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Extreme-tolerance mechanisms in meiofaunal organisms: a case study with tardigrades, rotifers, and nematodes

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Abstract

To persist in extreme environments, some meiofaunal taxa have adopted outstanding resistance strategies. Recent years have seen increased enthusiasm for understanding extreme-resistance mechanisms evolved by tardigrades, nematodes and rotifers, such as the capability to tolerate complete desiccation and freezing by entering a state of reversible suspension of metabolism called anhydrobiosis and cryobiosis, respectively. In contrast, the less common phenomenon of diapause, which includes encystment and cyclomorphosis, is defined by a suspension of growth and development with a reduction in metabolic activity induced by stressful environmental conditions. Because of their unique resistance, tardigrades and rotifers have been proposed as model organisms in the fields of exobiology and space research. They are also increasingly considered in medical research with the hope that their resistance mechanisms could be used to improve the tolerance of human cells to extreme stress. This review will analyse the dormancy strategies in tardigrades, rotifers, and nematodes with emphasis on mechanisms of extreme stress tolerance to identify convergent and unique strategies occurring in these distinct groups. We also examine the ecological and evolutionary consequences of extreme-tolerance by summarizing recent advances in this field.

Key words: anhydrobiosis; cryptobiosis, desiccation, diapause, dormancy, encystment

Introduction

Tardigrades, rotifers and nematodes are considered permanent and essential members of freshwater and terrestrial meiofaunal communities that can undergo dormancy during their life stages (Bertolani et al., 2019; Guidetti et al., 2018; Hengherr & Schill, 2018; Rundle et al., 2002; Schill & Hengherr, 2018).

Tardigrades, commonly called “water bears”, are micrometazoans categorized into two main classes (Eutardigrada and Heterotardigrada) with 1298 species described from marine, freshwater and terrestrial habitats (Degma et al., 2019). The highest number of species belong to the class eutardigrades and to the family Echiniscidae within the heterotardigrades and has been described from terrestrial habitats, where they are inactive unless surrounded by a film of water. The smallest numbers are true limnic species, but several species are limnoterrestrial and can colonize both terrestrial and freshwater habitats (Nelson et al., 2015). Rotifera, also called “wheel animals”, is a phylum of microscopic metazoans, comprising about 2000 species (Segers, 2007) traditionally divided in three main classes: (1) Bdelloidea live in freshwater and terrestrial ephemeral aquatic environments and only reproduce by apomictic parthenogenesis; (2) Monogononta live in freshwater and marine environments and reproduce by cyclical parthenogenesis; and (3) Seisonida, with only a few exclusively marine species (Ricci, 1987; Wallace and Snell, 1991; Melone et al., 1998; Mark Welch and Meselson, 2000; Ricci and Melone, 2000; Segers, 2007). A fourth class, the exclusively parasitic Acanthocephala, has recently been added, although its exact relationship with the other taxa is still debated (e.g. Sørensen et al., 2005; Sielaff et al., 2016). The majority of nematodes, also called “roundworms”, are small free-living animals inhabiting the thin layer of water surrounding soil particles and in aquatic sediments, although some taxa have become endoparasitic and can reach meters in length (Lee, 2002). Some taxa have evolved the ability to

resist desiccation during various stages of their life cycles (Ricci and Pagani, 1997; Womersley, 1987; Shannon et al., 2005, Erkut et al., 2011).

Tardigrades, rotifers and nematodes are meiofaunal aquatic animals common in lakes, rivers, streams, and ponds, but paradoxically they are able to colonize and persist in desiccation-prone environments, such as freshwater (e.g. temporary ponds, Antarctic lakes, cryoconite holes) and terrestrial (e.g. mosses and lichens) habitats where liquid water is not always available (Nelson et al., 2015, 2018; Rundle et al., 2002). In these habitats, water loss can occur via evaporation or freezing, with diel, seasonal, annual, or longer fluctuations in the duration of the wet phase. Since tardigrades, rotifers and nematodes are incapable of active migration to more suitable habitats, occupancy of these unpredictable habitats requires organisms to be versatile, tolerant, or to possess specific and exceptional resistance and adaptive strategies (Fontaneto, 2019). Accordingly, life in these environments is adapted to a dual existence, flourishing when the habitat contains liquid water, and dormant when liquid water is not available and dormant states are linked to a temporary suspension of active life with reduction or interruption of metabolism and/or arrested development. Dormancy includes any form of resting stage, regardless of the cues required for induction or termination (Hand, 1991; Cáceres, 1997). Tardigrades, rotifers, and nematodes exhibit both forms of dormancy: quiescence (cryptobiosis) and diapause (encystment, cyclomorphosis and resting eggs) (e.g. Crowe and Madin, 1975; Ricci, 1987; Guidetti et al., 2011a).

Among the various forms of dormancy, cryptobiosis ("hidden life", Keilin, 1959) is under exogenous control, being directly induced and maintained by adverse environmental conditions, and it is immediately reversed by the removal of the external stimuli. It originated independently several times in the history of life, as it is present in diverse groups of bacteria, metazoans, fungi and plants (Clegg, 2001). Cryptobiosis includes different strategies such as anhydrobiosis, cryobiosis, anoxybiosis and osmobiosis directly induced by desiccation, sub-zero temperatures,

low oxygen pressure and osmotic extremes, respectively (Keilin, 1959; Wright et al., 1992). Cryptobiosis allows tardigrades, rotifers and nematodes to survive periods of desiccation, whereas few freshwater and marine species are known to have this adaptive strategy (Ricci and Pagani, 1997; Ricci, 1998; Eyres et al., 2005 Guidetti et al., 2011a, b; Clausen et al., 2014). Conversely, encystment and the production of resting eggs are a state of diapause controlled by both exogenous and endogenous stimuli and is more common in freshwater and marine species. Although tardigrades, rotifers, and nematodes exhibit both forms of dormancy, there are differences among taxa. Tardigrades, as well as insects, can undergo both diapause (encystment and cyclomorphosis) (Guidetti and Møbjerg, 2019) and the production of resting eggs (Hansen and Katholm, 2002; Altiero et al., 2010). In comparison, in rotifers the two main types of dormancy are restricted to two separate taxa. The class Bdelloidea can resist adverse environmental conditions *via* quiescence and directly respond to environmental stimuli at any life stage, from eggs to adults, although with age-dependent degrees of resistance (Örstan, 1995, 1998; Ricci, 1987, 1998), while the other main class, the Monogononta, only engage in diapause *via* the production of resting eggs, which tend to stop at a specific and common developmental stage and are generally very resistant to various environmental stresses, including desiccation (e.g. Balompapueng et al., 1997; Cáceres, 1997; Schröder, 2005; Garcia-Roger et al., 2006, Boschetti et al., 2010, Ziv et al., 2017). Within nematodes, dormancy is more scattered across taxa. For example some genera or species can survive desiccation (e.g. Wharton, 1996; Tyson et al., 2012), while other species only have limited resistance at specific life stages (e.g. Erkut et al., 2011; Erkut and Kurzchalia, 2015).

