

The tardigrade cuticle

Michaela Czerneková*, Stanislav Vinopal*

Department of Biology, Jan Evangelista Purkyně University, Pasteurova 3632/15, 400 96 Ústí nad Labem, Czech Republic,
e-mail: Michaela.Czernekova@ujep.cz (M.C., *corresponding author), Stanislav.Vinopal@ujep.cz (S.V., *corresponding author)

Abstract: Tardigrades (phylum Tardigrada) are aquatic microecdysozoans that have adapted to survive extreme conditions through the formation of cysts or ametabolic tuns. Their body is covered by a cuticle that plays an important role in their life cycle, including their response and adaptation to environmental challenges. Cuticular characteristics are a critical component of tardigrade taxonomy. Therefore, research has often been focused on the relationship between cuticular morphology and ultrastructure and the evolutionary and phylogenetic positioning of the phylum and individual species herein. However, a deeper insight into the ultrastructural characteristics and chemical composition of the tardigrade cuticle is needed. This knowledge is important not only for a better understanding of tardigrade physiology and ecology but also for the development of efficient microinjection and/or electroporation techniques that would allow for genetic manipulation, opening new avenues in tardigrade research. Here, we review data on cuticle ultrastructure and chemical composition. Further, we discuss how the cuticle is affected during moulting, encystment, cyclomorphosis, and anhydrobiosis. Our work indicates that more systematic studies on the molecular composition of the tardigrade cuticle and on the process of its formation are needed to improve our understanding of its properties and functions.

Key words: cuticle, tardigrades, chemical composition, ultrastructure, chemical treatment

Introduction

Many organisms have evolved physiological, morphological, biochemical and behavioural strategies for surviving water loss. Desiccation avoidance strategists (including some true terrestrial arthropods) have a low-permeability cuticle to retain body water or locally specialized tissues to absorb atmospheric water, which helps to maintain their internal environment. On the other hand, organisms using the desiccation tolerance strategy can survive partial or even complete desiccation (Rebecchi 2013). Tardigrades belong to the latter group. These microscopic (50–1200 µm), essentially aquatic ecdysozoans have been found in marine, freshwater and terrestrial habitats (Nelson et al. 2018) and require a surrounding water film for the active stage of their life cycle (Møbjerg et al. 2018). Their taxonomic and phylogenetic position is still debated since they seem to be closely related to both phylum Arthropoda (Jørgensen et al. 2018) and Nematoda (Yoshida et al. 2017).

Tardigrades have developed remarkable adaptations to survive challenging environmental conditions. Several tardigrade species are known to form durable cysts (Rebecchi et al. 2020). Many tardigrades are able to enter an ametabolic state, cryptobiosis, at any stage of their life cycle (Jönsson et al. 2001; Rebecchi et al. 2007; Schill and Fritz 2008; Wełnicz et al. 2011; Czernekova and Jönsson 2016; Schill and Hengherr 2018). Some tardigrades can even tolerate extremely low temperatures (e.g., –196°C) in a fully hydrated state which is called cryobiosis (Hengherr and Schill 2018). Despite their remarkable survival abilities, only a few studies to date have investigated the relationship between the cuticle structure and the anhydrobiosis, the cryptobiotic state related to drastic water loss (Crowe 1970, 1972; Greven 1971; Wright 1989a; Wright et al. 1992). Detailed knowledge of the cuticular ultrastructure and chemical composition is important not only for understanding tardigrade physiology and ecology, but also for the improvement of useful modern biological techniques in tardigrade research

(e.g., efficient electroporation and/or microinjection). These techniques have so far been used only in *Hypsibius* sp. (Tenlen et al. 2013; Boothby et al. 2017; McGreevy et al. 2018; Tenlen 2018); however, they have great potential. Here, we present a review of the structure, composition and function of the tardigrade cuticle and discuss its possible relevance for the development of biotechnology.

General characteristics of the tardigrade integument

The integument of tardigrades consists of an acellular multilayered cuticle of varying thickness and

appearance (Fig. 1) and an underlying single layer of cuticle-secreting epidermal cells (Ramazzotti and Maucci 1983; Møbjerg et al. 2018). The epidermal cells rest on a thin, likely collagenous, basement membrane (Baccetti and Rosati 1971; Greven 1984). The cuticle covers the body externally, however, it also extends into the hind- and foregut (Greven 1984). The cuticle must be strong enough to mechanically support the organisms's soft body and to resist shrinkage and superficial abrasion. At the same time, it must be flexible enough to resist the stresses caused by tardigrade locomotion, feeding and oviposition (Wright 1988a).

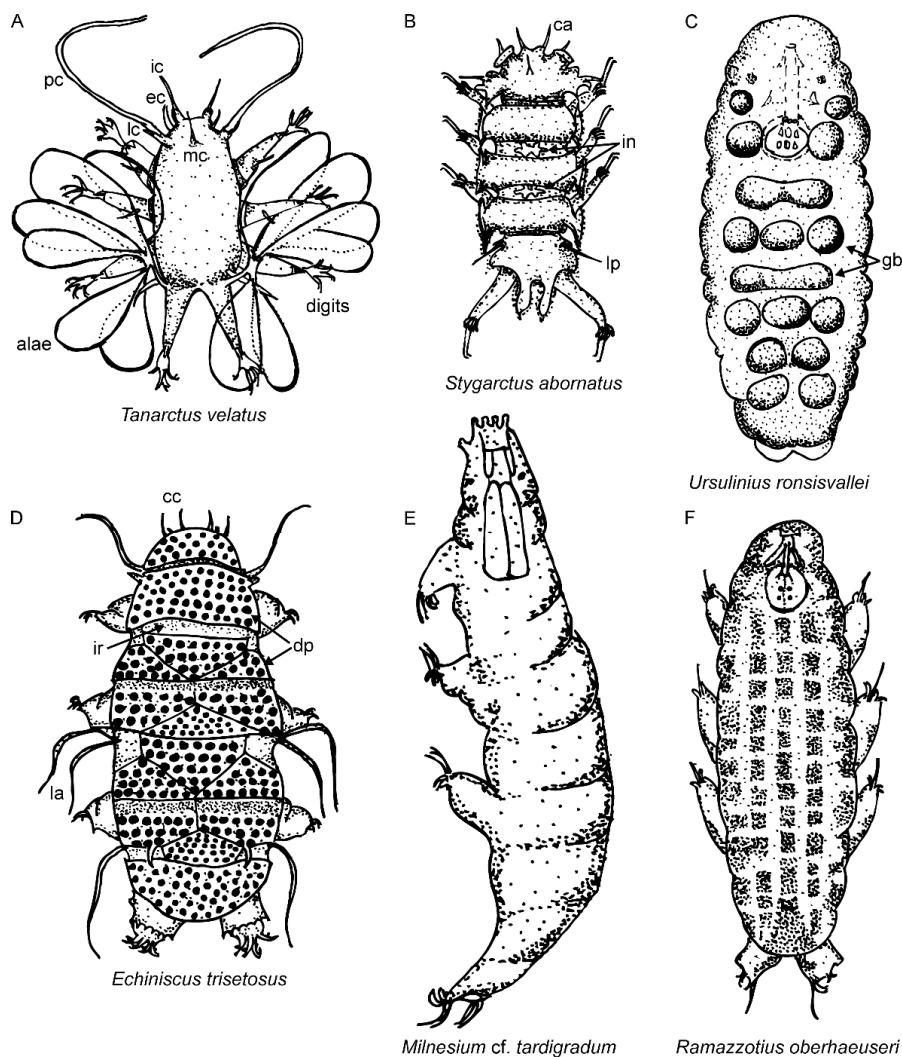


Fig. 1. Various morphologies of the tardigrade cuticle

A) *Tanarctus velatus* McKirdy, Schmidt & McGinty-Bayly, 1976 (~120 µm): cephalic cirri (internal cirrus, ic; external cirrus, ec; lateral cirrus, lc; median cirrus, mc) and clavae (primary clava, pc) on the head region, cuticular expansions ('alae'; seen also in genera *Florarctus*, *Actinarctus*, *Paradoxipus*, *Halechiniscus*), and digits instead of claws. B) *Stygarctus abornatus* McKirdy, Schmidt & McGinty-Bayly, 1976 (~95 µm): cuticle with lateral projections (lp), intersegmental nodes (in) between dorsal plates and prominent cephalic appendages (ca) – 'sensillae'. C) *Ursulinius ronsisvallei* (Binda & Pilato, 1969) (~275 µm): cuticle with large gibbosities (gb). D) *Echiniscus trisetosus* Cuénot, 1932 (~375 µm): thick dorsal plates (dp) with thinner intersegmental regions (ir), cephalic cirri (cc) and lateral appendages (la). E) *Milnesium* cf. *tardigradum* Doyère, 1840 (~600 µm): 'smooth' cuticle. F) *Ramazzottius oberhaeuseri* (Doyère, 1840) (~500 µm): cuticular ornamentation and pigmentation of epidermal cells give rise to this characteristic pattern. Modified from Greven (1980) and Dastyh (1988).

Importantly, somatic muscles are attached to the cuticle at specialized attachment points. The epidermal cells are modified at these sites and the associated cuticle contains distinct transversal cuticular fibres (Shaw 1974; Marchioro et al. 2013). The attachment sites are externally visible as a series of tiny holes, ridges or folds, and internally forms invaginations of the cuticle (Gross and Mayer 2019). Body and leg movements depend on somatic muscles and body cavity fluid pressure combined with cuticle resilience (Marchioro et al. 2013). Somatic muscles are also responsible for contraction of the cuticle during so-called "tun" formation in response to desiccation (Marcus 1929; Halberg et al. 2013a). The tun formation is a powerful morphological adaptation (Czerneková et al. 2017).

The cuticle has been proposed to play a role in excretion (Dewel et al. 1993; Kinchin 1994), however, experimental evidence for this is lacking. It has also been hypothesized that pigments in the integument might protect tardigrades from harmful radiation (Dastyk et al. 2003; Greven et al. 2005). Indeed, a recent work showed that a fluorescent pigment rendered *Paramacrobiotus* sp., designated as "*Paramacrobiotus* BLR", resistant to high doses of UV irradiation (Suma et al. 2020).

The cuticle is a major taxonomic characteristic used in the classification of tardigrades. Most of the studies on the tardigrade integument have aimed primarily at relating its morphology and ultrastructure to the evolution and phylogenetic positioning of the orders and of the phylum itself. Phylum Tardigrada is divided into three classes: Heterotardigrada, Eutardigrada and Mesotardigrada (Guidetti and Bertolani 2005; Degma and Guidetti 2007; Degma et al. 2020). The latter, however is comprised of the single species *Thermozodium esakii* Rahm, 1937; its existence is therefore considered dubious (Jørgensen et al. 2018). Two cuticular patterns have been traditionally described, the heterotardigrade-pattern and eutardigrade-pattern (Węglarska 1989).

Heterotardigrada is composed primarily of marine species, as well as some limno-terrestrial species. Their cuticles are diverse in their external appearance (Fig. 1A, B, D). In some groups (e.g., the terrestrial family Echiniscidae, including the genera *Echiniscus*, *Hypechiniscus*, *Pseudechiniscus* and the marine family Stygarctidae), the cuticle is thickened into cuticular plates or "armour". Heterotardigrades can also be characterized by the

presence of taxonomically significant spines and sensory appendages (cephalic cirri, clavae and papillae) or other body extensions (e.g., wing-like). Additionally, specifically patterned sculpture may be present all over the body or on specific body parts (Bertolani et al. 2009; Greven and Robenek 1983; Kristensen and Neuhaus 1999; Kristensen and Sørensen 2004; Nelson et al. 2015). In some species, cuticular pores have been detected (Li et al. 2007; Rocha et al. 2007).

In general, the cuticle of eutardigrades is simpler than that of heterotardigrades (Greven 1984). Eutardigrada comprise freshwater and other limno-terrestrial species without cuticular plates and cephalic cirri (Fig. 1C, E, F). Their cuticles can be smooth or sculptured, but without filaments and usually without spines (Ramazzotti and Maucci 1983; Kristensen and Neuhaus 1999; Bertolani et al. 2009; Nelson et al. 2010). Distinct and protruding sensory papillae are present only in Apochela (Nelson et al. 2010; Møbjerg et al. 2018; Morek et al. 2020). The eutardigrade cuticle can have shallow pores ("craters") on its surface called "pearls" (Marcus 1929; Baccetti and Rosati 1971; Wright 1988b; Nelson et al. 2010). Eutardigrade-type pores can be found, for example, in hatchlings of *Richtersius coronifer* (Richters, 1903) and in *Diaforobiotus islandicus islandicus* (Richters, 1904) (see Guidetti et al. 2016). The cuticle may also have pseudopores, which under phase contrast light microscopy appear as light circles (0.4–0.5 µm in diameter) on the cuticle (such as in *Milnesium* sp.; see Morek et al. 2019).

