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Graphical Review

New insights into survival strategies of tardigrades

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ABSTRACT

Life is set within a narrow frame of physicochemical factors, yet, some species have adapted to conditions far beyond these constraints. Nature appears to have evolved two principal strategies for living organisms to cope with hostile conditions. One way is to remain active, retaining metabolism through adaptations that enable the organism to match the physiological requirements of environmental change. The other is to enter a state of dormancy with metabolic suppression. One form of metabolic suppression, known as cryptobiosis, is a widespread state across life kingdoms, in which metabolism comes to a reversible standstill. Among animals, nematodes, rotifers and tardigrades, comprise species that have the ability to enter cryptobiosis at all stages of their life cycle. Tardigrades are microscopic cosmopolitan metazoans found in permanent and temporal aquatic environments. They are renowned for their ability to tolerate extreme stress and are particularly resistant after having entered a cryptobiotic state known as a “tun”. As new molecular tools allow for a more detailed investigation into their enigmatic adaptations, tardigrades are gaining increasing attention. In this graphical review, we provide an outline of survival strategies found among tardigrades and we summarize current knowledge of the adaptive mechanisms that underlie their unique tolerance to extreme or changing environments.

Tardigrades are microscopic bilaterians belonging to phylum Tardigrada within the protostome superclade Ecdysozoa (Jørgensen et al., 2018). These minute animals have an adult body length within the range of 50–1200 μm and are found in permanent and temporary aquatic environments across the globe (Møbjerg et al., 2018). The phylum comprises two major evolutionary lineages, i.e. eutardigrades (including apotardigrades) and the more diverse heterotardigrades. Limno-terrestrial species living in terrestrial habitats (e.g. on mosses or lichens) are only active when covered by a thin film of water. During desiccation, tardigrades quickly lose extra- and intra-cellular water retaining as little as 2–3% of their body water (Crowe, 1972) and reducing body volume by as much as 85–90% (Halberg et al., 2013), while entering the so-called cryptobiotic “tun” state (Fig. 1). The ability to enter cryptobiosis (latent or hidden life), where metabolism comes to a reversible standstill, is widespread across life kingdoms (Clegg, 2001). Nematodes, rotifers and tardigrades are metazoans with the ability to enter cryptobiosis at all stages of their life cycle, as eggs as well as in juvenile and adult stages. Cryptobiosis seems to provide animals with a potential to survive conditions that are far beyond any constraints set by their normal environment. Being particularly resilient in the cryptobiotic tun-state, tardigrades are renowned for their ability to tolerate a variety of extreme conditions, including desiccation, severe osmotic shock, freezing in liquid nitrogen, and even exposure to space vacuum and cosmic radiation (Jonsson et al., 2008). When excluding any dormancy periods, tardigrades generally live for a couple of months (Suzuki, 2003). However, cryptobiosis may extend their life span by numerous decades (Tsujimoto et al., 2016).

With reference to the “Krogh’s principle” (reviewed in Jørgensen, 2001)—that “for a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied” (Krogh, 1929)—and at the same time acknowledging Krogh’s warnings against generalizations and his profound call to study “vital functions in all their aspects throughout the myriads of organisms” (Krogh, 1929), tardigrades are obvious organisms for physiological studies. Cryptobiosis challenges our perception of the transition between life and death of an organism. Understanding the mechanisms that underlie the ability to stabilize biological structures, from macromolecules across cellular, tissue and organ levels to the whole animal, and subsequently restart life after years of metabolic suspension has great potential for translational and applied sciences. As new molecular tools allow for increasingly detailed investigations, tardigrades are indeed gaining attention by physiologists and biochemists.