This review analyses the dormancy strategies in tardigrades, rotifers, and nematodes with emphasis on mechanisms of stress tolerance in order to identify convergent strategies occurring in these animal taxa. The review also considers the ecological and evolutionary consequences of extreme-tolerance by summarizing recent advances in this field.

Diapause: encystment

In terrestrial and freshwater tardigrades, encystment is an adaptive strategy that involves profound morphological changes that occur during the molting process, resulting in the dormant organism lying within retained cuticular exuvia. During this state, the organism also presents a very low or undetectable metabolism, even if the cyst is not desiccated (Patil et al., 2013; Ziv et al., 2017), highlighting possible physiological similarities between diapause and quiescence. Although encystment is rare in moss-dwelling tardigrades, it has been confirmed in grassland and leaf litter habitats but is more common in freshwater sediments (Guidetti et al., 2006). Encystment has been verified in limnic eutardigrades and a few heterotardigrade and eutardigrade limnoterrestrial species, however the phenomenon may be widespread but relatively unstudied (Guidetti and Møbjerg, 2019; Bertolani et al., 2019). In addition, the marine intertidal heterotardigrade *Echiniscoides sigismundi* Plate, 1888, a cryptic species complex, produces two or three new cuticles during cyst formation (Clausen et al., 2014).

In response to the gradual onset of adverse environmental conditions (e.g. temperature, oxygen tension, pH), encystment in tardigrades begins with the ejection of the sclerified parts of the buccal-pharyngeal apparatus ("simplex stage"). Instead of undergoing normal ecdysis, however, one to three new cuticles are serially produced in addition to the retained external (old) cuticle (Fig. 1). The animal's size is reduced by longitudinal contraction, body movements cease completely, metabolism is significantly reduced, and the mouth and cloaca are closed. Modified claws and buccal-pharyngeal apparatus are synthesized, but non-functional. At this stage, the cyst resembles an onion or a Russian doll ("Matryoshka") (Guidetti et al., 2006), often with one cuticle becoming hardened and pigmented. Encystment ends as environmental conditions improve, and the

tardigrade gradually resynthesizes a normal cuticle, claws, and feeding apparatus and leaves the cyst. Unknown endogenous stimuli may also play a role in the process.

Ecology of cysts

Limnic eutardigrades that frequently encyst belong to the genera *Dactylobiotus*, *Pseudobiotus*, *Isohypsibius*, *Hypsibius*, *Thulinius*, and *Bertolanius* (See Table 9.1 in Guidetti and Mobjerg, 2019 for a list of encysting tardigrade species reported in the literature). Detailed steps in encystment in *Dactylobiotus* and moss-dwelling/freshwater *Bertolanius* were provided by Guidetti et al. (2006). *Dactylobiotus* has only one type of cyst, which is dark-reddish brown (Szymańska, 1995; Guidetti et al. 2006, 2008), whereas *Bertolanius*, which has both limnic and moss-dwelling species, forms two types of cysts ("white/type 1" in cold periods and "red/type 2" in warm periods) that Westh and Kristensen (1992) correlated with seasonal environmental changes in Greenland. Cyst formation in *Bertolanius* is cyclic and be a part of cyclomorphosis, defined as cyclic and reversible morphological modifications within a single species (Kristensen, 1982; Rebecchi and Bertolani, 1994; Hansen and Katholm, 2002). The production of extra cuticles isolates and protects the animals from environmental factors. Since the cysts remain viable for several months, encystment enhances tardigrade survival of freezing in winter and desiccation in summer (since limnic tardigrades often disappear in summer). Although encystment is best studied in limnic species, which do not undergo anhydrobiosis (but a few species can withstand cryobiosis), most of the species that produce cysts can also enter anhydrobiosis (Guidetti et al., 2011a). Since diapause (encystment) and cryptobiosis are dormancy states that can be present in a single species, their evolution was not mutually exclusive. Although we are beginning to understand the molecular mediators involved in cryptobiosis, the molecular mechanisms involved in encystment (see Rozema et al., 2019) are unknown.

Extreme resistance strategy: anhydrobiosis

The most widespread and best-known form of extreme-stress resistance evolved by tardigrades, rotifers and nematodes is the capability to tolerate complete desiccation (drying to < 0.1 g H_2O g^{-1} dry mass) by entering in a state of reversible suspension of metabolism called anhydrobiosis ("life without water") without the loss of viability. At the end of dehydration process, tardigrades have lost 97% of their body water (Westh and Ramløv, 1991; Horikawa et al., 2008), and similar values have been shown for the anhydrobiotic nematodes *Ditylenchus dipsaci* (Kuhn, 1857), *Aphelenchus avenae* Bastian, 1865 and *Panagrolaimus superbus* (Fuchs, 1930) (Crowe and Madin, 1975; Wharton, 1996; Banton and Tunnacliffe, 2012).

Anhydrobiosis indicates a fundamental concept about the nature of living systems since an anhydrobiotic organism lacks all dynamic features of living organisms due to the absence of detectable metabolism. In that sense it is not alive, but it is not dead because rehydration produces a living organism and a kind of resuscitation routinely occurs (Clegg, 2001; Tunnacliffe and Lapinski, 2003). Consequently, anhydrobiotic organisms have two distinct living physiological states: active and anhydrobiotic.

Despite its clear adaptive potentiality, anhydrobiosis can be found only in a restricted number of metazoans whose sizes generally do not exceed 1 mm, with the exception of a few taxa that can reach 5-7 mm in length, such as the larvae of the African midge *Polipedylum vanderplanki* Hinton 1951 (Watanabe et al., 2004). These apparent morphological and ecological characteristics could be linked to limiting factors required for tolerating physical and physiological constraints imposed by complete dehydration (Alpert, 2005). In animals, desiccation tolerance occurs either the whole animal at any stage of their life cycle, from the egg to the adult stage (tardigrades, bdelloid rotifers and nematodes), in which case the animals are defined as holo-anhydrobiotic (Jönsson, 2005;

Rebecchi et al., 2007), or at a specific life stage, usually egg/embryo/larval stage (shrimps, the midge *P. vanderplanki*, monogonont rotifers, some nematodes).