The cuticle ultrastructure

The terminology used to describe cuticular layers has not been standardized (alternative terminologies are summarized in Table 1). Here, we use the more commonly accepted terminology (Greven 1975, 1984; Greven and Robenek 1983; Wright 1988b; Guidetti et al. 2000), which describes the main layers of the cuticle from the outside to the inside as follows: an epicuticle (comprising a flocculent coat, an outer epicuticle and an inner epicuticle), a trilaminar layer, an intracuticle, a granular layer and a procuticle (Table 1, Fig. 2). In *M. cf. tardigradum* and *M. hufelandi*, there is an additional layer at the lower boundary of the intracuticle called the "inner flake of the intracuticle" (Baccetti and Rosati 1971; Greven 1975; Wright 1988b).

Table 1. Terminology of cuticular layers in Tardigrada

Cuticular layers and sublayers		Eutardigrada			Heterotardigrada	
		Apochela	Parachela	Arthrotardigrada	Echiniscoidea-dorsal	Echiniscoidea-ventral
Epicuticle ¹ /Exocuticle ¹⁰	Flocculent coat	Flocculent coat ^{1,5,10}	Mucous coat ^{2,4} , Flocculent coat ^{1,5,7,10,13}	Mucous layer/coat ^{3,11} , Flocculent coat ¹	Flocculent coat ^{1,5,13}	Flocculent coat ^{1,13}
	Outer epicuticle: - tripartite - multipartite*	Outer epicuticle ^{1,5} , Trilaminar epicuticle ¹⁰	Outer epicuticle ^{1,3,4,5,7,8,13} , Cuticulin ² , Outer membrane(s) ⁹	Outer epicuticle ^{1,3} , may be N/A in <i>P. sterner</i> ³ , Trilaminar layer ¹⁰ , Outer trilaminar layer ¹¹	Outer epicuticle ^{1,3,13} , Tripartite layer ¹²	Outer epicuticle ^{1,3,13}
	Inner epicuticle	Striations: N/A	Striations: N/A (with the exception of in <i>D. dispar</i>) ^{6,11} Inner epicuticle ^{1,3,4,5,6,7,8,13} (with pillars ^{4,6} in <i>Dactylobiotus</i> and <i>Murrayon</i>), Dense homogenous layer of the epicuticle ² , Cortical layer/cortex ³	Striation/Striated/Honeycomb (sub)layer ^{13,10,11} Inner epicuticle ³ with rods/pillars ^{3,10,11}	Striations: N/A Inner epicuticle ^{1,3,5,13} (labyrinthine/alveolar ⁵ structure), Outer layer ¹² (with lacunae) ¹²	Striated layer with honeycomb pattern ³ , (outer and inner) ¹² Inner epicuticle ^{1,3,5,13} , Transparent zone with dense rods ¹²
Trilaminar layer		Trilaminar layer ^{1,5} , Tripartite layer ⁵ , Inner trilaminar layer ¹⁰	Trilaminar layer ^{1,6,7,8,9} structure ³ , Outer membrane of intracuticle ² , Inner trilaminar layer ^{4,10} , Tripartite layer ⁵ , Convolute membrane ⁹ , Trilaminar layer ¹³	Trilaminar layer ^{1,3} /structure ³ , (inner) Trilaminar(y) layer ^{10,11}	Trilaminar layer ^{1,3} , Tripartite layer ⁵	Trilaminar layer ^{1,3} (with only a dense band in <i>V. viridis</i>) ¹ , Trilaminar layer ¹³ , Tripartite layer ⁵
	Intracuticle ** (Mesocuticle ¹⁰)	Intracuticle ^{1,2,5} , Basal cuticle ³	Intracuticle ^{1,2,3,4,5,7,8,13} , Basal cuticle ³	Intracuticle ¹ , Basal cuticle ³	Intracuticle ^{1,5,13}	Intracuticle ^{1,5,13}
Endocuticle ¹⁰	Granular layer	Granular/wax layer ^{1,5}	Granular layer ^{1,4,5,13} , Wax layer ^{2,3,7} , Basal cuticle (N/A in some species) ³	Basal cuticle (granular/wax layer; N/A in some species) ³	Granular layer ¹	Granular layer ¹³
	Procuticle	Procuticle ^{1,5}	Procuticle ^{1,2,4,5,6,7,8,13} , Basal cuticle ³ , Fibrous layer ⁹	Procuticle ¹ , Basal cuticle ³ , Endocuticle ¹⁰ , The homogeneous matrix layer ¹¹	Procuticle ^{1,5,13} , Basal cuticle ³ , Inner layer (everything under trilaminar layer) ¹²	Procuticle ^{1,5,13} , Basal cuticle ³ , Basal fiber layer ¹²

Dewel and Dewel (1979) and Dewel et al. (1993) coined the terms "exocuticle", "mesocuticle" and "endocuticle" to describe the three main layers of the cuticle in tardigrades. They also used the term "epicuticle" for the outer epicuticle together with the flocculent coat. "Basal cuticle", used by Kristensen and Neuhaus (1999), is an umbrella term that includes all layers between the epidermis and the trilaminar layer.

*Multipartite outer epicuticle occurs in the following species: *Batillipes mirus* Richters, 1909; *Echiniscus testudo* (Doyère, 1840), *Paramacrobiotus areolatus* (Murray, 1907), *Hypsibius dujardini* (Doyère, 1840), *Macrobiotus joannae* Pilato & Binda, 1983, *Paramacrobiotus richtersi* (Murray, 1911), *Mesobiotus harmsworthi* (Murray, 1907), *Macrobiotus hufelandi* C.A.S. Schultze, 1834 and *Halechiniscus* sp. (see Greven and Robenek 1983; Wright 1988b; Kristensen and Neuhaus 1999; Guidetti et al. 2000). **Some authors consider the striated layer to be part of the outer epicuticle (Greven 1975; Kristensen 1984; Weglarska 1989). ***Some tardigrades seem to lack the intracuticle (Greven 1975; Kristensen and Neuhaus 1999; Guidetti et al. 2000). An electron-dense layer in the cuticle of some marine heterotardigrades has also been called the "intracuticle" (Greven 1975, 1984; Greven and Grohe 1975) or "procuticle" (Hallas and Kristensen 1982; Kristensen 1982b). N/A – not applicable.

¹Greven (1975); *Halechiniscus* sp., *E. testudo*, *Viridiscus viridis* (Murray, 1910), *P. areolatus*, *M. hufelandi*, *M. cf. tardigradum*, *R. oberhauseri*, *Grevenius granulifer* (Thulin, 1928); ²Baccetti and Rosati (1971); *M. hufelandi*; ³Kristensen and Neuhaus (1999); *Parasigartus sterner* Renaud-Mornant, 1970, *Actinartus doryphorus* Schulz, 1935, *Actinartus physophorus* Grimaldi de Zio, D'Addabbo Gallo, Morone De Lucia, Vaccarella & Grimaldi, 1982, *Raiartus aureolatus* Renaud-Mornant, 1981, *Tholartus natans* Kristensen and Renaud-Mornant, 1983, *Halobiotus crispae* Kristensen, 1982; ⁴Guidetti et al. (2000); *Murrayon pullari* (Murray, 1907), *Murrayon hastatus* (Murray, 1907), *Dactylobiotus selenicus* Bertolani, 1982, *Dactylobiotus parthenogeneticus* Bertolani, 1982, *P. richtersi*, *M. harmsworthi*, *P. areolatus*, *M. joannae*, *R. cf. coronifer*, *Xerobiotus pseudohufelandi* (Iharos, 1966); ⁵Greven and Robenek (1983); *M. cf. tardigradum*, *E. testudo*, *M. hufelandi*; ⁶Weglarska (1989); *Dactylobiotus dispar* (Murray, 1907); ⁷Czerneková et al. (2017); *R. cf. coronifer*; ⁸Walz (1982); *M. hufelandi*; ⁹Crowe (1970); *P. areolatus*; ¹⁰Dewel et al. (1993); *Halechiniscus* sp., *M. cf. tardigradum*, *Echiniscoides* sp., *Stygartus* sp., *Viridiscus viridissimus* (Péterfi, 1956), *Macrobiotus* sp.; ¹¹Kristensen (1976); *Batillipes noerrevangi* Kristensen, 1978; ¹²Crowe et al. (1970); *V. viridis*; ¹³Wright (1988b); *M. hufelandi*, *H. dujardini*, *R. oberhauseri*, *E. testudo*.

The triple-layered epicuticle is the part of the cuticle that exhibits the most interspecific variation (Tables 1-2, Fig. 2) (Guidetti et al. 2000; Rocha et al. 2007). The outermost sublayer of the epicuticle is the flocculent (mucous) coat (Greven 1975, 1984; Wright 1988b). This coat may be continuous or discontinuous and can appear as granules or clustered floccules or spheres (Baccetti and Rosati 1971; Greven 1975; Wright 1988b). As it is in contact with the surrounding environment, bacteria, algae or debris might associate with this layer (Greven 1975). In marine species in particular, associated bacteria are assumed to be symbiotic (Kristensen 1984).

The presence of the flocculent coat seems to be species-dependent. It has been observed, for example, in *M. hufelandi*, *P. areolatus*, *R. oberhaseri* (see Baccetti and Rosati 1971; Greven 1975; Wright 1988b), *M. joannae* (see Guidetti et al. 2000), *E. testudo* (see Greven 1972; Wright 1988b), *Viridiscus rufoviridis* (du Bois-Reymond Marcus, 1944) (see Rocha et al. 2007), *B. noerrevangi* (see Kristensen 1976) and *T. natans natans* (see Kristensen and Neuhaus 1999), but not in *H. dujardini* (see Wright 1988b). Moreover, the presence of the flocculent coat can also be variable among the specimens of the same species. For example, the layer has been observed in desiccated specimens of *R. cf. coronifer*, but not in the active ones (Czerneková et al. 2017). The flocculent coat is of variable thickness in different species and even in different body parts of the same animal. The coat is thickest on the legs but is completely absent from the mouth region (Baccetti and Rosati 1971; Wright 1988a).

The second sublayer of the epicuticle is the thin outer epicuticle (~8-30 nm; Baccetti and Rosati 1971; Crowe et al. 1970; Greven 1975; Greven and Grohé 1975; Guidetti et al. 2000). It is tri- or multilaminar (Fig. 2; Table 1) (Greven 1975; Wright 1988b; Kristensen and Neuhaus 1999; Guidetti et al. 2000). The number of laminae/strata is inter- and intra-species variable and they appear as alternating dark and light zones (Wright 1988b; Guidetti et al. 2000). Some of the laminae seem to be of membrane-like origin (Crowe 1970; Kristensen 1976; Walz 1982).

The third sublayer of the epicuticle is the inner epicuticle. This sublayer exhibits the most structural variation among tardigrade taxa. It appears homogenous and amorphous in eutardigrades, but it can contain a striated layer supported by pillar/rod structures in heterotardigrades (Figs. 2A-B) and some eutardigrades (see below). The

inner epicuticle may also contain labyrinthine structures or lacunar system (Fig. 2A), which are observed primarily in the dorsal inner epicuticle of some heterotardigrades (Greven 1972, 1975; Dewel et al. 1993; Kristensen and Neuhaus 1999). In *M. cf. tardigradum* (Fig. 2C), vertical channels occur sporadically throughout the inner epicuticle (Greven 1975).

The heterotardigrade inner epicuticle is characterized by the presence of striations and pillars (Fig. 2A-B; Wright 1988b; Kristensen and Neuhaus 1999). However, a striated layer has also been found in the eutardigrade *D. dispar* (see Węglarska 1989). The striations are composed of fibrils (Greven 1972; Kristensen 1976; Kristensen and Neuhaus 1999). The fibrillar structures are organized in a hexagonal shape (Fig. 2A). Thus, this is frequently referred to as a "honeycomb layer" (Crowe et al. 1970; Dewel et al. 1993; Kristensen and Neuhaus 1999). In some cases (e.g., in the ventral inner epicuticle of *V. viridis*), the honeycomb layer is connected to the outer epicuticle by single fibrils (Fig. 2A) (Crowe et al. 1970; Greven 1975). In some echiniscids, including *E. testudo*, each hexagon contains an additional central fibril (Greven 1972; Wright 1988b; Kristensen and Neuhaus 1999). The striations vary greatly in thickness and may be limited to certain body regions (e.g., to the ventral epicuticle in *E. testudo* and *V. viridis*) (see Greven 1975).