Survival strategies among extant tardigrades include osmoregulation, as well as cryptobiosis and diapause in the form of encystment,
cyclomorphosis and resting eggs (Fig. 2). Extremotolerant tardigrades rapidly enter cryptobiosis in response to a sudden environmental change, and quickly revive from their ametabolic state upon removal of the stress factor. Cryptobiosis may be induced by a range of extreme conditions leading to different cryptobiotic sub-states, namely anhydrobiosis, osmobiosis, cryobiosis and possibly anoxybiosis and chemobiosis. Most notably are stress conditions that force the tardigrade to deal with water loss, i.e. desiccation (anhydrobiosis), rise in external osmotic pressure (osmobiosis) or freezing (cryobiosis). Lack of oxygen (anoxybiosis) and environmental toxicants (chemobiosis) may also induce quiescence; however, conclusive empirical evidence on these two forms of cryptobiosis is currently lacking. Anhydrobiosis, osmobiosis and possibly chemobiosis are cryptobiotic states characterized by tun formation. The ability to form tuns is present among all extant tardigrade lineages, indicating that this is an ancient and homologous trait (Hygum et al., 2016). Tun formation likely evolved within an ancient marine environment as a response to fluctuations in abiotic factors, such as external osmotic pressure. During desiccation, limno-terrestrial tardigrades generally enter the tun state within half an hour, and marine tidal species may enter the state in the course of seconds (Sørensen-Hygum et al., 2018). This is possibly due to the highly permeable nature of tardigrade epithelia and cell membranes as well as a strategy among extremotolerant species to constitutively express bioprotectants required for cryptobiotic survival.

Whereas cryptobiosis evolved as a quick response to sudden changes in the environment, encystment represents a slower emerging form of dormancy that likely is induced by endogenous stimuli as well as external changes in factors such as temperature and oxygen tension (Guidetti and Møbjerg, 2018). During encystment, tardigrades produce several protective cuticular coats, while undergoing profound morphological changes in the buccal-pharyngeal apparatus, claws and gonads (Guidetti and Møbjerg, 2018). Cyst formation in moss-dwelling tardigrades may occur as part of a seasonal cyclic event, and in the marine eutardigrade Halobiotus crispae cyclomorphosis includes the production of a cyst-like stage (Guidetti and Møbjerg, 2018). In addition, resting eggs have been reported. These diapausing eggs, which are laid in response to an environmental deterioration, require a sequence of stimuli (e.g. dehydration followed by rehydration) in order to hatch (reviewed in Guidetti et al., 2011).

Tardigrades can be extremely tolerant to environmental stress even in their active states (Møbjerg et al., 2011). The latter is exemplified by marine tidal species that osmoregulate over a range of external salinities (2–40 ppt) and avoid freezing by supercooling to around −20 °C (Halberg et al., 2009). Furthermore, limno-terrestrial species generally handle extreme levels of radiation with LD50 values for gamma and heavy ion radiation in adults of up 5 and 10 kGy, respectively (Jönsson, 2019). Interestingly, however, active tardigrades are sensitive to high temperatures with estimated LD50 values in the range 29–37 °C, depending on species and experimental approach (reviewed in Neves et al., 2020a). Indeed, tardigrades seem to have a restricted tolerance to high temperatures even in the tun state, with estimated LD50 values for desiccated Ramazzottius varieornatus of 83 °C, 63 °C and 56 °C following 1 h, 24 h and 1 week exposures, respectively (Neves et al., 2020a, 2020b). The latter may suggest that high temperatures destabilize and

Fig. 1. Light micrographs of the limno-terrestrial eutardigrade Ramazzottius varieornatus in the active state (dorsal view) and tun state (ventral view); anterior faces right in both aspects. With strong cryptobiotic abilities, this extremotolerant tardigrade endures a range of extreme conditions, including desiccation and high osmolalities (>2000 mOsm kg⁻¹) of non-electrolytes (Heidemann et al., 2016). During desiccation and exposure to high osmolality solutions the tardigrade will contract its body and withdraw its legs to form a tun. Upon return to favourable conditions (i.e., high water potential) the tun extends its body, draws its legs out and, eventually, resumes activity. The current tun was induced by a high osmolality sucrose solution.
denature proteins and other essential biological macromolecules.

