboratory-based investigation, a reliable method for establishing a multigenerational culture must be developed. One major issue associated with establishing such a culture is the high incidence of fungus-related deaths in individuals isolated from environmental samples. This study aims to determine a method by which fungal pathogen contamination can be minimised safely, using copper sulphate in the culture medium.

Results: Freshly collected tardigrades were exposed to a range of copper sulphate concentrations in solution for either 72 hours or 96 hours to determine the response of the tardigrades to the varying concentrations. Specimen activity and evidence of fungal growth was recorded every 24 hours. There was a general trend of decreased percentage of specimens demonstrating fungal growth with increasing concentrations of CuSO₄. Additionally, increased concentrations of CuSO₄ brought about a higher percentage of inactivity and at a quicker rate than lower concentrations.

Conclusions: High concentrations of copper sulphate inhibited tardigrade activity but it also inhibited fungal infections. Low concentrations had a negligible effect on tardigrade activity and fungal infections. A copper sulphate solution with an optimum concentration of 0.025 mg/l both inhibited fungal growth but enable a majority of tardigrade samples to remain active. This concentration could be added to the culture medium to reduce or prevent fungal contamination and thus improve the longevity and yield of the culture.

Keywords: copper sulphate, cultures, fungal pathogens, Tardigrada
Challenges of establishing terrestrial tardigrade cultures

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**Background:** Tardigrades are well known for their impressive ability to survive harsh environmental conditions such as extreme heat, cold, and radiation. But comparatively little is known about these organisms despite this. Producing long-term, sustainable, multi-generational colonies of tardigrades has proven difficult because of this lack of information about a taxa’s preferred environmental conditions. Consequently, there are very few active colonies being maintained globally. Issues involved in culturing tardigrades include fungal infection, sensitivity to water quality and contaminants. Literature detailing ideal conditions is surprisingly scarce. Experiments were performed to determine the most efficient source of acquiring tardigrades, comparing the numbers produced from different lichen and moss species, and the most effective ways of isolating tardigrades from these, comparing different water compositions and soaking times.

**Results:** Tap water versus KCM solutions versus bottled spring water, and soaking times of 1h, 4h, 24h, 48h, 72h, and 96h. The samples using chlorinated drinking water from the tap performed the worst, with the majority of animals becoming inactive over 96 hour period, whereas both the bottled spring water and the KCM solutions had active animals throughout the experiments. In addition, no significant difference was found in activity based on soaking times of lichen or moss. We also noted that there was a significant issue with specimens becoming infected with fungi during the experiments.

**Conclusions:** For small scale culturing, using either bottled spring water or a solution of KCM is possible. KCM, a fresh-water substitute, is usually cheaper to produce in bulk and is recommended. However, using chlorinated tap water resulted in all specimens becoming inactive. In addition, it is recommended to soak lichen for a 1h time period in contrast to the commonly accepted 24h+ as there is no significant difference in the number of organisms collected.

**Keywords:** cultures, Tardigrada, water quality
Use of tardigrades to implement classroom research projects

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Background: Next Generation Science Standards call for students to engage in scientific practices to better understand the natural world. This type of authentic learning experience was implemented by high school Biology students in Marion, IL as they investigated relationships between lichen/moss habitats and tardigrade populations. The goal of this classroom research was to make practices of scientific inquiry more attainable for school-aged children, improve the overall attitude of students towards science and research, and increase knowledge of tardigrades and their habitats. Tardigrade research in Illinois is limited, so students immediately recognized the opportunities for investigating these micro-animals. Students collected lichen/moss samples from 62 different Southern Illinois locations either at home or in natural areas. Tardigrades were isolated from the samples, and the genera were identified. The students then studied the trends of tardigrade abundance. They observed that samples collected from the ground consistently lacked tardigrades; however, every lichen sample collected from a tree had some tardigrades present. Based on these observations they developed the research question: How does the habitat (lichen versus moss) affect the tardigrade populations? Based on the initial observations made by students, classes hypothesized that the lichen samples would be more reliable than moss samples for observing tardigrade populations. Lichen and moss samples were collected from 21 vertical surfaces (trees or rocks) within the Crab Orchard National Wildlife Refuge.

Results: Of the 21 sampling sites, 7 sites produced more tardigrades on mosses, 6 produced more tardigrades on lichens, and 8 sites had an equal number of tardigrades in both lichen and moss samples. Therefore, students rejected their hypothesis. Many continued to further analyze the data and explored differences in tardigrade populations on different substrates, in natural versus developed areas, at the base of the tree versus breast height, and when other microorganisms such as nematodes, rotifers, and ciliates were present.

Conclusion: Students were engaged and invested in this research activity, and post-test scores increased by 45%. Inquiry based investigations with tardigrades were demonstrated to be an affordable and accessible strategy for meeting multiple Next Generation Science Standards in the classroom.

Keywords: classroom, education, inquiry-based, NGSS
Tardigrades are ideal for teaching scientific inquiry and research skills

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Background: Inquiry science and mentored scientific research are effective and empowering experiences for in-service teachers. During an intensive summer research program supported by the Robert Noyce Program at the National Science Foundation, we engaged 29 teachers in a unified team-based research project that involved tardigrade diversity in the Cache River wetland of Southern Illinois. Key elements to that summer program were 1) training by a dynamic professional in the focus area, 2) systematic and well-designed team efforts to collect, analyze and archive data, 3) development of lesson plans scaled for four grade bands (K-2, 3-5, 6-8, and 9-12), and 4) a combined research and pedagogy symposium. A STEM graduate student led each of five research teams that included at least one mathematics, one biology and one physical science teacher.

Results: Teachers have continued to work toward a scientific publication that reports their findings. Collectively, teachers have identified ten genera of tardigrades: Macrobiotus, Minibiotus, Milnesium, Echiniscus, Astatumen, Hypsibius, Isohypsibius, Ramazzottius, Pseudechiniscus and Doryphoribius, the latter two of which are new to Illinois. Based on participant responses to surveys, teachers valued the summer research experience, enjoyed working in teams and with scientists, and saw the experience as beneficial to their students. For example, the average response to “I understand better how science is done.” and “I feel more competent teaching my students the skills necessary to conduct scientific research.” was strongly agreed (4.68 and 4.7, respectively). In the ensuring academic year, teachers followed up in a systematic effort to incorporate research skills into their classrooms. They conducted Action Research to assess the impact on student performance.

Conclusions: We conclude that student involvement in research using charismatic tardigrades is a powerful method of enhancing teacher knowledge and inquiry teaching, which translate to improved student learning.

Keywords: inquiry science, lesson plans, research teams, STEM
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