The pillars/rods extend from the inner epicuticle and lift the overlying layers (Fig. 2A-B; Crowe et al. 1970; Greven 1975; Węglarska 1989; Kristensen and Neuhaus 1999; Guidetti et al. 2000). These pillars primarily occur in ~90% heterotardigrades (Kristensen and Neuhaus 1999; Nelson et al. 2015), although they appear to be absent from some genera such as *Echiniscoides* (Møbjerg et al. 2018). They are also found in the inner epicuticle of the eutardigrade genera *Dactylobiotus* and *Murrayon* and one species of *Isohypsibius* (see Greven 1982; Kristensen 1982a; Węglarska 1989; Guidetti 1998; Guidetti et al. 2000). The pillars usually have a broadened base and tip, with a circular cross-section in the centre, and are perforated internally by one or more channels of varying thickness (Greven 1975; Kristensen and Neuhaus 1999; Guidetti et al. 2000). Pillars may fuse together to form segmental cuticular plates, which are observed in taxa such as Echiniscidae (Kristensen and Neuhaus 1999). The wall of pillars is electron-dense, and their foot consists of fibres cross-connected by fibrils (Kristensen and Neuhaus 1999).

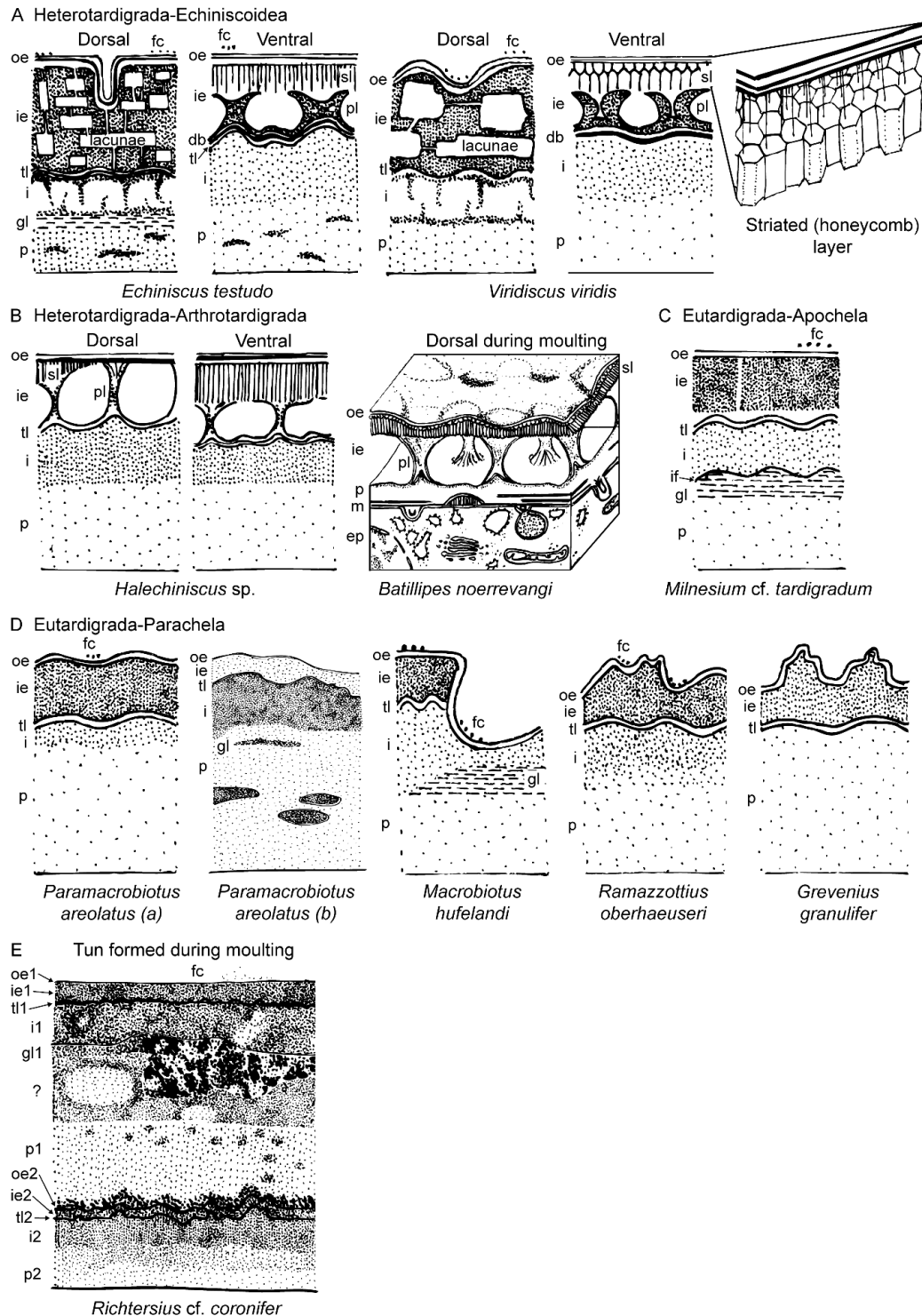


Fig. 2. Cuticle ultrastructure diversity in tardigrades

A) Heterotardigrada (Echiniscidae): dorsal cuticle with labyrinth-like lacunae and channels, ventral cuticle with the striated layer (outer, inner) and pillars. Striated layer detail based on Crowe et al. (1970). B) Arthrotardigrada (Halechiniscidae) differences in the striated layer between the dorsal and ventral cuticle; *B. noerrevangi*: example of dorsal cuticle at the initial stage of new cuticle formation starting with the production of membrane systems (m) (Kristensen 1976; Greven 1980). C) *M. cf. tardigradum* (Apochela): vertical channels in the epicuticle and a prominent granular layer. D) Various Parachela species: cuticles of two specimens of *P. areolatus*; two different (unspecified) parts of the body (a, b) differ in electron density of the epicuticle, in the electron-dense vesicles – ovoidal elements – of Guidetti et al. (2000) – of unknown composition, and discontinuous granular layer. E) The cuticle of a tun during the moulting process (*R. cf. coronifer*), with the old (1) and new (2) cuticle distinguished by numbers. Of note, the similar electron-dense bodies visible in the old procuticle of *R. cf. coronifer* have also been observed by Guidetti et al. (2000) in the procuticle of active *P. areolatus*. Based on Czerneková et al. (2017).

fc – floculent coat, oe – outer epicuticle, ie – inner epicuticle, tl – trilaminar layer, db – dense band, i – intracuticle, p – procuticle, if – inner flake of the intracuticle, gl – granular layer, ep – epidermis, sl – striated layer, pl – pillars.

Schematics of cuticular cross-sections. Figure inspired by and partially modified from Greven (1975).

Cuticular pillars vary in length and structure (Greven 1984; Kristensen and Neuhaus 1999; Guidetti et al. 2000). In general, longer pillars occur in the head and dorsal regions (Greven 1975). Inter-specific variation is minor but includes structural variations such as rod-shaped or hollow pillars, pillars with or without fused fibres, and hexagonal pillars (typical of Stygarctidae). In marine heterotardigrades in particular, the pillars may elevate the outer epicuticle by several micrometres; in case of special tubular pillars/spines, this may be up to 50 µm, e.g., in *A. doryphorus doryphorus* (see Kristensen and Neuhaus 1999). The interpillar space of the inner epicuticle may be open to the environment via cuticular pores, and can thus be filled with seawater and colonized by bacteria (Greven 1984; Kristensen and Neuhaus 1999). However, pillars may also be completely absent in certain species (e.g., *P. sterreri*) or body regions (Greven 1975). *Tetrakentron synaptae* Cuénot, 1892 possesses an epicuticular hyaline coat instead of cuticular pillars (Kristensen 1980). This species' inner epicuticle has a sponge-like structure and its substance appears to be homogenous within the genera (Guidetti et al. 2000). The thickness of the inner epicuticle varies greatly (in the range of a few tenths of a nanometre up to several micrometres), mainly due to the presence or absence of pillars and striations (Crowe et al. 1970; Greven and Grohé 1975; Kristensen and Neuhaus 1999).

The thin trilaminar layer ("*convoluted membrane*") is folded (Fig. 2, Table 2) and separates the epicuticle from the underlying layers in all examined species (Greven 1975; Węglarska 1989; Kristensen and Neuhaus 1999; Guidetti et al. 2000).

The intracuticle is the first layer underlying the trilaminar layer (Baccetti and Rosati 1971; Greven 1975; Greven and Robenek 1983; Wright 1988b). Nevertheless, it is not always distinguishable or even present (Greven 1982; Dewel et al. 1993; Kristensen and Neuhaus 1999; Guidetti et al. 2000). For instance, it has not been found in *D. dispar* (see Węglarska 1989), *Dactylobiotus ambiguus* (Murray, 1907) (see Greven 1982), *Murrayon dianae* (Kristensen, 1982) (see Guidetti et al. 2000; Kristensen 1982b) and in *G. granulifer* (see Greven 1975). The intracuticles of *E. testudo* and *M. cf. tardigradum* are arranged in a wavy pattern of microfibers that are 9 and 7 nm thick, respectively (Greven and Robenek 1983).

The granular (wax) layer is located at the boundary of the intracuticle and the procuticle (Fig. 2, Table 2). This layer has an interstitial granular structure and it is frequently discontinuous or absent. Even in species where it has been regularly detected (e.g.,

M. cf. tardigradum), it may be reduced or undetectable in the new cuticle after moulting and in exuvia (Greven and Robenek 1983). Discontinuous electron-dense granules similar to the granular layer have also been observed in desiccated specimens of *R. cf. coronifer* (see Czerneková et al. 2017). Of note, this layer has not been reported in active animals of the same species. The granular layer is not clearly detectable in arthrotardigrades (Greven 1984; Kristensen and Neuhaus 1999).

The procuticle overlies the epidermis and has been found in all tardigrade cuticles examined (Crowe 1970; Greven 1984; Dewel et al. 1993; Kristensen and Neuhaus 1999; Guidetti et al. 2000). This layer varies greatly in thickness (Table 2) and is composed of irregularly arranged microfibers (3.5–5.0 nm thick), as seen in *E. testudo*, *M. cf. tardigradum* and *M. hufelandi* (see Greven and Robenek 1983). The procuticle receives various inclusions containing lipids, pigments or waste material from epidermal cells, and can both store and distribute them (Dewel et al. 1993). It has been hypothesized that the material found in the procuticle might passively move through the cuticular pores and lacunar system into other cuticular layers (Wright 1988b; Dewel et al. 1993). The procuticle of *V. viridissimus* was suggested to have a liquid-like character. Thus, inclusions could be translocated to other cuticular layers through the animal's movement. In particular, such inclusions may move to the outer layers where they might serve as nutrients for symbiotic bacteria (Dewel et al. 1992).

The thickness of cuticular layers differs considerably among species (Fig. 2; Table 2). Nevertheless, it is hard to accurately measure it (Wright 1988a; Kinchin 1994). In general, the cuticle is thicker in freshwater species than in marine ones, in terrestrial species than in aquatic ones (Clothier 1963), in larger species than in smaller ones, in species that are more resistant to environmental stress than in less resistant species (Wright 1988a). Its estimates are summarized in Table 2. While the ventral and dorsal cuticles usually have the same structure and thickness in eutardigrades, they differ markedly in Echiniscoidea (Greven 1972).

The most structurally complex cuticles, containing all of the above layers and sublayers, are attributed to *M. cf. tardigradum* (Greven and Robenek 1983) and *E. testudo* (Greven 1972; Wright 1988b). Species such as *G. granulifer* (see Greven 1984) have a simpler cuticle (Fig. 2A–D), while the most structurally simple cuticles are ascribed to marine arthrotardigrades (Kristensen and Neuhaus 1999). In general, marine heterotardigrades have simpler cuticles than terrestrial ones in terms of the number of layers.