Tardigrades lack discrete respiratory and circulatory systems. Internal organs of active, hydrated tardigrades are surrounded by a fluid filled body cavity that may assist in movement of various solutes (Møbjerg et al., 2018). Somatic muscles attach to the cuticle (Fig. 3) and locomotion relies on regulation of body volume—tardigrades hyperregulate ensuring a constant influx of water with excess water likely being expelled by osmoregulatory epithelia of the epidermis, midgut and, in eutardigrades, Malpighian tubules (Møbjerg et al., 2018). Somatic as well as visceral muscles participate in tun formation with the latter directing internal organ rearrangement, while dorsal, ventral, and lateral muscle groups provide body contraction and the leg musculature retracts the four pair of legs (Halberg et al., 2013). Importantly, muscle protein filaments seem to play a vital role for stabilizing the three-dimensional structure of the desiccated tun state, with filaments locking in a rigor mortis-like state. In addition, actin and other cytoskeletal filaments within epithelia may stabilize anhydrobiotic cell structure.

Physiological and molecular studies on tardigrades have been challenged by their small size and the small (picogram) quantities of DNA and RNA available from single individuals, but a number of recent studies involve genomics, transcriptomics and proteomics. The compact genomes of the extremotolerant, Ramazzottius cf. varieornatus (~55 Mb), and the much less tolerant, but commercially available Hypsibius exemplaris (~100 Mb), were recently released (Hashimoto et al., 2016; Koutsovoulos et al., 2016) and candidate genes with proposed significance for tardigrade stress tolerance are currently being identified. The latter, for example, involve several groups of intrinsically disordered proteins found within the eutardigrade lineage (Hashimoto et al., 2016).
Very little is known of cell physiology in tardigrades. The available transcriptomic data indicate that tardigrades express a full repertoire of membrane transporters, including membrane pumps, numerous solute carriers, a variety of ion channels and aquaporins. For example, experimental evidence of organic anion secretion by the tardigrade midgut suggests a potential pathway for secretion of nitrogenous waste with apical H\(^+\) and basolateral Na\(^+/K\(^+\) pumps possibly delivering the driving force for transepithelial transport (Fig. 4; Halberg and Møbjerg, 2012). Importantly, transcriptomics as well as experimental data indicate that strong cryptobionts, such as *R. cf. varieornatus*, constitutively express bioprotectants necessary for cryptobiotic survival, whereas less tolerant species, such as *H. exemplaris* need a period of preconditioning in order to upregulate expression of essential protectants and survive extreme conditions. Generally, comparative genomics and transcriptomics reveal a high degree of divergence between tardigrade species, indicating that different evolutionary lineages exhibit unique physiological and molecular adaptations (Kamilari et al., 2019). Among the bioprotectants with relevance for cryptobiotic survival are disaccharides such as trehalose and sucrose, in addition to the polyol glycerol. Trehalose has traditionally been identified as a molecular stabilizer replacing water during desiccation in *Artemia* (brine shrimp) cysts, thereby protecting cellular structures such as plasma membranes, and at the same time stabilizing the cell through vitrification (reviewed in Hibshman et al., 2020). Some, but not all, tardigrades accumulate trehalose during desiccation, though in much lower concentration as compared with *Artemia*, and several tardigrade species appear to lack enzymes required for trehalose synthesis. Trehalose accumulation may, nevertheless, contribute to protection of cell structure in a synergistic effect with other bioprotectants, such as heat shock proteins (HSP) and late embryogenesis abundant (LEA) proteins. Intrinsic disordered LEA proteins seem conserved in tardigrades, including Hsp70, Hsp40 and various small heat shock proteins. More recently, it has been suggested that tardigrade-specific intrinsically disordered proteins can provide structural stabilization during desiccation through vitrification (Boothby et al., 2017). In addition, a highly effective antioxidant defense apparatus as well as high fidelity DNA repair systems are likely crucial for cryptobiotic survival (Kamilari et al., 2019). Accordingly, tardigrades seem to have a comprehensive number of genes encoding proteins involved in antioxidant defense. It was recently shown that the tardigrade damage suppressor protein (Dsup) binds to nucleosomes protecting chromosomal DNA from reactive hydroxyl radicals (Chavez et al., 2019). Interestingly, Dsup gene expression enhances tolerance of tobacco plants to genomutagenic stress (Kirke et al., 2020). Thus, while the secrets of latent life in many aspects still remain a mystery, recent research is beginning to unravel some of the underlying mechanisms.