As described above, anhydrobiosis allows tardigrades, rotifers and nematodes to colonise and persist in various otherwise unavailable environments. A high number of species colonise habitats subjected to periodic desiccation (e.g. lichens, mosses, and ephemeral lakes and ponds) that are prohibitive for most other animals. In these habitats, they perform all activities of routine life only when there is at least a small layer of water around the body of the animals. For example, mosses and lichens provide habitats featuring a myriad of small pockets of water; as their surroundings lose water through evaporation, animals lose water with them. Consequently, their life cycle consists of active periods for growth and reproduction, interrupted by periods of metabolic inactivity (Jönsson, 2005; Glime, 2017). When rehydrated by dew, rain or melting snow, they can return to their active state in a few minutes to a few hours. Therefore, during their life, holo-anhydrobiotic animals can enter anhydrobiosis several times (e.g. Ricci, 1987; Womersley, 1987). An experimental study evidenced that the moss-dwelling eutardigrade *Richtersius coronifer* (Richters, 1903) may survive up to 6 repeated desiccations, with a declining survival rate with an increasing number of desiccation events (Czernekova and Jönsson, 2016). Interestingly, repeated desiccation seems also to improve the long-term survival of rotifer populations. Populations that are regularly subjected to desiccation grow faster than permanently hydrated corresponding cohorts, suggesting that diapause is not only a strategy to survive harsh environmental conditions, but it also has ecological advantages to the organisms that managed to evolve this strategy (Ricci et al., 2007; Sommer et al., 2019).

The time for recovery to active life after a period of anhydrobiosis is directly related to the environmental condition during the desiccation phase (e.g. humidity rate during the desiccation process) in which higher stressors lead to longer recovery time, and to the time spent in

anhydrobiosis (Rebecchi et al., 2009a). The recovery time is probably function of the metabolic activities linked to the repair of damages caused by desiccation and/or to the restoration of metabolic pathways (see Mattimore and Battista, 1996).

Among anhydrobiotic tardigrade and rotifers studied, desiccation tolerance varied from zero to high tolerance (e.g. Ricci, 1987; Wright, 1989a; Bertolani et al., 2004; Rebecchi et al., 2006). These gradients are correlated with the abiotic factors (e.g. humidity) of the substrate inhabited since species living in constantly wet or submerged mosses usually show lower anhydrobiotic performance than those living in mosses growing on trees and rocks (Guidetti et al., 2011b; Eyres et al., 2015). In addition, anhydrobiotic capability is similar among species belonging to distant evolutionary lines, but they can be very different among closely related species. However, species with similar ecological requirements share a close similarity in anhydrobiotic performances (Wright, 1991, 2001; Guidetti et al., 2011b; Ricci, 1998, 2001; Ricci and Caprioli, 2005; Fontaneto et al., 2004; Fontaneto and Ambrosini, 2010; Eyres et al., 2015).

Therefore in both rotifers and tardigrades, we hypothesize that anhydrobiosis is more likely linked to local adaptations to habitats than to phylogenetic relationships suggesting that anhydrobiotic capabilities have been evolved once and secondarily lost in some lineages.

Some species of nematodes within the genus *Panagrolaimus* Fuchs, 1930 can survive immediate desiccation (e.g. Ricci and Pagani, 1997) and are referred to as fast-desiccation strategists, while others (e.g. *A. avenae*) require a period of slow-drying (pre-conditioning) and are referred to as slow desiccation strategists (e.g. Womersley, 1987; Shannon et al., 2005). Similar patterns were detected in tardigrade and rotifer species when experimentally desiccated under laboratory conditions (e.g. Ricci, 1987, 2001; Wright 1989a; Eyres et al., 2015; Hashimoto et al., 2016; Boothby et al., 2017). Full anhydrobiotic nematodes can undergo desiccation at any stage of their life cycles, but recent studies have suggested that some species, traditionally considered intolerant

to desiccation, can actually survive desiccation at least in some stages of their life cycle (e.g. the dauer larvae of the model species *Caenorhabditis elegans* (Maupas, 1900)) (Erkut et al., 2011). As in tardigrades and rotifers, the anhydrobiotic abilities of different taxa of nematodes seem do not appear to be related to their phylogeny, suggesting that the evolutionary processes have affected the loss or maintenance of this remarkable ability. Although traditionally less studied, the recent characterisation of some of the molecular strategies of diapause in nematodes, and especially in the well-known and well-characterised model organism *C. elegans*, allows a better understanding of how diapause is induced, maintained, and what its effects are, as well as common mechanisms to different organisms (e.g. Fielenbach and Antebi, 2008; Hand et al., 2016).

In the desiccated state, holo-anhydrobiotic animals are biostable for decades (e.g. tardigrades 20 years; Guidetti and Jönsson, 2002; Bertolani et al., 2004; Rebecchi et al., 2006; Jørgensen et al., 2007) even though recently the consistent long-term survival of at least some taxa under desiccation has been debated (Jönsson and Bertolani, 2001; Fontaneto et al., 2012a). For example, a comparative study of the survival rate of different taxa and the statistical model developed from it suggested that recovery of bdelloid rotifers, tardigrades and nematodes found in lichens within collections in museums decreases to almost zero after desiccation periods of up to 10 years; this is significantly longer than the life span of single individuals in the active state, but is not as long as anecdotally suggested by other studies, and not as long as in other taxa like resting eggs of monogonont rotifers (Cáceres, 1997; Fontaneto et al., 2012a). These data confirm that these organisms do survive long periods of desiccation but that the rate and general conditions of desiccation, as well as the substrate and the storage conditions during diapause, influence survival in a significant way (e.g. Ricci and Caprioli, 2001; Fontaneto et al., 2012a).

Other than its effect on longevity, anhydrobiosis can have an impact on ageing in meiofauna as illustrated by the “Sleeping Beauty” and “Picture of Dorian Grey” models derived from

experimental data on a few species of holo-anhydrobiotic organisms (for a review, see Kaczmarek et al., 2019). The first model predicts that anhydrobiotic organisms do not age during anhydrobiosis in at least some tardigrade and bdelloid rotifer species (Ricci and Covino, 2005; Hengherr et al., 2008a, b). The latter model predicts that anhydrobiotic organisms age, at least in the initial stages of the anhydrobiosis process, as in some species of nematodes (Ricci and Pagani, 1997). Nevertheless, a comprehensive comparative analysis that considers all taxa and strategies is still lacking.