Table 2. Estimated thickness of the cuticle in tardigrade species (in μm)

Species	Epicuticle	Trilaminar layer	Intracuticle	Granular layer	Procuticle	Total cuticle	References
<i>E. testudo</i> dorsal	0.77–1.8	0.015	0.1–0.5	0.07	0.1–0.2	1.21–2.00	Greven 1972; Wright 1988a
<i>E. testudo</i> ventral	0.27–0.44		0.27	0.09	0.21	0.74–1.3	Greven 1972; Wright 1988a
<i>Echiniscoides sigismundi sigismundi</i> (M. Schultze, 1865)	0.05–0.175	0.015	0.1–0.2		0.3–0.8		Greven and Grohé 1975
<i>V. viridis</i>	1.7 (dorsal) 0.53 (ventral)					2.9 (dorsal)	Crowe et al. 1970
<i>P. sterreri</i>		0.01			0.5–1.0 (ventral) 1.0–1.5 (dorsal)		Kristensen and Neuhaus 1999
<i>A. cf. doryphorus</i>					0.3–0.5		Kristensen and Neuhaus 1999
<i>A. cf. psysophorus</i>					0.2–0.4		Kristensen and Neuhaus 1999
<i>R. aureolatus</i>					0.2–0.3		Kristensen and Neuhaus 1999
<i>T. natans natans</i>					0.2		Kristensen and Neuhaus 1999
<i>Halechiniscus</i> sp.	0.3 (0.04–0.2: striated layer; 0.2–0.3: rod length)	0.01				0.3–1.0	Greven 1975
<i>B. noerrevangi</i>	0.78–1.24	0.01			0.3		Kristensen 1976
<i>R. oberhaeuseri</i>	0.025–0.62*		0.16	0.07	0.19	0.48	Greven 1972; Wright 1988a
<i>H. dujardini</i>	0.09		0.11		0.11	0.33	Wright 1988a
<i>Cryobiotus klebelsbergi</i> (Milhetič, 1959)						0.5–1	Greven et al. 2005
<i>Macrobiotus</i> spp., except <i>P. richtersi</i>	0.15–0.32	0.02	0.13–0.20	0.02–0.09	0.22–2.0		Baccetti and Rosati 1971; Bussers and Jeuniaux 1973a; Wright 1988a; Guidetti et al. 2000
<i>D. dispar</i>		0.01			0.5–2.0		Węglarska 1989
<i>P. areolatus</i>	0.15–0.41				0.7–5.3 (including intracuticle)		Crowe 1970
<i>M. cf. tardigradum</i>		0.015	0.3–0.4	1.5		4	Greven 1972; Bussers and Jeuniaux 1973a

* The upper value reflects the epicuticular elevations (Greven 1972)

Since the cuticle of the eutardigrade *D. dispar* greatly resembles the typical heterotardigrade-pattern (Węglarska 1989) and the cuticular pillars are present in the cuticles of various eutardigrade species, Guidetti et al. (2000) highlighted the inaccuracy of the hetero- and eutardigrade-pattern terminology based on the internal structure of the cuticle. Instead, the presence of cuticular pillars together with specific characteristics of the buccal tube and claws likely represent two evolutionary lines. The first, the "non-pillar" line, is represented by the genera *Macrobiotus*, *Richtersius* and *Xerobiotus* (all genera are xerophilous, with *Macrobiotus* and *Richtersius* being well-acknowledged cryptobionts), and possibly by some other genera whose cuticles have not been studied yet (*Calcarobiotus*, *Pseudohexapodibius*, *Adorybiotus*, *Minibiotus*, *Pseudodiphascon*). The second, the "pillar" line, includes the genera *Murrayon*, *Dactylobiotus* and *Macroversum* (Guidetti et al. 2000).

The cuticle during moulting

Like other ecdysozoans, tardigrades regularly shed their exoskeleton (Aguinaldo et al. 1997). Ecdysis is usually the result of increases in body size. However, tardigrades moult not only in response to their growth. Adverse environmental conditions or food shortages can trigger moulting as well, and animals may become even smaller than they were previously (Marcus 1929; Baumann 1961; Walz 1982; Greven 1984). In general, the first two moults in the life of a tardigrade are part of the developmental process (Walz 1982). Consequent moults are synchronized with oocyte maturation in females and hermaphrodites of non-marine species (Walz 1982; Rebecchi and Bertolani 1994; Rebecchi et al. 2000; Bertolani 2001).

The old cuticle, including claws and cuticular structures of the fore- and hindgut, is shed during moulting (Clothier 1963; Walz 1982). Ejection of the cuticular structures of the foregut (i.e., the buccopharyngeal apparatus) is a well visible signal that moulting has begun. Following this, the mouth opening is closed (Clothier 1963; Walz 1982; Greven 1984) and the animal is not able to feed until ecdysis is completed and the buccopharyngeal apparatus has been restored (Kinchin 1994). The buccal tube and the stylet system are resynthesized through secretions from the salivary glands, while the posterior part of the feeding apparatus is reconstructed by the pharyngeal cells and epithelial cells of the oesophagus (Crowe et al. 1971a; Guidetti et al. 2012). At the same time, a new body cuticle, including hindgut lining, a new cuticle in the oesophagus and new

claws are being synthesized (Nelson et al. 2010; Guidetti et al. 2012).

The phases of moulting in tardigrades can be distinguished by several consecutively appearing traits (the presence of gut content, the presence of the buccopharyngeal apparatus, oocyte size and the ultrastructure of the epidermal cells and the cuticle) (Crowe et al. 1971a; Kristensen 1976; Walz 1982; Dewel et al. 1993). All parts of the cuticle are secreted by epidermal cells or the surrounding epithelium (Nelson et al. 2010; Guidetti et al. 2012). Thus, the ultrastructure of those cells differs throughout the moulting cycle. The following description of moulting phases is based on observations of *B. noerrevangi* (see Kristensen 1976) and complemented by observations made in eutardigrades (Baccetti and Rosati 1971; Crowe et al. 1971a; Walz 1982).

At the beginning of the phase I, the gut fills with dark organic material. During this phase in *B. noerrevangi*, α -membrane systems are secreted into the old procuticle. These membranes are thought to be homologous to the arthropod ecdysial membrane (Kristensen 1976). During phase II, tardigrades eject the sclerified parts of the buccopharyngeal apparatus entering a "simplex stage" (Marcus 1929; Kristensen 1976). The epidermis becomes thicker and basophilic (Crowe et al. 1971a), and the first Golgi complexes (dictyosomes) become visible (Kristensen 1976; Walz 1982). The epidermal cells secrete membrane vesicles (*P. areolatus*; see Crowe et al. 1971a) or β -membranes (*B. noerrevangi*; see Kristensen 1976) or membrane "patches" (*M. hufelandi*; see Walz 1982) directly under the old cuticle. These membranous structures coalesce and ultimately form the outer epicuticle.

Phase III is the main cuticle synthesis stage. In this stage, a great number of Golgi apparatus-originating vesicles (forming clusters in the range of 20–300 nm; "moulting bodies") with proteinaceous material are discharged at the base of the old cuticle (Crowe et al. 1971a). Secretory vesicles carrying material for the new inner epicuticle are visible in the epidermal cells (Kristensen 1976; Walz 1982). Later in this stage, δ -membrane is secreted, separating the new epicuticle from newly forming matrix layer. This will give rise to the intracuticle (in species that possess this layer) and the procuticle at the end of phase III (Kristensen 1976). The procuticle grows continuously until the next moult (Greven 1984). The previously ejected parts of the buccopharyngeal apparatus are resynthesized during this phase as well (Kristensen 1976; Guidetti et al. 2012), while claw glands synthesize the new claws (Kristensen 1976; Walz 1982). Each claw gland extends thin

cellular processes that secrete the claws cuticle in a sequence identical to the rest of the body. When the claws are complete, the cellular processes of the claw glands retract (Greven 1984).

Individuals now have both the new cuticle and the old adherent cuticle. Both cuticles lack the flocculent coat, and the new intracuticle (if present) cannot be distinguished since it lacks sclerotization. The epidermal cells are rich in lipid droplets, which are transported to the upper cuticular layers. However, the newly formed cuticle is still missing the granular layer in species that usually have it (Baccetti and Rosati 1971). While the new cuticle is being secreted, the somatic muscle cells are still attached to the old cuticle. This attachment is made possible by cuticular filaments. The cuticular filaments run from the modified epidermal cells that are in contact with the muscles, continue through the newly forming cuticle up to the old cuticle, and terminate at the old trilaminar layer (Walz 1982; Greven 1984). Thus, the animal is still able to move during this stage (Crowe et al. 1971a; Greven 1984). Prior to ecdysis, the new cuticle is complete.

During phase IV, the animal shrinks inside the old cuticle. The attachments between the old cuticle and the muscles break, and in some species the animal defecates (Kristensen 1976). The procuticle of the old cuticle is either completely (Baccetti and Rosati 1971) or partially (Crowe et al. 1971a) solubilized and the new and the old cuticle separate (Baccetti and Rosati 1971; Walz 1982). It has been suggested that the decrease in body cavity fluid pressure following defecation or oviposition – two processes that are often synchronized with moulting – might facilitate the separation of the two cuticles (Kinchin 1994). Finally, the animal exits through the front side of the old cuticle (exuvium) with an empty gut and an open mouth which is described as phase V (Kristensen 1976). The newly formed cuticle is about one half of its final thickness. In *P. areolatus* it reaches its maximum thickness in 15–24 hours after separation of the exuvium (Crowe 1970). When the new cuticle comes in contact with the surrounding environment, large spheres of polysaccharides are released on top of the integument and a new flocculent coat is formed possibly as a result of the enzymatic activity of the epicuticle (Baccetti and Rosati 1971).

Encystment and cyclomorphosis

Tardigrades can secrete additional cuticles during the processes of encystment (especially limnic species but also moss-dwelling species) or cyclomorphosis (marine eutardigrades of the genus *Halobiotus* sp.) to protect themselves from unfavourable

environmental conditions, such as fluctuations in temperature, pH, oxygen level or osmotic stress. Encystment and cyclomorphosis may also be triggered by unknown endogenous factors (Møbjerg et al. 2007; Halberg et al. 2013b; Guidetti and Møbjerg 2018; Janelt and Poprawa 2020). In general, encystment is thought to be an optional response to environmental challenges where animals remain immobile inside their cysts (Węglarska 1957; Szymańska 1995; Clausen et al. 2014), whereas cyclomorphosis seems to be an obligate part of seasonal changes with animals remaining partially mobile (Kristensen 1982b; Møbjerg et al. 2007). The ecological and physiological aspects of both processes and their significance are reviewed in Guidetti and Møbjerg (2018).

During cyst formation, individuals decrease in size and, similarly to moulting, enter the “*simplex*” stage by ejecting the sclerified parts of the buccopharyngeal apparatus (Szymańska 1995; Guidetti et al. 2008; Janelt and Poprawa 2020). The newly serially synthesized cuticles (one to three, depending on the species) are, however, retained as protective coats (Węglarska 1957; Guidetti et al. 2006). These cuticles are not attached to the epidermis but are interconnected by a network of cuticular appendages and/or cuticular fibres of varying thickness (Szymańska 1995; Janelt and Poprawa 2020). The newly formed buccopharyngeal apparatus and claws might be structurally different from the previous ones (Guidetti and Møbjerg 2018). When the encystment is terminated, the intensely moving animal breaks through the cyst capsule/cuticular involucre (the series of cuticles enclosing the animal) using stylets (Hansen and Katholm 2002) and claws (Janelt and Poprawa 2020) and exits the cyst.

Following the terminology of Guidetti et al. (2006), the cuticular involucre include: (i) the old/external cuticle, (ii) the sarcophagus/middle cuticle and (iii) the mummy/internal cuticle. Since the term “*old cuticle*” is used for exuvia abandoned during moulting, but the external part of a cyst is also termed “*exuvia*” (Clausen et al. 2014), we use the term “*external cuticle*” instead to avoid confusion. The external cuticle has leg protuberances with claws. It is hardened and coloured in some species, such as *Bertolanius volubilis* (Durante Pasa and Maucci, 1975). When the external cuticle is absent, the sarcophagus cuticle is then the outermost cuticle and becomes hardened and coloured. The sarcophagus cuticle has wide clawless leg protuberances that sometimes terminate in small holes (Guidetti et al. 2006; Janelt and Poprawa 2020). Its surface appears as reticulated in light microscopy but it is smooth when viewed through scanning electron microscopy (SEM). The mummy cuticle has

no leg protuberances (except the type 2 cysts of *B. volubilis*, see below) and a wrinkled surface (Szymańska 1995; Guidetti et al. 2006; Janelt and Poprawa 2020).

Cyst formation has been discovered in 25 eutardigrade and some heterotardigrade (Echiniscoidea and Echniscidae) species (Guidetti and Møbjerg 2018). Some species, including limnic *Dactylobiotus* spp. and *Thulinus ruffoi* (Bertolani, 1982) are known to form only one type of cyst, whereas moss-dwelling species of *Bertolanius* spp. produce two types (Westh and Kristensen 1992; Hansen and Katholm 2002; Guidetti et al. 2006, 2008). "Type 1" cysts (white-cysts) are elongated with a yellow-pigmented or white-opaque surface. The cyst capsule is double-layered (the external and the sarcophagus cuticle) in *B. volubilis* (see Guidetti et al. 2006, 2008) and only single-layered (the external cuticle only) in *Bertolanius nebulosus* (Dasty, 1983) (see Hansen and Katholm 2002; Guidetti and Møbjerg 2018). "Type 2" cysts (red-cysts) are more complex. In the case of *B. nebulosus*, the "type 2" cyst capsule comprises (i) a thick red or dark brown tanned external cuticle and (ii) the mummy cuticle with a modified and non-functional buccopharyngeal apparatus and no protuberances for the legs or claws (Hansen and Katholm 2002; Guidetti and Møbjerg 2018). In contrast, the "type 2" cyst capsule in *B. volubilis* consists of (i) the external cuticle, (ii) the sarcophagus cuticle and (iii) the smooth mummy cuticle with clawless legs (Guidetti et al. 2006, 2008).

