Univerzita Karlova

1. lékařská fakulta

Studijní program: P1516

Studijní obor: Biologie a patobiologie buňky

UNIVERZITA KARLOVA
1. lékařská fakulta

Zásobní buňky a jejich role ve fyziologii želvušek

STORAGE CELLS AND THEIR ROLE IN TARDIGRADE PHYSIOLOGY

Disertační práce/Doctoral thesis

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Praha, 2020

CHARLES UNIVERSITY IN PRAGUE

FIRST FACULTY OF MEDICINE

PHD DEGREE PROGRAMME: P1516

BIOLOGY AND PATHOBIOLOGY OF THE CELL

UNIVERZITA KARLOVA
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Prague, 2020

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CZERNEKOVÁ, Michaela. *Zásobní buňky a jejich role ve fyziologii želvušek. [Storage cells and their role in tardigrade physiology].* Ústí nad Labem, 2020. 68 stran, 5 příloh. Disertační práce. Univerzita Karlova, 1. lékařská fakulta, Klinika / Ústav 1. LF UK 2020. doc. RNDr. Petr Svoboda, DrSc. / prof. K.I. Jönsson.

Protokol o úplnosti náležitostí disertační práce

Titul, jméno, příjmení: Mgr. Michaela Czerneková

Název práce: Zásobní buňky a jejich role ve fyziologii želvušek

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STORAGE CELLS AND THEIR ROLE IN TARDIGRADE PHYSIOLOGY

Abstract

Tardigrades possess remarkable tolerance to numerous stress conditions (e.g. almost complete desiccation, exposure to very low sub-zero temperature, heat stress and even exposure to space in low Earth orbit). Indeed, they are among the most radiation-resistant multi-cellular organisms. The body cavity of tardigrades is filled with the storage cells (SC). Their role in anhydrobiosis has been discussed. The main objectives of this work were to analyse (i) the occurrence of mitosis in SC, (ii) the factors constraining anhydrobiotic survival, and (iii) the general ultrastructure of SC and their ultrastructure concerning the stress conditions. Our model species, *R*. cf. *coronifer* is one of the most extensively studied tardigrades concerning anhydrobiosis. Comprehensive histochemical techniques were used in combination with SEM, TEM, and confocal microscopy. First, mitotic divisions of tardigrade SC occur with a higher frequency in juveniles than in adults and correlate with animal growth. Mitosis is more frequent in moulting tardigrades, but the overall mitotic index is low. Furthermore, tardigrades of *R*. cf. *coronifer* can survive the maximum of 6 repeated desiccation cycles with significantly declining survival rate with repeated desiccations and significantly lower number of SC and more incorrectly formed tuns ("semi-tuns") after the fifth desiccation cycle. Tardigrades of *R*. cf. *coronifer* survive 6 months of desiccation. Heat stress, however, decreases the survival rate of desiccated tardigrades. Only a few ultrastructural changes were observed concerning to desiccation: (i) change in pigmentation in epidermal cells, (ii) overall cellular shrinkage, (iii) increments of heterochromatin in SC, (iv) change in density and contents of reserve material in SC, (v) partially loss of nucleoli. The SC of active specimens contain a large nucleus, distinct nucleolus, ribosomes, mitochondria, RER, GA, large autophagosomes. Lipids and polysaccharides are the main stored material in SC. Finally, two cell-types with different ultrastructure were defined in tardigrades of *R*. cf. *coronifer*: (i) type I cells are metabolically active and store nutrients in form of reserve spheres and type II cells that might represent undifferentiated stem-cell-like cells.

Key words: storage cells, coelomocytes, mitosis, tardigrades, anhydrobiosis, tun formation, cryptobiosis, Tardigrada, Richtersius coronifer

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“Where awareness goes, energy flows.”

Dandapani

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PREFACE

The main objectives of studies were to analyze (A) the occurrence of mitosis in storage cells (*section 3.1*), (B) the factors constraining anhydrobiotic survival (*section 3.2*), and (C) the ultrastructure of storage cells and their ultrastructure in relation to survival of stress conditions (*section 3.3*). The aim was to document the internal morphology and ultrastructure of tissues and cells under desiccation stress. My thesis primarily aimed to address several aspects of the storage cells (coelomocyte-type cells) of the phylum Tardigrada with regard to stress tolerance, and hereby provide new insights into the general cell biology and stress biology of this animal group. This was done by applying different microscopic techniques, such as scanning and transmission electron microscopy, fluorescent and confocal microscopy, histochemical and cytochemical techniques which offered qualitative as well as quantitative data on tardigrade cell biology, and stress related survival analyses. The experiments were carried out mainly at the Kristianstad University in Kristianstad, Sweden. Part of the work was performed at the Department of Animal Histology and Embryology, University of Silesia in Katowice, Poland.