Numerous studies have focused on molecular changes during aging in tardigrades, rotifers and nematodes, especially from the molecular approach, and the potential "rejuvenation" of stressed animals, but the full picture is very complex and still poorly understood. Early studies highlighted general changes in protein patterns with age (Carmona et al., 1989), and recent advances have started uncovering specific changes in regulatory molecules (e.g. Snell et al., 2014), protein modifications like carbonylation (Krisko and Radman, 2019), and improved physiological characteristics like fecundity (Ricci and Covino, 2005: Ricci and Perletti, 2006). Based on these and other studies, rotifers can be added to the list of useful model organisms which can be used to study aging (Snell et al., 2015), although the exact links between molecular changes and aging are still not fully characterised. Even more obscure at the moment are the precise links between the ability of some types of dormancy to stop or reverse aging. For example, both desiccation and starvation seem to stop or reverse aging in bdelloid rotifers, allowing dormant bdelloids to "wake up" with similar or higher fitness than animals in the pre-stressed condition (Ricci and Covino, 2005; Ricci and Perletti, 2006; Sommer et al., 2019). Some recent advances suggest that some of the mechanisms and molecules involved in the organism's protection during desiccation, like antioxidants or LEA proteins, can also prevent at least some aspects correlated with aging (e.g. Kaneko et al. 2005; Snare et al., 2013). Aging is generally better characterised in nematodes,

although the majority of studies are limited to model species like *C. elegans* (e.g. Schaffitzel and Hertweck, 2006; Hughes et al., 2007; Mack et al., 2018) and therefore lack the more direct link between aging and dormancy in stress-resistant animals from natural habitats. Interestingly, where data are available, they suggest that the rejuvenation effect of desiccation is not present in at least some anhydrobiotic nematodes of the genus *Panagrolaimus* (Ricci and Pagani, 1997), making the understating of the relationship between desiccation resistance and aging even more fascinating and interesting.

Even though dehydration can have a major effect on survival, aging and longevity, the anhydrobiotic process *per se* can induce molecular damages that accumulate with time, reducing the viability of desiccated animals (França et al., 2007; Tyson et al., 2007; Neumann et al., 2009; Rebecchi et al., 2009a; Marotta et al., 2010; Hespeels et al., 2014). The amount of these damages is directly impacted by high temperature, high humidity level and high oxygen partial pressure. In tardigrades, the time required to recover active life after a period of desiccation is affected by these abiotic conditions and can be related to the metabolic activities necessary to repair molecular damages and to catabolise damaged molecules (Rebecchi et al., 2009a; Guidetti et al., 2011a). Different strategies and molecules seem to be involved in the reduction and/or repair of molecular damage (see below).

Even more striking, in the dry state, anhydrobiotic organisms show extraordinary resistance to physical and chemical extremes (very low sub-zero temperature, high pressure, radiation, extreme pH, toxic chemicals, lack of geomagnetic field) that may far exceed the tolerance ranges of active organisms (Wharton et al., 2003; Jönsson et al., 2005, 2013; Watanabe et al., 2006; Rebecchi et al., 2007; Gladyshev and Meselson, 2008; Rebecchi et al., 2009b; Altiero et al., 2011; Guidetti et al., 2011a; Krisko et al., 2012; Rebecchi, 2013; Hashimoto et al., 2016; Erdmann et al., 2017; Jönsson and Wojcik, 2017; Giovannini et al., 2018).

In tardigrades, a strong correlation between the capability to withstand desiccation and the capability to withstand sub-zero temperatures (-20°C, -80°C) was detected, and species that were not able to enter anhydrobiosis showed low or no capability to withstand sub-zero temperatures (Guidetti et al., 2011a, b). This direct relationship could be related to the fact that during both desiccation and freezing stresses, tardigrades are under the same selective pressure induced by a wide variation in body fluid osmolality and in cell volume (Sømme, 1996; Guidetti et al., 2011b). Nevertheless, the freeze resistance of anhydrobiotic tardigrades should be distinguished from cryobiosis, which is the ability of active hydrated animals in contact with water to freeze and survive after thawing (Guidetti et al., 2011b).

The aggregate of all these characteristics, especially radiation tolerance, has led to the characterization of tardigrades as the "toughest animals on the Earth" (Copley, 1999) and to make them an emerging model for space biology (Horikawa et al., 2008; Jönsson, 2007; Erdmann and Kaczmarek, 2017), more recently joined by bdelloid rotifers. Tardigrades and rotifers have been exposed to space stressors in Low Earth Orbit several times, on board of the International Space Station and FOTON (Ricci and Boschetti, 2003; Ricci et al., 2005; Leandro et al., 2007; Selch et al., 2008; Jönsson et al., 2008; Rebecchi et al., 2009b, 2011; Persson et al., 2011; Guidetti et al., 2012; Vukich et al., 2012).

Morphological, physiological and molecular adaptations enabling anhydrobiosis

The evolution of a series of behavioural, morphological, physiological and molecular/biochemical adaptations provided anhydrobiotic organisms with mechanisms to withstand the deleterious effects caused by the drastic loss of water. The majority of holo-anhydrobiont organisms cannot survive a desiccation rate that is too rapid (as shown in a few hours in laboratory experiments, even though the rate is species-dependent (Wright, 1989a, b, c; Wright et al., 1992; Jönsson and Järemo,

2003; Banton and Tunnacliffe, 2012; Boothby et al., 2017), so they have evolved different strategies to slow down the rate of water evaporation.

To reduce the rate, the tardigrade shrivels into a barrel-shaped structure ("tun"), about one-third of its original size, by contracting the body anterior-posteriorly and withdrawing the legs and head (Figs. 2-3). Tun formation produces a new spatial organization of some internal organs (such as the pharyngeal bulb), and epidermal cells, storage cells, ovarian cells, and digestive system cells undergo shrinkage, containing electron dense cytoplasm (Czernekova et al., 2016). Lipids and polysaccharides dominate in the reserve material of the storage cells, whereas the amount of protein is small (Czernekova et al., 2016). The tun minimizes the permeability and evaporative surface of the organism by removing the high permeability areas of the cuticle from direct contact with the air, resulting in a slow rate of desiccation (Wright, 1988a, b, 1989a, b, c, 2001). Differences in the reduction of cuticle permeability detected among tardigrade species are related to the level of desiccation tolerance of each species and to the morphology of the cuticle in eutardigrades and heterotardigrades (Wright, 1989a, b). The permeability slump of the cuticle permits animals to lose water slowly, allowing animals to produce bioprotectants. Somewhat similarly to tardigrades, bdelloid rotifers contract their body into a compact shape by withdrawing their cephalic and caudal extremities into the trunk, facilitated by muscle contractions and by a coordinated morphological arrangement of internal structures (Ricci, 2001; Ricci et al., 2003; Marotta et al., 2010; Fig. 4.) A decrease in permeability (the permeability slump) during the early stages of desiccation was detected in the anhydrobiotic plant-parasitic nematode *D. dipsaci* during which the surface of the animal body was coated with an extracuticular layer of lipid (triglyceride) that produced a slow rate of water loss necessary for its survival (Wharton et al., 2008). Nematodes tend to coil their body (Crowe, 1971) and certain nematodes are also reported to congregate into masses of "nematode wool", with better survival of specimens in the centre of the mass (Ellenby, 1968). The

aggregation effect has also been experimentally produced in tardigrades (Ivarsson and Jönsson, 2004), but not yet verified in nature.