In *D. parthenogeneticus*, the sarcophagus cuticle contains an external electron-lucent heterogeneous layer, a trilaminar layer and an internal wide layer with lacunae (Guidetti and Møbjerg 2018). The mummy cuticle contains two trilaminar layers separated by a non-homogenous layer, as well as an electron-lucent layer with electron-dense and convolute cordons that are either randomly oriented or organised in striations. However, it does not contain the procuticle observed in the mummy cuticle of *D. dispar* (see Guidetti et al. 2006). The cuticle of encysted *D. dispar* is composed of a sponge-like material containing striations, while the cuticle of active animals is composed of pillars (Szymańska 1995).

The cysts of the marine heterotardigrade *E. sigismundi sigismundi* are different from those of eutardigrades. The first phase of the encystment resembles moulting. The newly produced second cuticle (corresponding to the sarcophagus cuticle using the terminology for *B. volubilis*) is similar to the external cuticle and contains legs and claws. The new complete buccopharyngeal apparatus is present. Next,

the animal forms another cuticle (corresponding to the mummy cuticle) and contracts into a tun-like state. A set of discharged claws remains between the outermost and the second (sarcophagus) cuticle. The most developed cysts contain one more cuticle lining the animal in the tun-like shape with the discharged sclerified parts of the buccopharyngeal apparatus into the second cuticle. In general, encystment in *E. sigismundi sigismundi* does not involve colour changes, and in contrast to eutardigrades, the second (sarcophagus) cuticle has claws (Clausen et al. 2014; Guidetti and Møbjerg 2018).

Cyclomorphosis represents seasonal morphological and physiological changes, which are only partially understood and have been documented only for the marine eutardigrade genus *Halobiotus*, and the species *H. crispae* in particular (Kristensen 1982b; Halberg et al. 2009; Guidetti and Møbjerg 2018). The process starts with the expulsion of the sclerified parts of the buccopharyngeal apparatus, similar to moulting. However, the animal remains covered by the old/external cuticle, it possesses a modified buccopharyngeal apparatus and newly emerging claws, and its mouth and cloaca are closed. This state is called a pseudosimplex stage 1 (P1). In contrast to the encystment, the animal is able to move while in this state, since the muscles are still connected to the external cuticle. Gonads are reduced at this stage and the animal appears opaque (Kristensen 1982b; Halberg et al. 2013b; Guidetti and Møbjerg 2018). The individual, possessing the modified buccopharyngeal apparatus and small claws, then breaks through the external cuticle, entering an active stage of sexual maturation called a pseudosimplex stage 2 (P2). The mouth and cloaca are open in P2. After this stage, the animal can undergo the usual moulting process and enter the active stage with a fully developed buccopharyngeal apparatus, claws and gonads (Kristensen 1982b; Guidetti and Møbjerg 2018). The animals are translucent during the active stage, P2 and the simplex stage (Halberg et al. 2013b).

Cuticle chemical composition and mechanical properties

The cuticle is largely composed of polysaccharides (including acid mucopolysaccharides and chitin), proteins (including glycoproteins and lipoproteins) and lipids, which occur in varying quantities and proportion in different parts of the cuticle (Table 3). The main cuticular building blocks are proteins intertwined with polysaccharides (Crowe 1970; Baccetti and Rosati 1971) and lipids that likely function as diffusion barriers (Crowe 1970; Wright 1988b, 1989a).

Table 3. Chemical composition of the cuticle

Cuticle layers and sublayers	Eutardigrada		Heterotardigrada	Partially removed by	Significantly removed by	Resistant to
	Apochela	Parachela				
Flocculent coat		Acid mucopolysaccharides ^{2,4}		Pepsin and amylase (unfixed specimens only) ²	30% alcohol ³	Pepsin ² , trypsin ² , amylase ² , chloroform ² , xylene ²
Outer epicuticle		Chitin ¹ , neutral polysaccharides ² , poorly tanned lipoproteins ² , acid mucopolysaccharides ⁴ , lipoproteins (protein is the major constituent) ⁵ , Tyrosinase ⁶		Chloroform ²	Amylase ² , pepsin ²	Chitinase ²
Inner epicuticle		Chitin ¹ , proteins ² , neutral polysaccharides ² , acid mucopolysaccharides ⁴ , untanned lipoproteins ² , tyrosinase ⁶		Chloroform ² , amylase ² , xylene ²	Pepsin ²	Chitinase ²
Trilaminar layer		Tanned glycoprotein ² , lipoproteins ⁵				Amylase ² , pepsin ² , trypsin ² , chitinase ² , chloroform ²
Intracuticle	Chitin ¹	Chitin ^{1,7} , untanned glycoproteins ² , lipids ^{2,5} , proteins ⁴ , lipoproteins ² , polysaccharides ²	Chitin (only dorsal cuticle of <i>E. testudo</i>) ¹	Xylene ² , chloroform ²	Thymol + chloroform/methanol ⁵	Amylase ² , pepsin ² , trypsin ² , chitinase ²
Inner flake of the intracuticle ^{2,5}	Chitin ¹	Tanned glycoprotein and lipids ² , masked lipids or lipoproteins ⁵		Pepsin ²	Amylase ² , chloroform ²	Chitinase ²
Granular layer	Chitin ¹	Esterases ² , waxes ² , lipids ⁵ , chitin ⁷			Chloroform ² , xylene ²	Pepsin ² , trypsin ² , amylase ² , chitinase ²
Procuticle	Chitin ¹	Chitin ^{1,2,7} , polysaccharides ² , proteins ^{2,4,5}	Chitin ^{1,7}	Chitinase ²	Pepsin ² , chitinase ² , trypsin ² , pronase ⁴ , HNO ₃ ⁴	Amylase ² , chloroform ² , xylene ²

¹ Greven and Peters (1986): *M. hufelandi*, *M. cf. tardigradum*, *E. testudo*; ² Baccetti and Rosati (1971): *M. hufelandi*; ³ Kristensen (1976): *B. noerrevangi*; ⁴ Crowe (1970): *P. areolatus*; ⁵ Wright (1988b): *E. testudo*, *M. hufelandi*, *H. dujardini*, *R. oberhaeuseri*; ⁶ Volkman and Greven (1993): *M. hufelandi*; ⁷ Kristensen and Neuhaus (1999): *H. crispae*, *A. physophorus*, *T. natans natans*.

Wright (1988b) observed so-called lipid rosettes on the surface of tun cuticles fixed anhydrously by osmium vapour in *M. cf. tardigradum* and *M. hufelandi*. Those structures could be removed by chloroform.

Phospholipids and neutral fats are the main lipids found in the tardigrade cuticle (Crowe 1970; Wright 1989a). Lipids were originally thought to be evenly distributed throughout the cuticle (Crowe 1970; Baccetti and Rosati 1971; Greven 1984); however, more recent data indicate that lipids are especially prominent in the intracuticle and the granular layer (Wright 1988b). In some species, the epicuticle initially appeared to be lipid-free (Baccetti and Rosati 1971) but was later found to contain lipoproteins (Wright 1988b). Cuticular lipids may play an important role in anti-fungal defense or transpiration dynamics and for waterproofing properties of the cuticle (Crowe 1970; Crowe et al. 1971b; Hadley 1984; Wright 1989a).

The flocculent coat is composed of acid mucopolysaccharides, while the outer and inner epicuticle comprise neutral polysaccharides and lipoproteins (Baccetti and Rosati 1971; Wright 1988b). Tyrosinase (diphenoloxidase) has been detected in the epicuticle of *M. hufelandi* and may be involved in the sclerotisation of the cuticle (similar to in insects) or in a putative defence system (Volkmann and Greven 1993). Indeed, tanning has been detected in the outer epicuticle, trilaminar layer and sclerotized flake of the intracuticle of *M. hufelandi* (see Baccetti and Rosati 1971), in the cuticle of *M. cf. tardigradum* (see Bussers and Jeuniaux 1973a) and potentially in *E. testudo* (see Greven 1972). The trilaminar layer is mainly composed of tanned glycoproteins and lipoproteins (Baccetti and Rosati 1971; Wright 1988b). The intracuticle contains lipids (Wright 1988b), chitin (Greven and Peters 1986), lipoproteins (Baccetti and Rosati 1971) and untanned glycoproteins of a similar nature to those of insect cuticles. Finally, the procuticle is chitinous and proteinaceous (Crowe 1970; Baccetti and Rosati 1971; Greven and Peters 1986; Wright 1988b; Kristensen and Neuhaus 1999).

Chitin (poly- β -(1-4)-N-acetyl-D-glucosamine) is a common invertebrate polysaccharide that plays an important role in hardening the exoskeleton, making it resistant to penetration (Raabe et al. 2005). Whether chitin re-modelling is involved in the transition from the active state to tuns remains to be studied. However, the coding sequences of chitinase and its regulators have been documented (Förster et al. 2012) and suggest that active disintegration of cuticles might occur under specific circumstances such as moulting. Chromatographic analysis, histochemical staining and enzymatic digestion with chitinase failed to confirm the presence of a chitinous cuticle in *P. areolatus* (see Crowe 1970). However, chitin

has been detected in the cuticle, claws, and stomodaeum of species of *M. hufelandi*, *M. tardigradum*, *Echiniscus merokensis merokensis* Richters, 1904 and *E. testudo* through a similar process of enzymatic digestion with chitinase (Baccetti and Rosati 1971; Bussers and Jeuniaux 1973b). The presence of chitin in the tardigrade cuticle has also been demonstrated using controlled experiments with wheat germ agglutinin (WGA)-gold conjugates (Greven and Peters 1986; Kristensen and Neuhaus 1999) and Fourier transform infrared spectroscopy (Greven et al. 2016). The latter study showed that chitin in the cuticle of *M. hufelandi* is organized as antiparallel chains (α -chitin). This finding supports the hypothesis that the presence of α -chitin in cuticle layers close to the epidermis might be an apomorphic trait of the Ecdysozoa clade. The subcuticular localization of chitin varies among species (Greven and Peters 1986; Kristensen and Neuhaus 1999), but the procuticle is always chitinous (Table 3). Various chemical treatments also have different effects on the different layers of the cuticle (Table 3). Such treatments are of particular interest for establishing new techniques that could support studies on the cell and molecular biology of tardigrades. For example, chitinase treatment has been used to increase the permeability of the tardigrade integument before immunolabelling in *Hypsibius exemplaris* Gąsiorek, Stec, Morek and Michalczyk, 2018 (see Gross and Mayer 2015).

The mechanical strength of the cuticle increases with its thickness, while the permeability decreases. Thicker cuticular areas, which are often in direct contact with the surrounding environment, serve as a permeability barrier during desiccation. In contrast, thinner and more permeable areas occur in the intersegmental parts of the cuticle and can be folded inside the body to avoid desiccation (Crowe 1970, 1972). The external flocculent coat is deformable and provides no mechanical resistance. On the other hand, the outer epicuticle and the trilaminar layer provide strong mechanical barriers and protect the cuticle from penetration and mechanical stress. The inner epicuticle found between these layers tends to fold, giving the cuticle plasticity and resilience; the lipidic and glycoproteinaceous intracuticle is also deformable. The procuticle is formed by a chitin-proteinaceous mix and it seems to be the most rigid and tension-resistant cuticular layer (Wright 1988a). In general, quinone-tanning and chitin presence increase the mechanical resistance of the cuticle (Crowe 1970; Crowe et al. 1971b).

Cuticular properties related to anhydrobiosis

Tardigrades exhibit interspecific disparity in their anhydrobiotic abilities, which are based on various morphological, physiological, and biochemical processes (Wright 1989b; Welnicz et al. 2011; Hygum et al. 2016). Morphologically, cryptobiotic states such as anhydrobiosis lead to changes in body volume during tun formation (Crowe 1972; Greven and Greven 1987; Halberg et al. 2013a; Nelson et al. 2015). The development of cuticular plates, sclerotization and generally higher ultrastructural complexity of the cuticle (e.g. in echiniscids) might have developed as a result of adaptation to new environmental conditions (Kristensen 1987).

Since the tardigrade cuticle is highly permeable to water (Marcus 1929; Crowe 1972; Greven and Greven 1987), it has been hypothesized that one of the main prerequisites for their anhydrobiotic survival is the reduction of high-permeability areas of their body surface (Crowe 1972), such as the intersegmental and thinner cuticular parts (Crowe 1972; Czerneková et al. 2017), through folding. Similarly to rotifers (Ricci et al. 2003), such folding could be used to control evaporative water loss in tardigrades (Crowe et al. 1971b; Crowe 1972). However, Wright (1989b) suggested that the effectiveness of tun formation in limiting evaporative water loss is simply due to a general reduction in surface area, rather than the reduction in areas with higher permeability. The most efficient surface area reduction correlates with the highest desiccation tolerance, with the notable exception of species having rigid and thick epicuticular plates such as *E. testudo* (see Wright 1989b). In such species, air-filled inner epicuticular spaces and a thick intracuticle likely compensate for their limited ability to reduce their surface area (Wright 1989a). Interestingly, another marine heterotardigrade *E. sigismundi sigismundi* tolerates dehydration even without tun formation (Hygum et al. 2016).