My thesis consists of an introduction to the general biology of tardigrades: their morphology, ecology and classification, as well as the biology of storage cells and cryptobiosis with a focus on anhydrobiosis. All studies were performed on the tardigrade model species *Richtersius* cf. *coronifer* (Richters, 1903) described in the Materials and methods-section in detail. My thesis further provides an extensive study on mitosis occurence in tardigrade storage cells. It also brings new data on the ultrastructure of tardigrade tun formation and storage cells. It focuses on potential ultrastructural changes connected with tardigrade stress tolerance, especially desiccation and heat stress. An overview of the results is presented thematically based on four manuscripts that form the basis of this thesis. All publications are published in peer-reviewed journals with myself as the first author. One side stude in form of poster presentation is included as well. One additional manuscript was published during my Ph.D. studies, but is not included in the thesis.

This work was supported by Kristianstad University, Mobility Fund of the Charles University in Prague and Academy of Sciences of the Czech Republic.

Ústí nad Labem, the 22th of January 2020

Michaela Czerneková

LIST OF PUBLICATIONS

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Czernekova M, Jönsson KI (2016a). Mitosis in storage cells of the eutardigrade *Richtersius coronifer* - Zoological Journal of the Linnean Society 178 (4): 888 – 896.

II Czernekova M, Jönsson KI (2016b). Experimentally induced repeated anhydrobiosis in the eutardigrade *Richtersius coronifer* - PLoS ONE 11 (11): e0164062.

III Czerneková M, Jönsson KI, Chajec L, Student S, Poprawa I (2017). The structure of the desiccated *Richtersius coronifer* (Richters, 1903) - Protoplasma 254 (3): 1367-1377.

IV Czerneková M, Janelt K, Student S, Jönsson KI, Poprawa I (2018). A comparative ultrastructure study of storage cells in the eutardigrade *Richtersius coronifer* in the hydrated state and after desiccation and heating stress. PLoS ONE 13(8): e0201430.

1 INTRODUCTION

1.1 Tardigrades (phylum Tardigrada) as model organisms.

Tardigrades, commonly called water bears, are multicellular hydrophilous micrometazoans (0.1 – 1.2 mm) that belong to protostomes with close affinities to the euarthropod complex (Giribet et al., 1996; Aguinaldo et al., 1997; Halberg et al., 2009b). They are often described as a lesser-studied group as they have no impact on the human economy (Nelson, 2002; Guil & Cabrero-Sañudo, 2007). The first description of tardigrades was reported in notes of J. A. Goeze "Über den kleinen Wasserbären" ("About the small water bear", named due to their resemblance to a tiny bear), but the current name Tardigrada was given by the Italian naturalist Spallanzani in 1776 (Lat. tardus - slow, grado - walker).

Some of the tardigrade species live in oceans, but most of the ~1300 tardigrade species (Degma et al., 2019) occur in freshwater, terrestrial or semi-terrestrial environments from continental Antarctica to the icecap of Greenland, some of which are the most extreme natural habitats on the Earth (Kinchin, 1994; Sømme and Meier, 1995). They inhabit mostly mosses and lichens where they constitute a major component of the microfauna (Halberg et al., 2009b). Their active life proceeds only in the presence of a moist environment and water surrounding their bodies with a liquid layer (Ramazzotti and Maucci, 1983; Kinchin, 1994). However, tardigrades are fascinating organisms for biologists because they can withstand a variety of extreme stress conditions (see below) by entering an ametabolic state called cryptobiosis (Crowe and Cooper, 1971; Crowe, 1972; Wright et al., 1992). Once conditions become favourable, they reactivate metabolism and continue their life. They share this ability with several species from the phyla Rotifera, Nematoda and Arthropoda. Although the cryptobiotic ability of tardigrades has been known and investigated for quite a long time (Broca, 1860; Keilin, 1959; Wright et al., 1992; Wright, 2001), the molecular and cellular mechanisms of this ability, as well as its limits, are still poorly understood.

Tardigrades can survive almost complete desiccation (Westh and Ramløv, 1991). They can remain in a desiccated cryptobiotic state for as much as 20 years (Jørgensen et al., 2007) and be retrieved from a moss sample frozen for over 30 years (Tsujimoto et al., 2016). Furthermore, anhydrobiotic (desiccated) tardigrades have been reported to survive and tolerate multiple extreme environmental conditions, including fast cooling to sub-zero temperature (Hengherr et al., 2009b, 2010), -80°C for up to three decades without loss

of viability (Tsujimoto et al., 2016) and even the temperatures close to absolute zero (Ramløv and Westh, 1992; Guidetti et al., 2012). They can also survive temperatures as high as 70°C for 1h (Ramløv and Westh, 2001), high external osmotic pressure (Heidemann et al., 2016), treatments with alcohols of varying polarity (Ramløv and Westh, 2001) and biocide methyl bromide gas (Jönsson and Guidetti, 2001). Tardigrades can further resist low and high hydrostatic pressure (up to 600-1200 MPa; Ono et al., 2008; Horikawa et al., 2009), several thousand grey (Gy) of gamma irradiation (Jönsson et al., 2005; Horikawa et al., 2006), heavy ion irradiation in the form of ^{4}He (Horikawa et al., 2006), protons (Nilsson et al., 2010), alpha particles ^{4}H (Horikawa et al., 2012), iron ions and helium ions (Jönsson and Wojcik, 2017), high doses of UV radiation, including UV_A (Jönsson et al., 2008), UV_B (Altiero et al., 2011), and UV_C (Horikawa et al., 2013), and exposure to space in low Earth orbit (Jönsson et al., 2008; Persson et al., 2011). Although several hypotheses explaining tardigrade tolerance to environmental stress have been formulated, they have not been united in a comprehensive theory yet mainly because the underlying molecular and physiological mechanisms are largely unknown.