As water evaporates and dry conditions set in, holo-anhydrobiotic organisms start generating a variety of protective agents, collectively termed bioprotectants, which they accumulate in and around the cells of their body. It was initially thought that non-reducing disaccharides, like trehalose, were solely responsible for preventing damage (e.g. Crowe et al., 1984, 1992), but more recent studies point to a complex picture of molecular adaptations. These bioprotectants molecules include: sugars, mostly disaccharides such as trehalose; a unique repertoire of proteins generally lacking persistent tertiary structure classified as intrinsically disordered proteins (IDPs) or proteins with intrinsically disordered regions (IDRs) and represented by Late Embryogenesis Abundant proteins (LEAp), Heat Shock proteins (HSPs), cytoplasmic abundant heat soluble (CAHS) proteins, secretory abundant heat soluble (SAHS) proteins, and mitochondrial abundant heat soluble (MAHS) proteins; antioxidants, and molecules involved in protection from or repair of DNA damage (e.g. Lapinski and Tunnacliffe, 2003; Schill et al., 2004; Altiero et al., 2007; Jönsson and Schill, 2007; Pouchkina-Stantcheva et al., 2007; Förster et al., 2009, 2011; Schokraie et al., 2010; Boschetti et al., 2011; Yamaguchi et al., 2012; Boschetti et al., 2013; Rebecchi 2013; Wang et al., 2014; Tanaka et al., 2015; Hashimoto et al., 2016; Boothby et al., 2017: Schill and Hengherr, 2018). A recent study showed that one of the most stress-tolerant tardigrade species (*Ramazzottius varieornatus* Bertolani and Kinchin, 1993) has a tardigrade-unique DNA-associating protein, termed Dsup, which is able to suppress the incidence of DNA breaks caused by radiation (Hashimoto et al., 2016). Accumulation of these xeroprotectants is generally slow and gradual, taking place in parallel with the drying process, although a few taxa, like the nematode *P. superbus*, seem to express a full repertoire of protective molecules and have therefore been defined as fast-desiccation strategist (Shannon et al., 2005; Banton and Tunnacliffe, 2012). Even though many

organic compounds have been identified in tardigrades, rotifers, and nematodes, the biochemical and molecular mechanisms involved in complete desiccation tolerance are currently little known and constitute an intriguing challenge for biologists. For instance, it is well known that in some species of tardigrades the synthesis of the disaccharide trehalose counteracts the loss of water, as well as other environmental extremes (Westh and Ramløv 1991; Hengherr et al., 2008b, Jönsson and Persson, 2010; Wełnicz et al., 2011; Cesari et al., 2012; Schill and Hengherr, 2018). In any case, the absolute trehalose levels detected in tardigrades are much lower than those reported for other anhydrobiotic organisms. This sugar has a double role in desiccation tolerant organisms. As the trehalose replaces water, it protects biomolecules and the integrity of membranes during dehydration, and participates in the formation of a glassy matrix that reduces the rates of chemical reactions and inhibits free radical production (Crowe et al., 1984; Teramato et al., 2008). The CAHS, SAHS and MAHS proteins have been detected so far only in eutardigrades (Tanaka et al., 2015; Boothby et al., 2017). However, the distribution of the encoding genes of these proteins is scattered among tardigrades, suggesting species- or at least taxon-specific adaptations (Yoshida et al., 2017; Kamilari et al., 2019). The CAHS and SAHS proteins probably form a molecular shield inside and outside cells, respectively, whereas MAHS proteins are defined as potent specific mitochondrial protectants (Boothby et al., 2017). The heterologous expression of some CAHS proteins in both prokaryotic and eukaryotic cells allows an increase in their tolerance to desiccation, and purified CAHS proteins protect desiccation-sensitive proteins *in vitro* (Boothby et al., 2017). The very low or null metabolic activity of anhydrobionts limits the production/accumulation of products of metabolism such as Reactive Oxygen Species (ROS). Even though the origin of ROS in anhydrobiosis is not yet well known, their production can occur both during the dehydration, in a permanent desiccated state as well as during rehydration, so an efficient antioxidant mechanism is necessary (França et al., 2007; Cornette et al., 2010; Rebecchi, 2013). For example, in desiccated

specimens of the eutardigrade *Paramacrobiotus spatialis* Guidetti, Cesari, Bertolani, Altiero and Rebecchi, 2019, glutathione peroxidase was the most abundant antioxidant enzyme in hydrated animals, followed by the enzyme superoxide dismutase and glutathione content (Rizzo et al., 2010). With regard to the repair of DNA damages, desiccation enhanced the expression of DNA-repair proteins in tardigrades (Wang et al., 2014; Kamilari et al., 2019).

Rotifers possess similar strategies, but there are also marked differences. Neither trehalose nor the metazoan genes for its synthesis have been found in bdelloid rotifers (Lapinski and Tunnaclife, 2003), although trehalose has been found in monogonont rotifers (Caprioli et al., 2004), suggesting that non-reducing disaccharides are not necessary for successful recovery from desiccation. Instead, other molecules are now thought to be essential to protect molecules, cells and tissues and to repair any damage caused by anhydrobiosis. It is becoming clear that no single class of protectant/repair molecules is sufficient, but successful desiccation depends on the co-ordinated action of all of them. These molecules, which are usually upregulated upon desiccation, include different types of LEA proteins which perform different and often still uncharacterised functions, other (non-LEA) protein families, often at least partially unstructured (IDPs and proteins containing IDRs) and with still uncharacterised functions, other types of hydrophilins or chaperones, different types antioxidants, molecules involved in DNA repair as well as, probably, other still unknown molecules and mechanisms (e.g. Browne et al., 2002; Browne et al., 2004; Goyal et al, 2005; Pouchkina-Stantcheva et al., 2007; Denekamp et al., 2010, 2011; Boschetti et al., 2011, 2012; Hanson et al., 2013; Hespeels et al., 2014).

All these molecules, some of which are taxon-specific while others are common to all analysed taxa (e.g. Denekamp et al., 2010; Mali et al., 2010; Boschetti et al., 2011, 2012; Hanson et al., 2013; Hashimoto et al., 2016; Boothby et al., 2017; Hashimoto and Kunieda, 2017; Kamilari et al.,

2019; Kamilari et al, 2019), seem to be necessary for an integrated and effective response to anhydrobiosis.