Another important prerequisite for the anhydrobiotic survival of limno-terrestrial tardigrades is a relatively slow pace of desiccation (Crowe 1970, 1972, 1975; Greven and Greven 1987; Wright 1989b, 1989a). Entering anhydrobiosis at a slow pace allows for (i) the synthesis of surface and/or membrane protectants such as trehalose and glycerol in species that utilize them for this purpose (Crowe 1975), (ii) the proper packing and relocation of the inner organs, preventing structural disruption (Czerneková et al. 2017), (iii) the expression of parachelan tardigrade-specific

intrinsically disordered proteins (TDPs), protecting cellular content via vitrification (Boothby et al. 2017) and (iv) the rearrangement of cuticular lipids, significantly decreasing the permeability of the cuticle (Wright 1989a).

In one study, tardigrades that were consistently subjected to successive desiccation-rehydration cycles were unable to form tuns properly (individuals entered a "semi-tun" state), resulting in lower survival rates (Czerneková and Jönsson 2016). Further comparison of desiccated and active specimens revealed ultrastructural changes in the integument. Desiccated specimens lacked pigment granules in the epidermal cells, however, they exhibited a discontinuous flocculent coat and an interstitial granular layer (Czerneková et al. 2017).

Knowledge of the chemical nature of the cuticle is essential for our understanding of anhydrobiosis. The role of lipids in anhydrobiosis, similarly to pigments and other integumental inclusions, has been debated (Dewel et al. 1992). While Krusberg (1967) and Womersley et al. (1982) found no significant correlation between lipid content and anhydrobiotic survival in nematodes, Wharton et al. (2008) showed the importance of the extra-cuticular lipid layer in nematodes and Hadley (1984) did the same in arthropods. Cuticular lipids are expected to play a similarly important role in anhydrobiosis in tardigrades (Crowe 1972; Wright 1989b, 1989a). In terrestrial insects, the extra-cuticular lipids form the a water-proof layer that reduces transpiration and prevents desiccation as well (Chung and Carroll 2015). Lipids were also implied in the production of bioprotectants such as glycerol and trehalose, however, the studies are contradictory (Madin and Crowe 1975; Womersley et al. 1982).

Although their precise role in anhydrobiosis has not yet been determined, lipids are thought to be the main transpiration barrier in tardigrades (Hadley 1984; Wright 1988b, 1989a). Small lipid rosettes or discs have been detected on the tardigrade body surface; these likely enclose the cuticular pores and might form an additional permeability barrier (Wright 1988b). Lipids are also the main component of the intracuticle and granular layers, which have been proposed as the main permeability barriers in species of *M. tardigradum*, *M. hufelandi* and *R. oberhaueseri* (see Baccetti and Rosati 1971; Greven and Robenek 1983; Greven and Greven 1987; Wright 1989a; Wright et al. 1992). The importance of the intracuticle is supported by the fact that desiccation-tolerant species such as *R. oberhaueseri* have the thickest intracuticles (Wright 1988a). Together with lipids,

chitin might also play an important role in limiting the permeability of the cuticle in both tardigrades and arthropods (Crowe 1970; Hadley 1984). In addition, if certain tardigrade species lack extracuticular lipids (lipid rosettes) or granular layers, cuticular lipoproteins might form an important permeability barrier. Likewise, the material deposited in the procuticle can be distributed through the pores or channels to other cuticular layers or remain in the procuticle to decrease the rate of evaporative water loss (Greven 1972; Greven and Greven 1987; Wright 1988b). Moreover, epidermal cells, which are thought to regulate the water permeability of the integument in arthropods (Hadley 1984), contain a large number of lipid vacuoles in tardigrades (Czerneková et al. 2017).

The effect of tardigrade ecdysis on desiccation survival is largely unexplored. In nematodes, moulting has been observed to slow the rate of desiccation and thus improved their survival after desiccation (Gaur and Perry 1991). In tardigrades, moulting does not restrict the organism's ability to form tuns (Fig. 2E; Czerneková et al. 2017). Studies on moulting and the secretion activity of epidermal cells might thus bring new insight into tardigrade physiology.

Future perspectives and possible biotechnological relevance

The ultrastructure of the tardigrade cuticle has received much attention with respect to taxonomy and phylogenetic analysis. This review demonstrates that such knowledge, together with information about its chemical composition, can also contribute to our understanding of desiccation tolerance and the mechanical properties of the cuticle. Although past research has uncovered some information about the chemical composition of various cuticular layers, we are still missing a more detailed and comprehensive picture (Table 3). We also lack information about the biochemical pathways that regulate the formation of various cuticular structures.

Systematic studies on ways to soften and/or permeate the cuticles of living specimens without severe adverse effects on survival would greatly facilitate the development of improved micromanipulation and/or electroporation techniques (Tenlen et al. 2013; Boothby et al. 2017; McGreevy et al. 2018; Tenlen 2018). Such techniques would allow researchers to perform controlled loss- and gain-of-function experiments in living animals. This is necessary for the establishment of tardigrades as a genetic model and would bring

deeper insight into tardigrade biology. Research on the molecular mechanisms that drive the remarkable extremotolerant abilities of tardigrades may also help boost innovations in biotechnology. The preservation of cellular integrity under desiccation, freeze-drying and radiation are of particular interest. Such knowledge could enable the engineering of increased tolerance in sensitive species and may advance the development of efficient xeroprotection technology (Rebecchi et al. 2020).

The great biotechnological potential of tardigrade research is already being applied in a variety of ways. There are three known parachelan TDP families – Cytosolic-, Secreted- and Mitochondrial Abundant Heat Soluble proteins (CAHS, SAHS and MAHS, respectively; see Yamaguchi et al. 2012; Tanaka et al. 2015) – that are either highly expressed constitutively or whose expression is substantially increased in response to desiccation (Hesgrove and Boothby 2020). These proteins are lacking a persistent tertiary structure and do not denature upon exposure to various abiotic stresses (Boothby et al. 2017; Hesgrove and Boothby 2020). Although the precise mechanisms of action of these proteins are unknown, their protective function has been demonstrated in several systems. Overexpression of several MAHS proteins in human cells increased their resistance to hyperosmotic shock (Tanaka et al. 2015), while overexpression of CAHS proteins improved desiccation tolerance in yeasts and bacteria (Boothby et al. 2017). Recently, CAHS has been successfully used to stabilize pharmaceutical excipients such as lactate dehydrogenase and lipoprotein lipase (Piszkiewicz et al. 2019), which suggests that they have a great potential to stabilize protein-based therapeutics.

Research on radiation tolerance in tardigrades has so far focused on dose-responses, survival and fertility (Jönsson 2019). Different tardigrade species are known to exhibit varying tolerances to genotoxic stress (Horikawa et al. 2013), but no studies to date have documented the amount of DNA damage induced by low doses of non-lethal radiation. Tardigrade studies aimed at turning off/on specific genes involved in DNA repair and protection and in cellular responses to radiation such as cell cycle arrest could eventually contribute to cancer research (Jönsson 2019).

Another promising example of a biotechnological application of a tardigrade-specific protein involves the novel DNA binding protein damage suppressor (Dsup). Dsup was first identified in *Ramazzottius varieornatus* Bertolani & Kinchin, 1993 and has been found to decrease X-ray-induced damage to DNA in human cells by 40%

(Hashimoto et al. 2016). Furthermore, heterologous expression of Dsup in tobacco plants was found to enhance their tolerance to genotoxic stresses, such as treatment with ethylmethane sulfonate or bleomycine, and UV- and X-ray irradiation (Kirke et al. 2020). Loss of function studies would help to elucidate whether the same cell-protection systems evolved to cope with the cell and DNA damage caused by both desiccation and radiation (the cross-tolerance hypothesis). While the above examples already demonstrate the tremendous biotechnological potential of tardigrades, their implications are likely even greater, as many tardigrade-specific proteins are yet to be discovered, described or functionally characterized (Förster et al. 2012; Schokraie et al. 2012; Hashimoto et al. 2016).

Several non-tardigrade cuticular enzymes and chitin derivatives are being increasingly used in medicine, pharmacology and biotechnology (Saini et al. 2020). In particular, chitosan is a non-toxic, biocompatible compound that protects against pathogenic fungi and bacteria. It has many applications in medicine (e.g., drugs and vaccines delivery, wound healing, haemostasis) and great potential in food preservation and packaging technology (Aider 2010; Akca et al. 2018; Zhao et al. 2018). The precise structure and activity of tardigrade chitosans and chitinases remain to be studied, but these compounds could have similar practical applications. Finally, recent work on a protective pigment found in a novel species of *Paramacrobiotus* (Suma et al. 2020) suggests that systematic research on the pigmentation of the tardigrade integument could also be very fruitful.

In conclusion, gaining deeper insight into the structure and composition of the tardigrade cuticle could lead to many new important biological findings. It would also facilitate the development of improved techniques for the genetic manipulation of tardigrades. Research on tardigrade cell and molecular biology have implications for a range of biological disciplines, from evolutionary and developmental biology to biotechnology and even medicine.

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Author contributions

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References

- Aguinaldo A.M.A., Turbeville J.M., Linford L.S., Rivera M.C., Garey J.R., Raff R.A., Lake J.A., 1997, Evidence for a clade of nematodes, arthropods and other moulting animals, *Nature* 387(6632): 489–493.
- Aider M., 2010, Chitosan application for active bio-based films production and potential in the food industry: Review, *LWT - Food Sci. Technol.* 43(6): 837–842.
- Akca G., Özdemir A., Öner Z.G., Şenel S., 2018, Comparison of different types and sources of chitosan for the treatment of infections in the oral cavity, *Res. Chem. Intermed.* 44(8): 4811–4825.
- Baccetti B., Rosati F., 1971, Electron microscopy on tardigrades. III. The integument, *J. Ultrastruct. Res.* 34(3-4): 214–243.
- Baumann H., 1961, Der Lebenslauf von *Hypsibius (H.) convergens* Urbanowicz (Tardigrada) (The life history of *Hypsibius (H.) convergens* Urbanowicz (Tardigrada)), *Zool. Anz.* 362–381 (in German).
- Bertolani R., 2001, Evolution of the Reproductive Mechanisms in Tardigrades – A Review, *Zool. Anz.* 240(3-4): 247–252.
- Bertolani R., Altiero T., Nelson D.R., 2009, Tardigrada (Water Bears), [in:] Likens G.E. (ed.), *Encyclopedia of Inland Waters*, Elsevier, Amsterdam: 443–455.
- Boothby T.C., Tapia H., Brozena A.H., Piszkiwicz S., Smith A.E., Giovannini I., Rebecchi L., Pielak G.J., Koshland D., Goldstein B., 2017, Tardigrades Use Intrinsically Disordered Proteins to Survive Desiccation, *Mol. Cell* 65(6): 975–984: e5.
- Bussers J.C., Jeuniaux C., 1973a, Structure et composition de la cuticule de *Macrobiotus* sp. et de *Milnesium tardigradum* (Structure and Composition of the cuticle of *Macrobiotus* sp. and *Milnesium tardigradum*), *Ann. Soc. R. Zool. Belg.* 103: 271–279 (in French).
- Bussers J.C., Jeuniaux C., 1973b, Chitinous cuticle and systematic position of tardigrada, *Biochem. Syst. Ecol.* 1(1): 77–78.
- Chung H., Carroll S.B., 2015, Wax, sex and the origin of species: Dual roles of insect cuticular hydrocarbons in adaptation and mating, *BioEssays* 37(7): 822–830.
- Clausen L.K.B., Andersen K.N., Hygum T.L., Jørgensen A., Møbjerg N., 2014, First record of cysts in the tidal