In general, cell biology of tardigrades have been a neglected field, even though a few ultrastructural studies have been conducted (Walz, 1973; Węglarska, 1975; Dewel et al., 1993; Avdonina et al., 2007; Persson et al., 2012; Hyra et al., 2016b). Tardigrades have sometimes been characterised as organisms with constant cell numbers in their adult lives (Gabriel et al., 2007; Guidetti et al., 2012; Wright, 2014), however, occasionally, some cell divisions were observed (Bertolani, 1970a, b; Gross et al., 2018). Conceivably, this assumption may have discouraged any attempts to culture tardigrade cells. As detailed above, tardigrades exhibit an impressive capacity to survive under the most hostile environmental conditions. Actually, they are among the most radiation-resistant multi-cellular organisms, not very far from the abilities of bacteria in terms of short-term post-irradiation survival (Jönsson and Guidetti, 2001; Schill et al., 2004; Jönsson et al., 2005; Horikawa et al., 2006; Halberg et al., 2009b; Altiero et al., 2011; Nilsson et al., 2013).

Research on tardigrades, therefore, has profound potential for the development of novel technologies in the field of radioprotection, cryopreservation, and preservation of dried biological material. Although possible applications of tardigrade research findings in the fields of medicine and health protection have been recognized (Keilin, 1959; Clegg, 2001), very few studies investigating the effects of various stressors on living organisms used tardigrades as model organisms (Hashimoto and Kunieda, 2017; Jönsson, 2019), especially in biomedical sciences (Guidetti et al., 2012). Nevertheless, several interesting studies have

emerged recently. Expression of tardigrade-specific proteins, such as RvLEAM (mitochondrial heat-soluble) and MAHS, in human cells (Hep-2) increased their tolerance to hyperosmotic stress (Tanaka et al., 2015). Another tardigrade-specific protein, Dsup, associating with DNA and protecting it from hydroxyl radicals (Hashimoto et al., 2016; Chavez et al., 2019) was able to reduce X-ray induced DNA damage by 40% when overexpressed in human embryonic kidney cells (HEK293) and it improved their post-irradiation viability.

Garcia-Arrarás and Dolmatov (2010) highlighted a paradox in biomedical science: "The range and accuracy of scientific techniques and tools available to explore the solutions to research questions are increasing, but meanwhile there is a smaller number of model organisms to apply these techniques". The "classical model systems" were represented mostly by organisms amenable to genetic manipulations, including Tobacco mosaic virus, *Escherichia coli* (Escherich, 1885), yeasts *Saccharomyces cerevisiae* (Meyen ex E.C. Hansen, 1883), and some invertebrates. Widely used invertebrate model organisms have been the multicellular nematode *Caenorhabditis elegans* (Maupas, 1899) and the fruit fly *Drosophila melanogaster* (Meigen, 1830) that serve as models for developmental biology, molecular or population genetics, and neuropharmacological research as well (Manev et al., 2003; Govind, 2011).

Many animal groups, including tardigrades, were, however, left behind, despite possessing special properties that may provide important insights into the systematic, organ, cellular and molecular bases of general physiology and pathophysiology. For instance, the earthworms have been traditionally used in Chinese medicine for thousands of years, but the research and development of biochemical technologies and pharmaceutical effects of earthworms only started just in the past few decades (Dinesh et al., 2013). Tardigrades have been proposed as model organisms for astrobiological research (Jönsson, 2007; Horikawa, 2008; Guidetti et al., 2012) and are becoming the model organisms for studies of invertebrate cryptobiosis (Jönsson et al., 2019). Encouraging report have been emerging also in other fields, such as food preservation (Colaço and Roser, 1994; Saragusty and Loi, 2019), cryopreservation of mammalian cells (Eroglu et al., 2000), preservation of macromolecules (Piszkiewicz et al., 2019), sperms, blood cells and tissues (Crowe, 1971; Clegg, 2001). The research potential of invertebrate model organisms was documented by recent investigations of invertebrates with coelomocyte-type cells regarding cancer. The studies on earthworms revealed the existence of coelomic fluid humoral proteins released from various coelomocytes with cytotoxic, antibacterial, agglutinating, proteolytic and mitogenic activities (Dinesh et al., 2013; Mácsik et al., 2015). It was shown that cytolytic

factors of coelomic fluid cause apoptosis of tumour target cells and HeLa cells *in vitro* (Mácsik et al., 2015; Augustin et al, 2017). Another example is demonstrated in the study of Homa (2018) who showed the function of earthworm coelomocytes in the production of extracellular traps that are structurally and functionally similar to those produced by human neutrophils. Coelomocyte-type cells occur also in other phyla, such as in nematodes, echinoderms, annelids, rotifers, and tardigrades (Tahseen, 2009).