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Fig. 3.** Schematic representation of somatic muscles (ventral view) involved in tun formation of a eutardigrade, *Ricthersius cf. coronifer* (modified from Halberg et al., 2013); anterior faces upwards in both aspects. The somatic musculature attaches to the cuticle at distinct attachment sites, not all visible in the current representation. Muscle fibers involved in locomotion of the hydrated, active tardigrade (A) participate in body contraction during tun formation (B). Indeed, the contraction of dorsal, lateral and ventral muscles as well as leg muscles mediate a structural reorganization of the tardigrade during formation of the desiccated tun state. Muscle protein filaments likely play a vital role for stabilizing the three-dimensional structure of the desiccated tun state, locking filaments in a *rigor mortis*-like state. The body musculature is also essential for maintaining the structural integrity when the tardigrade resumes activity following rehydration. Note the relative position of the pharyngeal bulb (pb) and the midgut (mg) both in the extended and contracted body. Visceral muscles (not shown) likely participate in tun formation with the latter directing internal organ rearrangement.
Fig. 4. Schematic illustration of cellular and molecular mechanisms potentially involved in epithelial transport and anhydrobiotic survival of tardigrades as exemplified by a midgut epithelial cell (based on several sources, including Boothby et al., 2017, Chavez et al., 2019, Halberg and Mejberg, 2012, Hashimoto et al., 2016, Hibshman et al., 2020; Kamilari et al., 2019). The Na⁺/K⁺-ATPase and the V-type H⁺-ATPase are likely candidates providing electrochemical driving force for transepithelial movement of ions and organic molecules over cell membranes of hydrated active tardigrades. Experimental evidence in the eutardigrade Halobiotus crispae indicates that solute carriers may transport organic anions (AO⁻), providing a potential pathway for secretion of nitrogenous waste. An assortment of bioprotectants may play important roles in tardigrade desiccation tolerance, protecting cell membranes, proteins and DNA against damage during desiccation. Among the proposed protectants are disaccharides such as trehalose, heat-shock proteins, LEA (Late Embryogenesis Abundant) proteins and tardigrade-specific intrinsically disordered proteins. Trehalose may help stabilize proteins, nucleic acids and membrane lipids by replacing water during desiccation, but also by immobilizing macromolecules following formation of a glassy matrix through vitrification. Heat-shock and LEA proteins are suggested to have a role preventing protein aggregation during desiccation, acting as molecular shields and chaperones, and/or by being involved in the repair process of damaged proteins during rehydration. Recently identified, heat soluble tardigrade-specific intrinsically disordered proteins (CAHS, MAHS, SAHS) appear to be unique to the eutardigrade lineage. These proteins have been associated with anhydrobiotic survival and are constitutively expressed during dehydration and rehydration in Ramazzottius cf. varieornatus. In this species, RvLEAM (a group 3 LEA protein) and MAHS have been identified as potent mitochondrial protectants, while the unstructured-nuclear protein Dsup binds to nucleosomes and protects DNA from reactive hydroxyl radicals. Potential cell damage due to reactive oxygen species is furthermore prevented by superoxide dismutases, glutathione peroxidases and catalases (catalases are not present in all tardigrades).

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