Interestingly, the majority of the previously mentioned studies were based on the analyses of one or a relatively small subset of genes, but recent technological advances have allowed analyses of whole genomes and transcriptomes and are uncovering an even more fascinating story, suggesting that some animals, and bdelloid rotifers in particular, have been acquiring genes, which code for protective/repair molecules, from organisms that are not direct ancestors and can even belong to different taxa, in a process known as Horizontal Gene Transfer (HGT, also called lateral gene transfer, LGT). Horizontal gene transfer was previously known only in bacterial and archaeal organisms and was thought to be absent in eukaryotic organisms, but recent studies suggest that it is more widespread than previously thought (e.g. Dunning Hotopp, 2011; Boto, 2014, Drezen et al., 2017) and that these "foreign" genes can indeed contribute to the resistance to desiccation of some organisms, especially bdelloid rotifers. Initial suggestions that bdelloid rotifers possess a very high percentage of genes acquired *via* HGT (Gladyshev et al., 2008; Boschetti et al., 2012) and "domesticated" (Barbosa et al., 2016) have now been confirmed and expanded (Flot et al., 2003; Eyres et al., 2015; Hespeels et al., 2015; Nowell et al., 2018). This unusual characteristic is made even more interesting by the recent understanding of the role that these foreign genes play in stress resistance: many foreign genes are over-expressed during desiccation or rehydration and might therefore at least partially responsible for their successful anhydrobiotic capabilities (Boschetti et al., 2011, 2012; Eyres et al., 2015), although the precise link between desiccation resistance and levels of HGT is still unclear (e.g. Eyres et al., 2015; Nowell et al., 2018). Indeed, recent studies have found genes involved in trehalose synthesis in bdelloids, but they seem to have been originated by HGT (Hespeels et al., 2015), while other foreign genes can add various biochemical capabilities, some of which might improve the desiccation abilities of bdelloids (Boschetti et al.,

2012; Szydlowski et al., 2015). This unusual high level of foreign genes seems to be a characteristic only of bdelloid rotifers: other taxa have been analysed and, although a few foreign genes are present (e.g. Boothby et al., 2015; Bemm et al., 2016; Koutsovoulos et al., 2016; Nowell et al., 2018), they are not so abundant, and the details of their contribution to successful desiccation is still being characterised (e.g. Yoshida et al., 2017; Nowell et al., 2018; Kamilari et al., 2019).

Ecological and evolutionary consequences of extreme tolerance of meiofaunal organisms

The evolution of anhydrobiosis is the result of trade-offs between the selective advantages of this adaptive strategy, the energetic costs, and the physical and physiological constraints related to the process (Jönsson, 2005; Guidetti et al., 2011a). Energy is probably necessary to produce and accumulate bioprotectants during the initial phase of anhydrobiosis and to catabolize them during the exit phase (rehydration). There are few data on anhydrobiotic energetic costs, but a substantial energetic cost of anhydrobiosis was shown in the tardigrade *Richtersius coronifer* (Jönsson and Rebecchi, 2002) and in some species of nematodes (Madin and Crowe, 1975; Demeure et al., 1978). Little is known about rotifers, but the presence of lipid droplets (Wurdak et al., 1978) and the differential expression of some genes potentially involved in lipid metabolism or protection (Denekamp et al., 2009) in monogonont resting eggs and in desiccated bdelloids (Marotta et al., 2010) suggest that costs are present in these taxa as well.

The anhydrobiotic process requires energy that is withdrawn from other physiological functions such as growth and reproduction. This should have strong effects on the life histories of holo-anhydrobiotic organisms. Even though there is no direct evidence for a trade-off between anhydrobiosis and fitness, the few ecological studies on this topic are consistent with the hypothesis that fitness of desiccation tolerant organisms is lower (Jönsson, 2005; Alpert, 2006; Guidetti et al., 2007). In tardigrades, both positive and negative relationships between body size (as an indication

of age) and desiccation performance have been demonstrated at the intraspecific and interspecific levels. In species living in the same moss and with high anhydrobiotic performance, desiccation survival increases in *R. coronifer* with an increase of the body size, whereas it decreases in *Ramazzottius oberhaeuseri* (Doyère, 1840) (Jönsson et al., 2001; Jönsson and Rebecchi, 2002). These contrasting models could be due to genetic differences and/or contingent factors, such as nutritional state, level of molecular protectants and some life history traits, including age, reproductive stage, and phenotypic plasticity. Lastly, differences in anhydrobiotic performances among geographically isolated populations of eutardigrades have been reported, but the literature data are conflicting (Horikawa and Higashi, 2004; Jönsson et al., 2001) probably due to the presence of cryptic species and differences in ecological conditions of the microhabitats.

Interestingly, as previously mentioned, bdelloid rotifers seem to be different, i.e. desiccation improves individual and population fitness (Ricci et al., 2007; Sommer et al., 2019), but with still unknown mechanisms.

In addition, low fitness associated with a long-lifespan could slow down rates of evolution in comparison to organisms with similar lifespans but without the capability to perform anhydrobiosis. Furthermore, ancestral genetic traits may reappear after a long time in anhydrobiosis, jumping generations, and contributing to the longer existence of unchanged traits (Kaczmarek et al., 2019). Anhydrobiosis represents an ‘‘escape in time’’ from habitat conditions hostile to active life, opposed to an ‘‘escape in space’’ performed by organisms with an ability to migrate away from unfavorable conditions (Jönsson, 2005). In addition, it limits selection and creates a ‘‘seedbank’’ for maintaining haplotypes in time and space (environment) (Guidetti et al., 2011a). These advantages are reinforced by the ageing models (“Sleeping Beauty” and “Picture of Dorian Grey”) allowing organisms to withstand adverse conditions for a long time and the capability to restore active life and reproduction when environmental conditions become suitable

(Kaczmarek et al., 2019). Such scenarios are in line with the hypothesis that anhydrobiotic organisms almost avoid environmental selection since they are active only under favourable environmental conditions (Pilato, 1979). Therefore, anhydrobiotic periods could have an impact on generation time, which in turn influences the potential rate of evolution. This could be the cause of the surprising morphological uniformity at the species, genera and family level of terrestrial anhydrobiotic tardigrades in contrast to marine species that, in practice, are not able to enter anhydrobiosis (Kaczmarek et al., 2019). Interestingly, molecular analyses have suggested that bdelloid and monogonont rotifers might have different diversification and mutation rates, although it is still unclear if this is due to the different dormancy patterns (quiescence *vs* dormancy, respectively) or the different reproductive strategies (obligately *vs* cyclical parthenogenesis, respectively) of these taxa or other, still unknown, factors, and if they are indeed common (Barraclough et al., 2007; Swanstrom et al., 2011; Fontaneto et al., 2012b).