Tardigrade coelomocyte-type cells are called storage cells (or body cavity cells) which occur inside the body cavity filled with coelomic fluid (Węglarska, 1975; Reuner et al., 2010a). Much of tardigrade physiology depends on these cells and they are assumed to play a role in cryptobiosis of tardigrades (Węglarska, 1975). Several studies showed a role of storage cells in starvation (diminishing reserves or size of storage cells after starvation; Węglarska, 1957) and anhydrobiosis (Jönsson and Rebecchi, 2002). Coelomocytes of other, less stress-tolerant, invertebrate groups were found to play an important role in cancer research (Dinesh et al., 2013; Mácsik et al., 2015). Recently, Buis et al. (2019) revealed the role of nematode coelomocytes in the regulation of starvation-induced fat catabolism.

Still, we know very little about the tardigrade storage cells´ ultrastructure and physiology. Because the properties of tardigrade cells are mostly unknown, and the primary cell culture has not been established yet, it makes any studies difficult and time-consuming. Moreover, the conditions of cell division and cell cycle, in general, remain puzzling (**Paper I**; Gross et al., 2018; Jönsson et al., 2019).

To introduce tardigrades as a model organism in cell biology research, it is necessary to know whether and under which conditions these cells do divide. **Paper I** provides an extensive evaluation of an occurrence of mitotic division in storage cells and analyses the possible connections between the occurrence of mitosis and the different phenotypic characteristics of the animal.

The stress tolerance of tardigrades with a specific focus on the storage cells was also investigated. To understand factors constraining dehydration tolerance in tardigrades, (1) their survival patterns under repeated cycles of desiccations/rehydration, (2) the potential effect of each desiccation cycle on morphometric traits, and (3) the storage cell divisions after each desiccation/rehydration cycle were researched in **Paper II**.

In paper III, the morphological and ultrastructural changes related to desiccation were studied through an analysis of the gross morphology and tissue organisation

of tardigrades during so-called tun formation. The ultrastructure of the body wall, ovary, midgut and storage cells was analyzed in desiccated samples of *R*. cf. *coronifer*, a species with pronounced anhydrobiotic abilities. This study includes the first analysis of cuticle organisation in moulting and non-moulting tardigrades in a desiccated state. A 3D reconstruction of the tun stage is presented.

The aims of **paper IV** were to (1) compare the ultrastructure of storage cells in active and desiccated specimens, and (2) evaluate the effect of temperature stress on tardigrade cells.

My thesis is divided into four chapters. First, I include a brief introduction to the phylum Tardigrada with general characteristics of the phylum and the storage cells. Description of model species used in all studies, the study area, data collection and analyses are included in *Material and method* section. *Results and discussion* section is ordered into three subdivisions: 1) *Mitosis in storage cells and eutely in tardigrades* (**Paper I** and **II**), 2) *Anhydrobiosis in R.* cf. *coronifer* (**Paper II**, **III**, and **IV**), 3) *Ultrastructure of storage cells* (**Paper III** and **IV**). The storage cells are the main focus of this thesis and of all presented manuscripts. The detailed methodology used in my studies can be found directly in my publications. each study is described in the separate publications, attached to this thesis. *Conclusions and future perspectives* section brings then the main conclusions of my work.

1.2 THE GENERAL BIOLOGY AND MORPHOLOGY OF TARDIGRADES

The bilaterally symmetrical bodies of tardigrades have five segments: a head and four trunk segments, each with a pair of lobopod legs (**Fig. 1A**), usually terminating with claws and/or sucking discs of varying number and shape. The total body length, excluding the 4th pair of legs, varies between 50 µm up to 1200 µm (Møbjerg et al., 2019).

Tardigrades are relatively complex invertebrates with well-developed musculature and nervous system, as well as a complex alimentary canal and specialized excretory and reproductive system (Rebecchi and Bertolani, 1994; Nelson et al., 2005; Halberg and Møbjerg, 2012). Possibly due to their miniaturisation, they lack respiratory and circulatory organs (Gross et al., 2019). The gas exchange occurs via diffusion across the epidermis and the thick cuticle (Nelson et al., 2015). The circulation is carried out via a large body cavity filled with coelomic fluid containing body cavity cells (storage cells) varying in numbers and size (**Fig. 1A**, **Paper I and IV**). Their specific function is discussed below. The body cavity sometimes contains reserved material (Ramazzotti and Maucci, 1983; Nelson, 2002). The cells are usually translucent or opaque, but some species exhibit colouration in cells of the gut, epidermis, cuticle, or in storage cells (Ramazzotti and Maucci, 1983).

The muscular system consists of dorsal, ventral and lateral somatic muscles arranged dorsoventrally or longitudinally, pharyngeal stylet and visceral muscles, circular muscles are, however, absent (Nelson et al., 2005; Halberg et al., 2009a, b; Marchioro et al., 2013; Møbjerg et al., 2018). The entire muscle consists of a single cell. While eutardigrades have longitudinal muscle strands in all muscle groups with several additional transverse muscles, heterotardigrades exhibit lower complexity in somatic muscles with a lower number of fibres and barely recognizable longitudinal strands (Marchioro et al., 2013). The cuticular and hydrostatic skeleton works as antagonists to muscle contraction.