- tardigrade *Echiniscoides sigismundi*, Helgol. Mar. Res. 68: 531–537.
- Clothier G.W., 1963, Water Bears, Bios 34(3): 121–128.
- Crowe J.H., 1970, Cryptobiosis in the tardigrade, *Macrobiotus areolatus*: structure and function of the cuticle [Thesis], University of California, 134 pp.
- Crowe J.H., 1972, Evaporative water loss by tardigrades under controlled relative humidities, Biol. Bull. 142(3): 407–416.
- Crowe J.H., 1975, The physiology of cryptobiosis in tardigrades., Mem. Ist. Ital. Idrobiol. 32(Supp.): 37–59.
- Crowe J.H., Newell I.M., Thomson W.W., 1970, *Echiniscus viridis* (Tardigrada): Fine Structure of the Cuticle, Trans. Am. Microsc. Soc. 89(2): 316–325.
- Crowe J.H., Newell I.M., Thomson W.W., 1971a, Cuticle formation in the tardigrade *Macrobiotus areolatus* Murray, J. Microsc. 11(1): 121–132.
- Crowe J.H., Newell I.M., Thomson W.W., 1971b, Fine structure and chemical composition of the cuticle of the tardigrade, *Macrobiotus areolatus* Murray, J. Microsc. 11(1): 107–120.
- Czerneková M., Jönsson K.I., 2016, Experimentally Induced Repeated Anhydrobiosis in the Eutardigrade *Richtersius coronifer*, PLoS One 11(11): e0164062.
- Czerneková M., Jönsson K.I., Chajec L., Student S., Poprawa I., 2017, The structure of the desiccated *Richtersius coronifer* (Richters, 1903), Protoplasma 254(3): 1367–1377.
- Dastych H., 1988, The Tardigrada of Poland, PWN, Warszawa-Kraków, 255 pp.
- Dastych H., Kraus H., Thaler K., 2003, Redescription and notes on the biology of the glacier tardigrade *Hypsibius klebelsbergi* MIHELČIČ, 1959 (Tardigrada), based on material from the Ötztal Alps, Austria, Mitt. Hamb. Zool. Mus. Inst. 100: 73–100.
- Degma P., Bertolani R., Guidetti R., 2020, Actual checklist of Tardigrada species (2009–2020, Ver. 39: 17–12–2020), 59 pp. Retrieved from <https://iris.unimore.it/retrieve/handle/11380/1178608/308574/%20Actual%20checklist%20of%20Tardigrada%20%2039th%20Edition%2017-12-20.pdf>
- Degma P., Guidetti R., 2007, Notes to the current checklist of Tardigrada, Zootaxa 1579: 41–53.
- Dewel R.A., Dewel W.C., 1979, Studies on the tardigrades. IV. Fine structure of the hindgut of *Milnesium tardigradum* Doyère, J. Morphol. 161(1): 79–109.
- Dewel R.A., Dewel W.C., Roush B.G., 1992, Unusual cuticle-associated organs in the heterotardigrade, *Echiniscus viridissimus*, J. Morphol. 212(2): 123–140.
- Dewel R.A., Nelson D.R., Dewel W.C., 1993, Tardigrada, [in:] Harrison F.W., Rice M.E. (eds), Microscopic Anatomy of Invertebrates. Vol. 12: Onychophora, Chilopoda, and Lesser Protostomata, Wiley-Liss, New York: 143–183.
- Förster F., Beisser D., Grohme M.A., Liang C., Mali B., Siegl A.M., Engelmann J.C., Shkumatov A. V., Schokraie E., Müller T., Schnölzer M., Schill R.O., Frohme M., Dandekar T., 2012, Transcriptome analysis in tardigrade species reveals specific molecular pathways for stress adaptations, Bioinform. Biol. Insights 6: 69–96.
- Gaur H., Perry R., 1991, The role of the moulted cuticles in the desiccation survival of adults of *Rotylenchus reniformis*, Rev. Nématol. 14(4): 491–496.
- Greven H., 1971, Zur morphologie der Tardigraden. Rasterelektronenmicroscopische Untersuchungen an *Macrobiotus hufelandi* und *Echiniscus testudo* (On the Morphology of Tardigrades. A Stereoscan Study of *Macrobiotus hufelandi* and *Echiniscus testudo*), Forma Funct. 4: 283–302 (in German, English abstract).
- Greven H., 1972, Vergleichende Untersuchungen am Integument von Hetero- und Eutardigraden (Comparative Studies on the Integument of Hetero- and Eutardigrada), Z. Zellforsch. 135: 517–538 (in German, English summary).
- Greven H., 1975, New results and considerations regarding the fine structure of the cuticle in tardigrades, Mem. Ist. Ital. Idrobiol. 32(Supp.): 113–131.
- Greven H., 1980, Die Bärtierchen (Water Bears), A. Ziemsen Verlag, Wittenberg Lutherstadt, 101 pp (in German).
- Greven H., 1982, Homologues or analogues? A survey of some structural patterns in tardigrada, [in:] Nelson D.R. (ed.), Proc. of the Third International Symposium on the Tardigrada, East Tennessee State University Press, Johnson City: 55–76.
- Greven H., 1984, Tardigrada, [in:] Bereiter-Hahn J., Matoltsy A.G., Richards K.S. (eds), Biology of the Integument: Invertebrates, Springer-Verlag, Berlin-Heidelberg: 714–727.
- Greven H., Dastych H., Kraus H., 2005, Notes on the integument of the glacier-dwelling tardigrade *Hypsibius klebelsbergi* Mihelčič, 1959 (Tardigrada), Mitt. Hamb. Zool. Mus. Inst. 102: 11–20.
- Greven H., Greven W., 1987, Observations on the permeability of the tardigrade cuticle using lead as an ionic tracer, [in:] Bertolani R. (ed.), Biology of Tardigrades. Vol. 1. Mucchi, Modena: 35–43.
- Greven H., Grohé G., 1975, Die Feinstruktur des Integumentes und der Muskelansatzstellen von *Echiniscoides sigismundi* (Heterotardigrada) (The fine structure of the integument and the muscle attachments in *Echiniscoides sigismundi* (Heterotardigrada)), Helgoländ. Wiss. Meer. 27(4): 450–460 (in German, English abstract).
- Greven H., Kaya M., Baran T., 2016, The presence of α -chitin in Tardigrada with comments on chitin in the Ecdysozoa, Zool. Anz. 264: 11–16.
- Greven H., Peters W., 1986, Localization of chitin in the cuticle of tardigrada using wheat germ agglutinin – Gold conjugate as a specific electron-dense marker, Tissue Cell 18(2): 297–304.
- Greven H., Robenek H., 1983, Freeze-fracture studies on tardigrade cuticle, Tissue Cell 15(2): 329–340.
- Gross V., Mayer G., 2015, Neural development in the tardigrade *Hypsibius dujardini* based on anti-acetylated α -tubulin immunolabeling, EvoDevo 6: 12.
- Gross V., Mayer G., 2019, Cellular morphology of leg musculature in the water bear *Hypsibius exemplaris* (Tardigrada) unravels serial homologies, R. Soc. Open Sci. 6(10): 191159.

- Guidetti R., 1998, Two new species of Macrobiotidae (Tardigrada, Eutardigrada) from the United States of America, and some taxonomic considerations of the genus *Murrayon*, *Proc. Biol. Soc. Washington* 111: 663–673.
- Guidetti R., Altiero T., Marchioro T., Sarzi Amadé L., Avdonina A.M., Bertolani R., Rebecchi L., 2012, Form and function of the feeding apparatus in Eutardigrada (Tardigrada), *Zoomorphology* 131: 127–148.
- Guidetti R., Bertolani R., 2005, Tardigrade taxonomy: an updated check list of the taxa and a list of characters for their identification, *Zootaxa* 845(1): 1.
- Guidetti R., Boschini D., Altiero T., Bertolani R., Rebecchi L., 2008, Diapause in tardigrades: a study of factors involved in encystment, *J. Exp. Biol.* 211(14): 2296–2302.
- Guidetti R., Boschini D., Rebecchi L., Bertolani R., 2006, Encystment Processes and the "Matrioshka-like Stage" in a Moss-dwelling and in a Limnic Species of Eutardigrades (Tardigrada), *Hydrobiologia* 558(1): 9–21.
- Guidetti R., Møbjerg N., 2018, Environmental Adaptations: Encystment and Cyclomorphosis, [in:] Schill R.O. (ed.), *Water Bears: The Biology of Tardigrades*, Springer, Cham: 249–271.
- Guidetti R., Rebecchi L., Bertolani R., 2000, Cuticle structure and systematics of the Macrobiotidae (Tardigrada, Eutardigrada), *Acta Zool.* 81: 27–36.
- Guidetti R., Rebecchi L., Bertolani R., Jönsson K.I., Kristensen R.M., Cesari M., 2016, Morphological and molecular analyses on *Richtersius* (Eutardigrada) diversity reveal its new systematic position and lead to the establishment of a new genus and a new family within Macrobiotidea, *Zool. J. Linn. Soc.* 178(4): 834–845.
- Hadley N.F., 1984, Cuticle: Ecological Significance, [in:] Bereiter-Hahn J., Matoltsy A.G., Richards K.S. (eds), *Biology of the Integument*, Springer, Berlin-Heidelberg: 685–693.
- Halberg K.A., Jørgensen A., Møbjerg N., 2013a, Desiccation tolerance in the tardigrade *Richtersius coronifer* relies on muscle mediated structural reorganization, *PLoS One* 8(12): e85091.
- Halberg K.A., Persson D., Ramløv H., Westh P., Kristensen R.M., Møbjerg N., 2009, Cyclomorphosis in Tardigrada: adaptation to environmental constraints, *J. Exp. Biol.* 212(17): 2803–2811.
- Halberg K.A., Persson D.K., Jørgensen A., Kristensen R.M., Møbjerg N., 2013b, Ecology and thermal tolerance of the marine tardigrade *Halobiotus crispae* (Eutardigrada: Isohypsibiidae), *Mar. Biol. Res.* 9(7): 716–724.
- Hallas T.E., Kristensen R.M., 1982, Two new species of the tidal genus *Echiniscoides* from Rhode Island, U.S.A. (Echiniscoideae, Heterotardigrada), [in:] *Proc. of the Third International Symposium on the Tardigrada*, Tennessee State University Press, Johnson City: 179–192.
- Hansen J.G., Katholm A.K., 2002, A study on the genus *Amphibolus* from Disko Island with special attention on the life cycle of *Amphibolus nebulosus* (Eutardigrada: Eohypsibiidae), *Arct. Biol. F. Course, Quqertarsuaq*: 129–163.
- Hashimoto T., Horikawa D.D., Saito Y., Kuwahara H., Kozuka-Hata H., Shin-I T., Minakuchi Y., Ohishi K., Motoyama A., Aizu T., Enomoto A., Kondo K., Tanaka S., Hara Y., Koshikawa S., Sagara H., Miura T., Yokobori S., Miyagawa K., Suzuki Y., Kubo T., Oyama M., Kohara Y., Fujiyama A., Arakawa K., Katayama T., Toyoda A., Kunieda T., 2016, Extremotolerant tardigrade genome and improved radiotolerance of human cultured cells by tardigrade-unique protein, *Nat. Commun.* 7: 12808.
- Hengherr S., Schill R.O., 2018, Environmental Adaptations: Cryobiosis, [in:] Schill R.O. (ed.), *Water Bears: The Biology of Tardigrades*, Springer, Cham: 295–310.
- Hesgrove C., Boothby T.C., 2020, The biology of tardigrade disordered proteins in extreme stress tolerance, *Cell Commun. Signal.* 18: 178.
- Horikawa D.D., Cumbers J., Sakakibara I., Rogoff D., Leuko S., Harnoto R., Arakawa K., Katayama T., Kunieda T., Toyoda A., Fujiyama A., Rothschild L.J., 2013, Analysis of DNA Repair and Protection in the Tardigrade *Ramazzottius varieornatus* and *Hypsibius dujardini* after Exposure to UVC Radiation, *PLoS One* 8(6): e64793.
- Hygum T.L., Clausen L.K.B., Halberg K.A., Jørgensen A., Møbjerg N., 2016, Tun formation is not a prerequisite for desiccation tolerance in the marine tidal tardigrade *Echiniscoides sigismundi*, *Zool. J. Linn. Soc.* 178(4): 907–911.
- Janelt K., Poprawa I., 2020, Analysis of Encystment, Excystment, and Cyst Structure in Freshwater Eutardigrade *Thulinus ruffoi* (Tardigrada, Isohypsibiidea: Doryphoribiidae), *Diversity* 12(2): 62.
- Jönsson K.I., 2019, Radiation Tolerance in Tardigrades: Current Knowledge and Potential Applications in Medicine, *Cancers (Basel)* 11(9): 1333.
- Jönsson K.I., Borsari S., Rebecchi L., 2001, Anhydrobiotic Survival in Populations of the Tardigrades *Richtersius coronifer* and *Ramazzottius oberhaeuseri* from Italy and Sweden, *Zool. Anz.* 240(3-4): 419–423.
- Jørgensen A., Kristensen R.M., Møbjerg N., 2018, Phylogeny and Integrative Taxonomy of Tardigrada, [in:] Schill R.O. (ed.), *Water Bears: The Biology of Tardigrades*, Springer, Cham: 95–114.
- Kinchin I.M., 1994, *The biology of tardigrades*, Portland Press, London, 186 pp.
- Kirke J., Jin X.-L., Zhang X.-H., 2020, Expression of a Tardigrade Dsup Gene Enhances Genome Protection in Plants, *Mol. Biotechnol.* 62(11-12): 563–571.
- Kristensen R.M., 1976, On the fine structure of *Batillipes noerrevangi* Kristensen 1976 1. Tegument and moulting cycle, *Zool. Anz.* 197: 120–150.
- Kristensen R.M., 1980, Zur Biologie des marinen Heterotardigraden *Tetrakentron synaptae* (On the biology of the marine heterotardigrade *Tetrakentron synaptae*), *Helgoländ. Meer.* 34(2): 165–177 (in German, English abstract).
- Kristensen R.M., 1982a, New Aberant Eutardigrades from Homothermic Springs on Disko Island, West Greenland, [in:] *Proc. of the Third International Symposium on the Tardigrada*, Tennessee State University Press, Johnson City: 203–220.