Further selective advantages of anhydrobiosis can be cited. Anhydrobiosis allows the reduction of predators, competitors, and parasites since stochastic habitats are colonized only by a reduced number of species (Wilson and Sherman, 2013; Guidetti et al., 2011a). Since holo-anhydrobiotic organisms are aquatic animals, desiccation tolerance allows them to colonize and persist in terrestrial habitats other than in stochastic and extreme habitats. Moreover, the capability to withstand extreme conditions by entering anhydrobiosis increases the number of possible ‘‘refugia’’ that can be utilized by the species during long harsh environmental conditions, with a decrease in the rate of extinction and the loss of diversity (Guidetti et al., 2011a). Anhydrobiosis increases passive dispersal capability since dormant anhydrobiotic animals and eggs can act as propagules, be transported over long distances, and cross physical barriers for months without losing viability, which active animals cannot do, and establish new populations in new territories (Guidetti et al., 2011a; Mogle et al., 2018; Fontaneto, 2019). This capability is aided by the fact

that mostly holo-anhydrobionts reproduce via telytokous parthenogenesis, a reproductive strategy favourably adapted to colonise new and isolated habitats with a single individual (Bertolani, 2001, Ricci and Fontaneto, 2009; Fontaneto, 2019). This could influence the biogeographical pattern of holo-anhydrobionts that supports the hypothesis that "everything is everywhere". This hypothesis was confirmed for many bdelloid rotifers (Fontaneto et al., 2008), monogonont rotifers (e.g. Gómez et al. 2002; Mills et al., 2017) and for few tardigrade species (Kaczmarek et al., 2019), although some caution should be exercised, as other variables, including sampling effort or hidden diversity, might influence results and should therefore be carefully considered (Fontaneto et al., 2007, 2008, 2009; Mills et al., 2017). Nevertheless, despite that tardigrades are able to disperse by wind as are other terrestrial anhydrobionts (Nkem et al., 2006; Rivas et al., 2019), most tardigrade species have a narrow species range, with a large number of endemic species (e.g. Pilato and Binda, 2001; Guidetti et al., 2019). In contrast, biogeographic patterns were detected in several anhydrobiotic taxa of nematodes (Faurby and Barber, 2015; Zullini, 2018). In any case, the paucity of faunistic data, the presence of cryptic species, and the high level of confounding factors make the distribution patterns more complex with the exigency to collect further experimental and faunistic data. Some of these effects on the life history, like the ability to "escape in time", aging, "refugia", generation time, selection, and avoidance of predators, are also valid for other dormant stages, for example resting eggs and cysts, even when they are not desiccated, highlighting common ecological effects of dormancy, irrespective of the physiological adaptations of each taxon or response to stress.

These conditions, together with the capability of desiccation-tolerant organisms to repopulate habitats when liquid water returns, affect community dynamics and produce substantial modifications in the structure of biological communities, even leading to modifications in the functional integrity of the ecosystems (Irons et al., 1993; Walsh et al., 2104).

Implications/application of extreme tolerance of meiofaunal organisms

A better understanding of the life strategies of anhydrobiotic animals both at the ontogenetic and phylogenetic levels can provide answers to many fundamental questions as well as useful practical outcomes in many branches of applied sciences. Understanding desiccation tolerance in anhydrobiotic organisms will enable us to induce or engineer tolerance in sensitive species and to produce subsequent long-term stabilization and preservation of biological material in a dry state This is a topic of considerable practical importance both in medical and commercial fields since drying is widely used in the food and pharmaceutical industries as a long-term preservation technique (Saragusty and Loi, 2019).

Based on knowledge accumulated from anhydrobiotic organisms, much of the research on stabilising cellular membranes and proteins has centered on trehalose, which preserves cell membranes, and proteins, which can allow fluids to solidify without forming crystals through glass transition or vitrification, forming a large number of hydrogen bonds with membranes and proteins, and by replacing water molecules during the drying process (Hengherr et al., 2009), although many other protein families, as well as molecules, perform many functions, some of which still uncharacterised (e.g. Tompa, 2002; Tunnacliffe and Wise, 2007; Tunnacliffe et al., 2010). Some anhydrobiotic organisms naturally possess the molecular mechanisms to produce these sugars and load and unload them to and from the cells of the body or the intracellular spaces. Since the first report that biomolecules, membranes, and organisms can be stabilized in a dry state, due to the presence of trehalose, an array of possible applications for trehalose have been reported, ranging from the stabilization of vaccines, lysosomes, platelets, spermatozoa and oocytes to the hypothermic storage of human organs (Chen et al., 2001; Crowe et al., 2005; Schill et al., 2009; Saragusty and Loi, 2019). The determination of the properties of trehalose and the debate whether trehalose alone is sufficient to preserve biomolecules (e.g. Garcia de Castro and Tunnacliffe, 2000;

Ratnakumar and Tunnacliffe, 2006; Pouchkina-Stantcheva et al., 2007; Tapia et al., 2015, Chau et al., 2016) have stimulated the continuation of basic research to discover the secret of life without water. Recently, Boothby and co-workers (2017) indicated that the heterologous expression of some CAHS proteins in both prokaryotic and eukaryotic cells is sufficient to increase desiccation tolerance in these sensitive systems, and purified CAHS proteins protect desiccation-sensitive proteins *in vitro*. Moreover, Hashimoto et al. (2016) found that tardigrade DNA-associating protein (Dsup) suppresses X-ray-induced DNA damage by 40% and improves radiotolerance of human cultured cells. The story of the already known bioprotectants tells us that the tolerant ability of anhydrobiotic animals could be transferred to more sensitive organisms at least partly by transferring the corresponding genes. Recent rapid progress of molecular analyses should accelerate the elucidation of the mechanisms at the basis of extreme stresses, including complete desiccation stress, providing novel clues that open new avenues to confer stress resistance to intolerant species, including humans.

Conclusions

Various extreme-tolerance mechanisms have evolved in meiofauna, enabling micrometazoans like tardigrades, rotifers, and nematodes, to reduce or interrupt metabolism and survive stressful environments. In response to the gradual onset of adverse environmental conditions (e.g. water availability, temperature, oxygen tension, pH), these organisms undergo complex molecular, physiological, morphological and behavioural changes, which can share common characteristics but also present some differences. For example, tardigrades undergo encystment, an adaptive strategy that involves profound morphological changes that occur during the molting process, resulting in the dormant organism lying within retained cuticular exuvia. On the other side, cryptobiosis happens at any stage of the life cycle of the organisms and includes different strategies

such as anhydrobiosis, cryobiosis, anoxybiosis and osmobiosis directly induced by desiccation, sub-zero temperatures, low oxygen pressure and osmotic extremes, respectively. The most widespread and best-known form of these is anhydrobiosis, the capability evolved by tardigrades, rotifers and nematodes to tolerate complete desiccation by entering in a state of reversible suspension of metabolism without the loss of viability.