Tardigrade digestive system consists of a foregut with buccal-pharyngeal apparatus and oesophagus lined with cuticle, a large midgut, and a short hindgut with cuticular lining (Nelson, 2002; Nelson et al., 2015). The hindgut is divided into an anterior part (rectum) and it is terminating in a cloaca (in eutardigrades) or anus (as in heterotardigrades reproductive and digestive system are separated) (Nelson et al., 2005). The midgut (consisting of ~40 cells) is lined with a cuticle and has a digestive function (Nelson et al., 2015; Gross et al., 2019). However, it is also involved in oogenesis (Hyra et al., 2016a). The hindgut is associated with osmoregulatory functions (Dewel and Dewel, 1979).

Excretion and osmoregulation are associated with the cuticle and/or Malpighian organs (three osmoregulatory glands, two lateral and one dorsal, in eutardigrades), or with ventral organs associated with the cuticle (in some heterotardigrades) (Dewel and Dewel, 1979; Møbjerg and Dahl, 1996; Nelson et al., 2015). Excretion occurs through the buccal glands at moulting by shedding cuticle with accumulated excretory granules together with the wall of midgut and via active transport over folded plasma membrane in the initial segment of the Malpighian tubules (in eutardigrades). Malpighian tubules are considered secretion-reabsorption “kidneys”; however, they can also serve as nutrient (glycogen or lipid) storage (Møbjerg and Dahl, 1996; Halberg et al., 2009b).

Morphology and ultrastructure of Malpighian organs reveal dissimilarities within tardigrades. The Malpighian tubules can be divided into three groups: (i) long monomorphous tubules in xerophilic species, e.g. *Milnesium tardigradum*, (ii) short monomorphous tubules in freshwater and semi-terrestrial species, e.g. *Isohypsibius granulifer* (Thulin, 1928) and (iii) dimorphous tubules, e.g. *Halobiotus crispae* (Kristensen, 1982; Møbjerg and Dahl, 1996). The tubules empty into the digestive tract in the transition zone of the midgut and rectum (with osmoregulatory function; Halberg et al., 2009b) and can be divided into an initial segment (3 cells or 6 cells in *M. tardigradum*), and a proximal and a distal part that is composed of the canal system. The tubules completely lack ciliary structures typical for proto- and meta-nephridia (Møbjerg and Dahl, 1996).

The central nervous system (containing 440 – 650 cells in total) consists of a dorsal tripartite brain (consisting of 200-370 neurons) and ventral nerve chain with four bilobed segmented ganglia (each containing 60 – 70 cells) linked by somata-free connectives (Mayer et al., 2013; Nelson et al., 2015; Gross et al., 2019). Many eutardigrades have a pair of eyespots (cup-shaped photoreceptors with pigment granules on the surface of the brain) composed of 3-5 cells per eye (ciliary and rhabdomeric receptor cells; Greven, 2007). Heterotardigrades have various external cephalic sensory organs, while eutardigrades have receptors under the cephalic cuticle. The gonads in females and hermaphrodites have one gonoduct, and two gonoducts in males. Gonoducts terminate internally in a cloaca in eutardigrades or externally in a gonopore in heterotardigrades (Nelson et al., 2015).

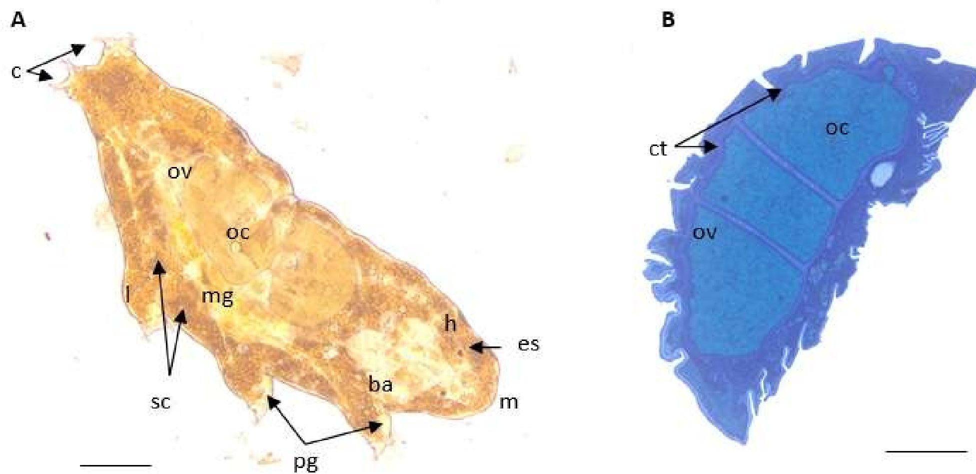

Figure 1. General morphology of the tardigrade *Richtersius* cf. *coronifer*.