- Kristensen R.M., 1982b, The first record of cyclomorphosis in Tardigrada based on a new genus and species from Arctic meiobenthos, *J. Zool. Syst. Evol. Res.* 20(4): 249–270.
- Kristensen R.M., 1984, On the biology of *Wingstrand-arctus corallinus* nov. gen. et spec., with notes on the symbiotic bacteria in the subfamily Florarctinae (Arthrotardigrada), *Vidensk. Meddel. Naturhist. Foren.* 145: 201–218.
- Kristensen R.M., 1987, Generic revision of the Echiniscidae (Heterotardigrada), with a discussion of the origin of the family, [in:] Bertolani R. (ed), *Biology of Tardigrades*. Vol. 1. Mucchi, Modena: 261–335.
- Kristensen R.M., Neuhaus B., 1999, The ultrastructure of the tardigrade cuticle with special attention to marine species, *Zool. Anz.* 238: 261–281.
- Kristensen R.M., Sørensen M. V., 2004, Tardigrada, [in:] Schlager N. (ed), *Grzimek's Animal Life Encyclopedia*, Thomson-Gale, Detroit: 115–123.
- Krusberg L.R., 1967, Analyses of total lipids and fatty acids of plant-parasitic nematodes and host tissues, *Comp. Biochem. Physiol.* 21(1): 83–90.
- Li X.C., Wang L.Z., Yu D., 2007, The tardigrada fauna of China with descriptions of three new species of Echiniscidae, *Zool. Stud.* 46(2): 135–147.
- Madin K.A.C., Crowe J.H., 1975, Anhydrobiosis in nematodes: Carbohydrate and lipid metabolism during dehydration, *J. Exp. Zool.* 193: 335–342.
- Marchioro T., Rebecchi L., Cesari M., Hansen J.G., Viotti G., Guidetti R., 2013, Somatic musculature of Tardigrada: phylogenetic signal and metameric patterns, *Zool. J. Linn. Soc.* 169(3): 580–603.
- Marcus E., 1929, Tardigrada, [in:] Bronn H.G. (ed.), *H.G. Bronn's Klassen Und Ordnungen Des Tierreichs: Bd. 5. Arthropoda. Abt. 4. Arachnoidea. Tardigrada*, Akademische Verlagsgesellschaft, Leipzig (in German).
- McGreevy K.M., Heikes K.L., Kult S., Tharp M.E., Goldstein B., 2018, *Fluorescent Cell Staining Methods for Living Hypsibius exemplaris* Embryos, *Cold Spring Harb. Protoc.* 2018: pdb.prot106021.
- Morek W., Ciosek J.A., Michalczyk Ł., 2020, Description of *Milnesium pentapapillatum* sp. nov., with an amendment of the diagnosis of the order Apochela and abolition of the class Apotardigrada (Tardigrada), *Zool. Anz.* 288(4): 107–117.
- Morek W., Stec D., Gąsiorek P., Surmacz B., Michalczyk Ł., 2019, *Milnesium tardigradum* Doyère, 1840: The first integrative study of interpopulation variability in a tardigrade species, *J. Zool. Syst. Evol. Res.* 57: 1–23.
- Møbjerg N., Jørgensen A., Eibye-Jacobsen J., Halberg K.A., Persson D., Kristensen R.M., 2007, New records on cyclomorphosis in the marine eutardigrade *Halobiotus crispae* (Eutardigrada: Hypsibiidae), *J. Limnol.* 66(Suppl. 1): 132–140.
- Møbjerg N., Jørgensen A., Kristensen R.M., Neves R.C., 2018, Morphology and Functional Anatomy, [in:] Schill R.O. (ed.), *Water Bears: The Biology of Tardigrades*, Springer, Cham: 57–94.
- Nelson D.R., Bartels P.J., Guil N., 2018, Tardigrade Ecology, [in:] Schill R.O. (ed.), *Water Bears: The Biology of Tardigrades*, Springer, Cham: 163–210.
- Nelson D.R., Guidetti R., Rebecchi L., 2010, Tardigrada, [in:] Thorp J.H., Covich A. (eds), *Ecology and Classification of North American Freshwater Invertebrates*, Academic Press, New York: 455–484.
- Nelson D.R., Guidetti R., Rebecchi L., 2015, Phylum Tardigrada, [in:] Thorp J.H., Rogers D.C. (eds), *Ecology and general biology*. Thorp and Covich's freshwater invertebrates, Elsevier, Amsterdam: 347–380.
- Piszkievicz S., Gunn K.H., Warmuth O., Propst A., Mehta A., Nguyen K.H., Kuhlman E., Guseman A.J., Stadtmiller S.S., Boothby T.C., Neher S.B., Pielak G.J., 2019, Protecting activity of desiccated enzymes, *Protein Sci.* 28(5): 941–951.
- Raabe D., Sachs C., Romano P., 2005, The crustacean exoskeleton as an example of a structurally and mechanically graded biological nanocomposite material, *Acta Mater.* 53(15): 4281–4292.
- Ramazzotti G., Maucci W., 1983, Il phylum Tardigrada (The phylum Tardigrada), *Mem. Ist. Ital. Idrobiol. Dott. Marco Marchi* 41: 1014 (An English translation, C. Beasley).
- Rebecchi L., 2013, Dry up and survive: the role of antioxidant defences in anhydrobiotic organisms, *J. Limnol.* 72(s1): 62–72.
- Rebecchi L., Altiero T., Guidetti R., 2007, Anhydrobiosis: the extreme limit of desiccation tolerance, *Invertebr. Surviv. J.* 4(2): 65–81.
- Rebecchi L., Bertolani R., 1994, Maturative pattern of ovary and testis in eutardigrades of freshwater and terrestrial habitats, *Invertebr. Reprod. Dev.* 26(2): 107–117.
- Rebecchi L., Boschetti C., Nelson D.R., 2020, Extreme-tolerance mechanisms in meiofaunal organisms: a case study with tardigrades, rotifers and nematodes, *Hydrobiologia* 847(12): 2779–2799.
- Rebecchi L., Guidi A., Bertolani R., 2000, Maturative pattern of the ovotestis in two hermaphrodite species of eutardigrades, *Invertebr. Reprod. Dev.* 37(1): 25–34.
- Ricci C., Melone G., Santo N., Caprioli M., 2003, Morphological response of a bdelloid rotifer to desiccation, *J. Morphol.* 257(2): 246–253.
- Rocha A.M., Izaguirre M.F., Moly de Peluffo M.C., Peluffo J.R., Casco V.H., 2007, Ultrastructure of the cuticle of *Echiniscus rufoviridis* [Du Bois - Raymond Marcus, (1944) Heterotardigrada], *Acta Microsc.* 16(1-2): 16–21.
- Saini A., Kamboj P., Sharma P., Sharma I., 2020, Chitin, Chitinases and Chitin Derivatives in Biopharmaceutical, Agricultural and Environmental Perspective, *Biointerface Res. Appl. Chem.* 11(3): 9985–10005.
- Schill R.O., Fritz G.B., 2008, Desiccation tolerance in embryonic stages of the tardigrade, *J. Zool.* 276(1): 103–107.
- Schill R.O., Hengherr S., 2018, Environmental Adaptations: Desiccation Tolerance, [in:] Schill R.O. (ed.), *Water Bears: The Biology of Tardigrades*, Springer, Cham: 273–293.
- Schokraie E., Warnken U., Hotz-Wagenblatt A., Grohme M.A.A., Hengherr S., Förster F., Schill R.O., Frohme M., Dandekar T., Schnölzer M., 2012, Comparative proteome analysis of *Milnesium tardigradum* in early

- embryonic state versus adults in active and anhydrobiotic state, *PLoS One* 7(9): e45682.
- Shaw K., 1974, The fine structure of muscle cells and their attachments in the tardigrade *Macrobiotus hufelandi*, *Tissue Cell* 6(3): 431–445.
- Suma H.R., Prakash S., Eswarappa S.M., 2020, Naturally occurring fluorescence protects the eutardigrade *Paramacrobiotus* sp. from ultraviolet radiation, *Biol. Lett.* 16(10): 20200391.
- Szymańska B., 1995, Encystment in the tardigrade *Dactylobiotus dispar* (Murray, 1907) (Tardigrada: Eutardigrada). Part 1: Observation of living animals and structure of cyst, *Zool. Pol.* 40(1-4): 91–102.
- Tanaka S., Tanaka J., Miwa Y., Horikawa D.D., Katayama T., Arakawa K., Toyoda A., Kubo T., Kunieda T., 2015, Novel Mitochondria-Targeted Heat-Soluble Proteins Identified in the Anhydrobiotic Tardigrade Improve Osmotic Tolerance of Human Cells, *PLoS One* 10(2): e0118272.
- Tenlen J.R., 2018, Microinjection of dsRNA in Tardigrades, *Cold Spring Harb. Protoc.* 2018: pdb.prot102368.
- Tenlen J.R., McCaskill S., Goldstein B., 2013, RNA interference can be used to disrupt gene function in tardigrades, *Dev. Genes Evol.* 223(3): 171–181.
- Volkman A., Greven H., 1993, Ultrastructural localization of tyrosinase in the tardigrade cuticle, *Tissue Cell* 25(3): 435–438.
- Walz B., 1982, Molting in Tardigrada. A review including new results on cuticle formation in *Macrobiotus hufelandi*, [in:] Nelson D.R. (ed.), *Proc. of the Third International Symposium on the Tardigrada*, East Tennessee State University Press, Johnson City: 129–142.
- Welnicz W., Grohme M.A., Kaczmarek Ł., Schill R.O., Frohme M., 2011, Anhydrobiosis in tardigrades – The last decade, *J. Insect Physiol.* 57(5): 577–583.
- Westh P., Kristensen R.M., 1992, Ice formation in the freeze-tolerant eutardigrades *Adorybiotus coronifer* and *Amphibolus nebulosus* studied by differential scanning calorimetry, *Polar Biol.* 12: 693–699.
- Węglarska B., 1957, On the encystation in Tardigrada, *Zool. Pol.* 8(4): 315–324.
- Węglarska B., 1989, Ultrastructure of tardigrade *Dactylobiotus dispar* (Murray 1907) cuticle, *Acta Biol. Cracoviensia Ser. Zool.* 31: 57–62.
- Wharton D.A., Petrone L., Duncan A., McQuillan A.J., 2008, A surface lipid may control the permeability slump associated with entry into anhydrobiosis in the plant parasitic nematode *Ditylenchus dipsaci*, *J. Exp. Biol.* 211(18): 2901–2908.
- Womersley C., Thompson S.N., Smith L., 1982, Anhydrobiosis in Nematodes II: Carbohydrate and Lipid Analysis in Undesiccated and Desiccated Nematodes, *J. Nematol.* 14(2): 145–153.
- Wright J.C., 1988a, Structural correlates of permeability and tun formation in tardigrade cuticle: An image analysis study, *J. Ultrastruct. Mol. Struct. Res.* 101(1): 23–39.
- Wright J.C., 1988b, The tardigrade cuticle. I. Fine structure and the distribution of lipids, *Tissue Cell* 20(5): 745–758.
- Wright J.C., 1989a, The tardigrade cuticle II. Evidence for a dehydration-dependent permeability barrier in the intracuticle, *Tissue Cell* 21(2): 263–279.
- Wright J.C., 1989b, Desiccation Tolerance and Water-Retentive Mechanisms in Tardigrades, *J. Exp. Biol.* 142: 267–292.
- Wright J.C., Westh P., Ramløv H., 1992, Cryptobiosis in tardigrada, *Biol. Rev.* 67(1): 1–29.
- Yamaguchi A., Tanaka S., Yamaguchi S., Kuwahara H., Takamura C., Imajoh-Ohmi S., Horikawa D.D., Toyoda A., Katayama T., Arakawa K., Fujiyama A., Kubo T., Kunieda T., 2012, Two Novel Heat-Soluble Protein Families Abundantly Expressed in an Anhydrobiotic Tardigrade, *PLoS One* 7(8): e44209.
- Yoshida Y., Koutsovoulos G., Laetsch D.R., Stevens L., Kumar S., Horikawa D.D., Ishino K., Komine S., Kunieda T., Tomita M., Blaxter M., Arakawa K., 2017, Comparative genomics of the tardigrades *Hypsibius dujardini* and *Ramazzottius varieornatus*, *PLoS Biol.* 15(7): e2002266.
- Zhao D., Yu S., Sun B., Gao S., Guo S., Zhao K., 2018, Biomedical Applications of Chitosan and Its Derivative Nanoparticles, *Polymers (Basel)* 10(4): 462.