When dormant, these taxa show extraordinary resistance to physical and chemical extremes that may far exceed the tolerance ranges of active organisms, therefore the two dormancy strategies, quiescence and diapause, allows tardigrades, rotifers and nematodes to colonise and persist in various otherwise unavailable environments. Interestingly, while dormant, some taxa do not age, although the specific effects of dormancy on aging varies with the taxa and is poorly understood but this ability make tardigrades, rotifers, and nematodes very useful model organisms that can be used to study the aging process. Furthermore, the evolution of anhydrobiosis resulted in selective advantages but also in energetic costs with effects on growth, reproduction, life history, and fitness, in turn affecting the rate of evolution, but more studies are needed to fully understand the ecological and evolutionary implications of these resistance strategies on these taxa.

Furthermore, novel findings have also contributed to expand other aspects of these taxa, with potential exciting applications in other fields: the evolution of a series of behavioural, morphological, physiological and molecular/biochemical adaptations provided anhydrobiotic organisms with different unusual mechanisms to withstand desiccation. To prevent cell damage during dehydration, bioprotectant molecules that accumulate in and around the cells of their body are generated; the identification of these molecules and their mechanisms are the focus of much current research, including the role of horizontal gene transfer. It is becoming clear that no single class of protectant/repair molecules is sufficient, but successful desiccation depends on the co-ordinated action of all of them. The understating of the detailed mechanisms and consequences of

extreme tolerance, these meiofauna taxa are becoming popular model organisms in the fields of exobiology and medical research, with the hope that they might also help to improve the tolerance of human cells to extreme stress in the future.

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FIGURE LEGENDS

Fig. 1. A) *In toto* cyst of the freshwater eutardigrade *Hypsibius* sp. (phase contrast). B) *In toto* cyst of the freshwater eutardigrade *Dactylobiotus parthenogeneticus* (phase contrast). Bar = 100 μm.

Fig. 2. A) *In toto* specimen of the limnoterrestrial eutardigrade *Acutuncus antarcticus* (*in vivo* and Nomarski contrast). B) *In toto* female of the lichen-dwelling eutardigrade *Ramazzottius* cf. *oberhaeuseri;* the ovary containing three oocytes (asterisk). (*in vivo* and Nomarski contrast). C) Tun (desiccated animal) of the eutardigrade *Ramazzottius* cf. *oberhaeuseri* (*in vivo*).
Arrow: buccal-pharyngeal apparatus; arrowhead: midgut; cross: gonad. Bar = 100 μm.

Fig. 3. Scanning electron micrographs of the moss-dwelling heterotardigrade *Echiniscus* sp. A) Dorsal view of an *in toto* and hydrated specimen. B) Dorsal view of an *in toto* desiccated specimen (tun). C) Ventral view of an *in toto* desiccated specimen (tun). Bar = 100 μm.

Fig. 4. Scanning electron micrographs of the rotifer *Adineta tuberculosa*. A) *In toto* and hydrated specimen. B) *In toto* desiccated specimen (tun). Bar = 100 μm. (Courtesy of Giulio Melone and Diego Fontaneto).

Figure 1

Figure 2

Figure 3

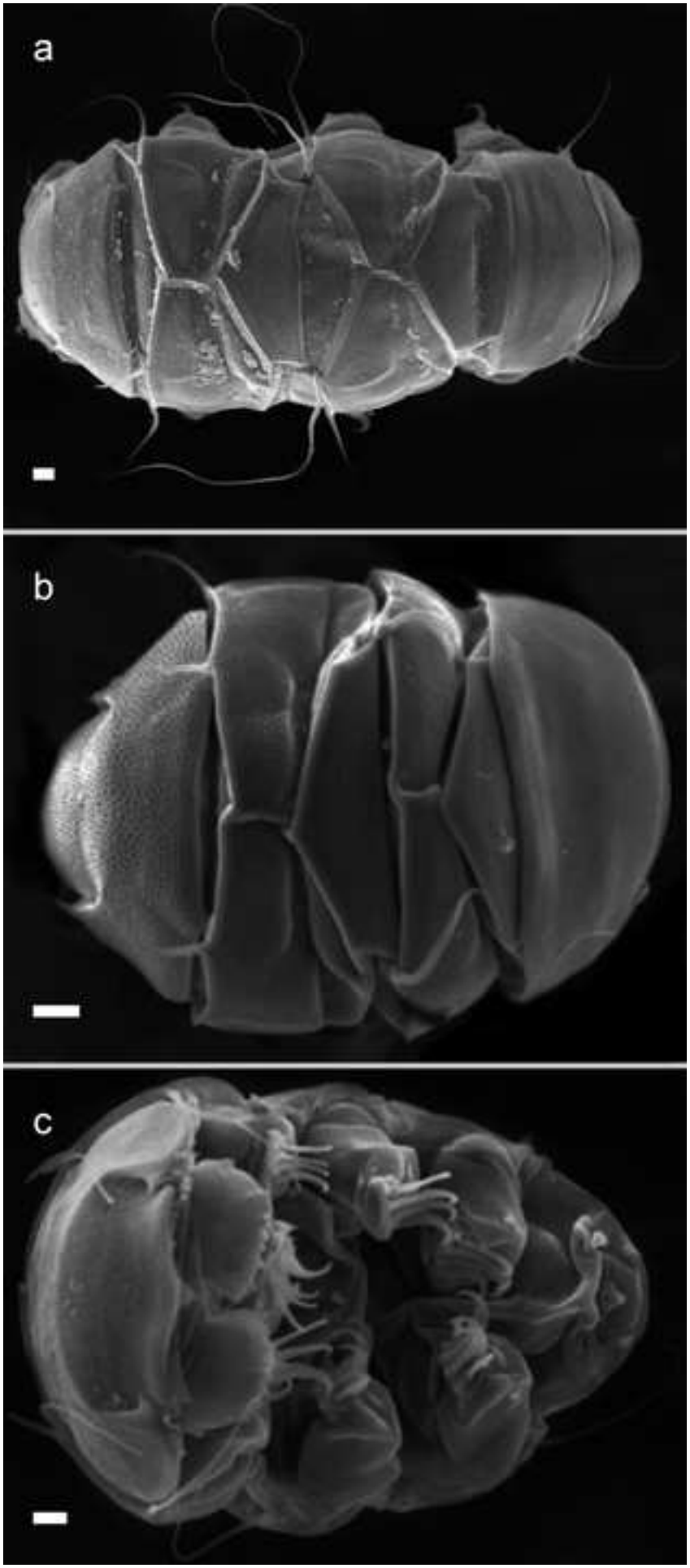

Figure 4