(A) An adult specimen of *R.* cf. *coronifer*, light microscopy*:* the head (h) with mouth (m) and eye spots (es) and four trunk regions with four paired legs (l) ended with claws (c). Under the cuticle are the pedal glands (pg). The stylet of buccopharyngeal apparatus (ba) is not present due to moulting. The transparent cuticle allows observation of free storage cells (sc), filling the free space among the inner organs, such as ovaries (ov) with two large oocytes (oc) and midgut (mg), in the body cavity. Bar = 20 µm; **(B)** Tun formation of *R.* cf. *coronifer*, the longitudinal plane, histochemical staining (bromophenylblue), light microscopy: ov = ovary, ct = folded cuticle. Bar = 50 µm

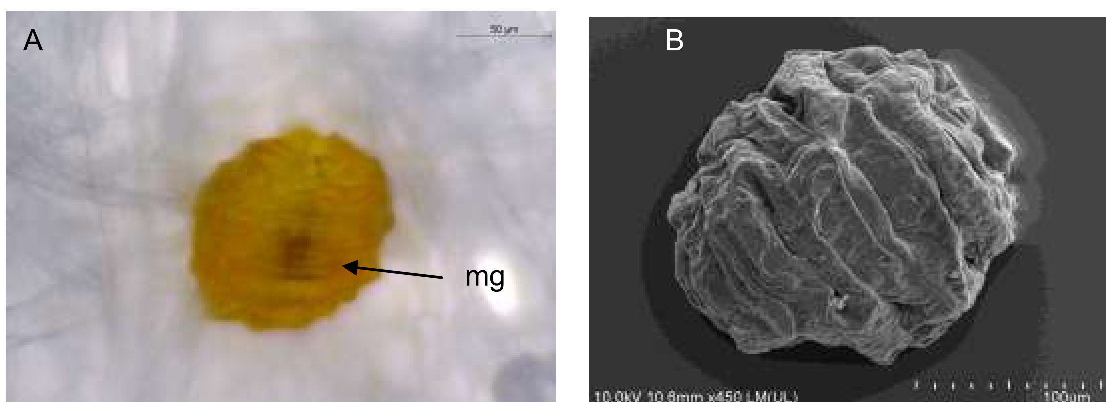

Figure 2. Tun formation of *R.* cf. *coronifer*. **(A)** A light microscopy of a tun, when the animal was desiccated on filter paper. The brownish midgut (mg) is visible through the cuticle. **(B)** Scanning electron microphotograph of a tun, with a dorsal view showing the cuticular folding.

1.3 TARDIGRADE PHYLOGENY AND EVOLUTION

Although tardigrades have been known to science since the 18th century, their phylogenetic status has been difficult to resolve (Giribet and Edgecombe, 2017; Jørgensen et al., 2018). Previously, tardigrades were considered a part of a group known as Aschelminthes (Crowe et al., 1970), however, their relation to arthropods was also recognised (Garey et al., 1996). Currently, tardigrades constitute a separate phylum Tardigrada, which belong to Protostomia, specifically, to one of its subgroups, to the monophyletic clade Ecdysozoa (Aguinaldo et al., 1997). All ecdysozoans have the moulting of their exoskeleton (the cuticle) in common. The clade Ecdysozoa (**Fig. 3**) includes Cycloneuralia with a ring-like brain (the phyla Nematomorpha, Nematoda, Priapulida, Khinorhyncha, Loricifera) and Panarthropoda with a ganglionic brain (the phyla Euarthropoda, Onychophora and Tardigrada; Mallatt and Giribet, 2006; Telford et al., 2008; Edgecombe, 2010; Smith et al., 2017). Based on 18S mRNA, tardigrades are a sister group of Euarthropoda. According to Dunn et al. (2008) and Yoshida et al. (2017), tardigrades are closely related to nematodes.

Molecular and morphological studies influenced the current taxonomy identifying the three classes: (i) Heterotardigrada (orders Arthrotardigrada and Echiniscoidea), (ii) Eutardigrada (orders Parachela and Apochela) shown in **Fig. 3** and **4**. and (iii) Mesotardigrada. This sorting is based on taxonomy of claws, cuticle, cephalic appendages, buccal apparatus and reproductive structures (Ramazzotti et Maucci, 1983; Nelson, 2002; Sands et al., 2008; Jørgensen et al., 2018). The class Heterotardigrada has both marine and limnoterrestrial representatives (order Arthrotardigrada and family Echiniscoidea), while the class Eutardigrada with few exceptions is represented by limnoterrestrial or freshwater species. The class Mesotardigrada is considered dubious, represented by only one species, *Thermozodium esakii* (Rahm, 1937) that was reportedly found in a Japanese thermal spring and which has never been found again despite recent efforts (Grothman et al., 2017).

The tardigrades´ taxonomy relies upon the analysis of morphological characteristics, bucco-pharyngeal apparatus, cuticular ornamentation, claws, and egg morphology (Bertolani, 2001; Nichols et al., 2006). The actual checklist is made available by Degma et al. (2019).

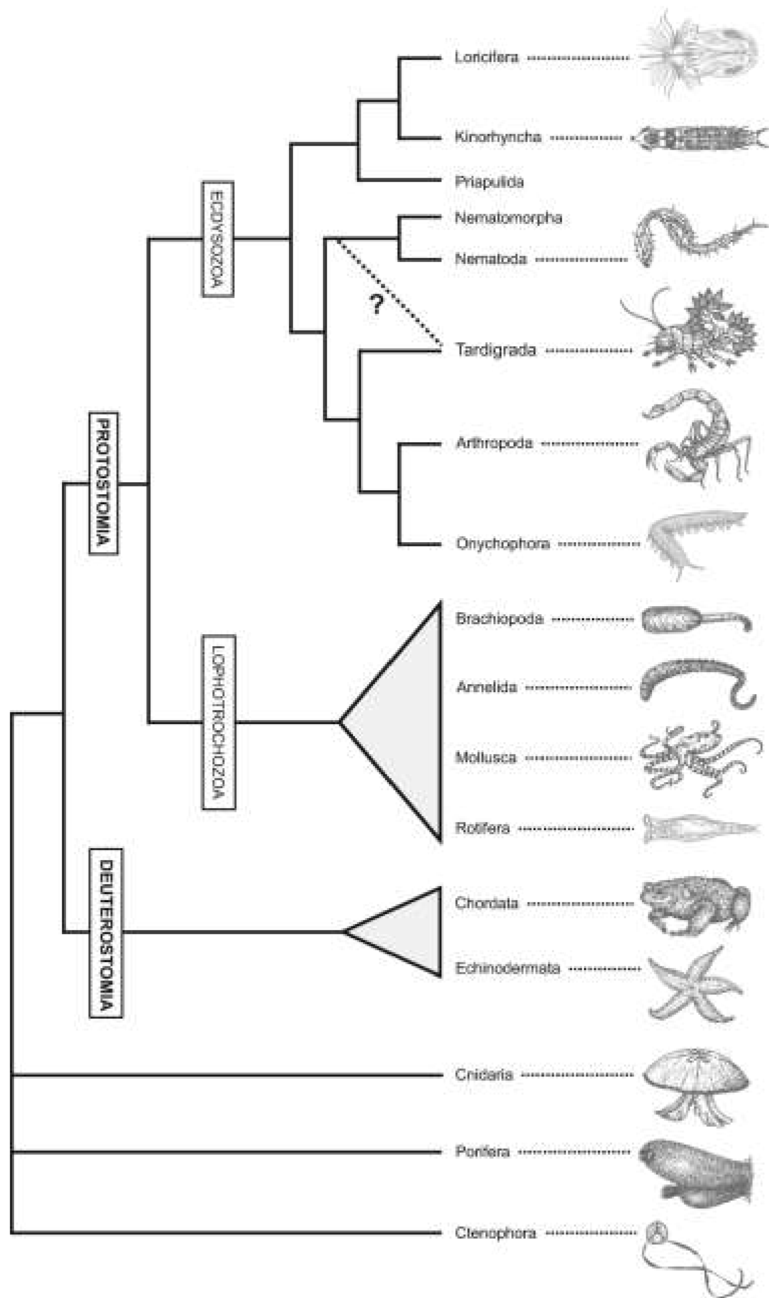

Figure 3. Phylogenetic position of Tardigrada. Reprinted from Jørgensen et al. (2018).

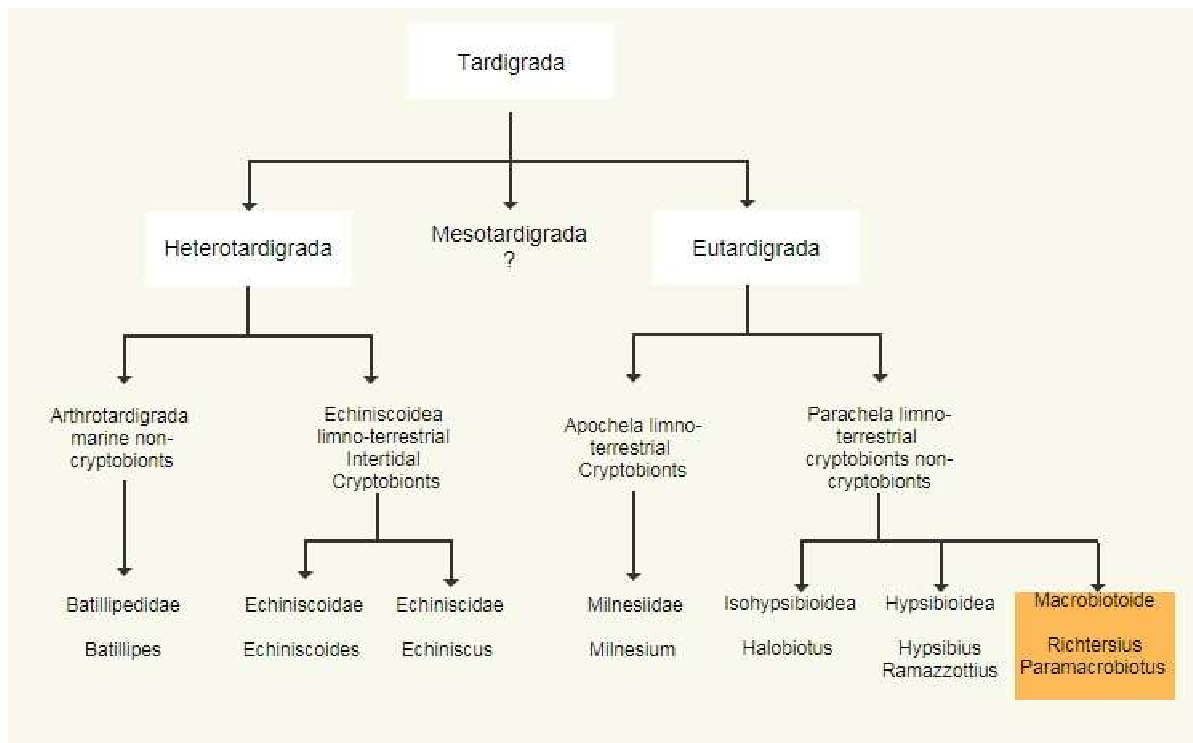

Figure 4. Tardigrade phylogeny. Major clades and position of model species, including *R.* cf. *coronifer* (adapted from Møbjerg et al., 2011).

1.4 GENOME AND GENETICS

Although the first data about tardigrade chromosomes derived from histological sections by Henneke (1911) and von Wenck (1914), the first precise karyotype of a tardigrade species was obtained by Ammermann (1967), who reported a diploid number of ten chromosomes in *Hypsibius exemplaris* (previously *H. dujardini* Doyère, 1840; Gąsiorek et al., 2018). Karyological studies were later performed mainly on oocytes, which have larger chromosomes compared to those observed during mitosis in gametogonia or somatic cells. Studies on Feulgen-DNA content and genome size confirmed the presence of diploid and polyploid populations and the presence of nuclei with varying amounts of DNA arising from endoreplication (Rebecchi and Bertolani et al., 1994; Jönsson et al., 2019). In diploid strains, the haploid chromosome number is 5 or 6. Polyploid (often triploid but also tetraploid) populations were also observed in some species, mostly in eutardigrades. Triploidy (17-18 chromosomes in total) was detected in females of parthenogenetic species of genera *Paramacrobiotus, Macrobiotus, Xerobiotus, Pseudobiotus, Eremobiotus,* and *Ramazzottius*

(Bertolani and Rebecchi, 2018). The chromosomes described so far have been acrocentric. Crossing-over was described only in female oocytes and not in spermatocytes (Altiero and Rebecchi, 2003). Diploid populations of tardigrades have meiotic (apomictic) parthenogenesis with the pairing of homologous chromosomes.

The genome size of tardigrades is relatively small and ranges from 55 Megabases (Mb) up to 800 Mb (Møbjerg et al., 2011; Yoshida et al., 2017). Feulgen-DNA content of spermatozoa ranges from 0.08 pg tp 0.73 pg (Bertolani and Rebecchi, 2018) that is among the lowest in invertebrates (Bracken-Grissom et al., 2014). Estimated genome size for model organisms *Caenorhabditis elegans* or *Drosophila melanogaster* is 100.4 Mb (Fierst et al., 2015) and 175 Mb (Hjelmen et al., 2019).

Several omics studies contributed to our understanding of genetics, and thus physiology, phylogeny and evolution of tardigrades (Mali et al., 2010; Levin et al., 2016, Hashimoto et al., 2016; Boothby et al. 2017; Yoshida er al., 2017). Precise genome analysis revealed investments in stress-specific adaptations, such as protein, DNA and redox-protection, maintenance and protein recycling (Förster et al., 2009). An excellent tardigrade proteome map was published by Schokraie et al. (2010). Tenlen et al. (2013) performed pioneering experiments suggesting the applicability of RNA interference (RNAi) in species *H. exemplaris* using microinjection of several dsRNA into its intestines or gonads. Although the reduction of intracellular levels of target mRNA and encoded proteins were not verified, the phenotypic analysis indicated that using RNAi in tardigrades is viable. The same method was later used to study the role of tardigrade-specific internally disordered proteins in desiccation tolerance of *H. exemplaris* (Boothby et al., 2017).

1.5 CRYPTOBIOSIS

Organisms living in extreme habitats have developed specific adaptations to environmental stressors. The most widespread adaptation is dormancy, i.e. any form of a resting stage (temporary decrease of activity, temporary cessation of growth and reproduction, reduced or suspended metabolism, developmental standstill). Dormancy includes two forms: diapause and quiescence. Both types of dormancy are associated with the low but still measurable metabolic activity (hypo-metabolism), albeit with temporary cessation of growth, development and animal activity (Lees, 1955; Neumann, 2006; Rebecchi et al., 2019). Diapause is endogenously and centrally mediated temporary interruption

of activity, which may persist even after environmental conditions return to favourable range and is related to seasonal alterations between favourable and unfavourable conditions (e.g. photoperiod) (Womersley, 1981; Watanabe, 2006; Rebecchi et al., 2007; Record et al., 2018). Quiescence is a decrease of metabolic activity under exogenous control, induced directly by environmental stressors, which is immediately reversed by removal of external stressors (Rebecchi et al., 2007). Dormancy is well documented in the invertebrates, plants and microbes of extreme environments (tolerance to freezing or drying; Lees, 1955; Guidetti et al., 2011; Košťál, 2011). However, some organisms can enter an extreme form of quiescence called cryptobiosis reducing their metabolism to undetectable levels (Rebecchi et al., 2007). The concept of cryptobiosis (or "hidden life") was introduced by David Keilin in 1959. Keilin (1959) defined cryptobiosis as "...the state of an organism when it shows no visible signs of life and when its metabolic activity becomes hardly measurable or comes reversibly standstill" (pp. 166). Consequently, during this latent state metabolism, growth, reproduction, repair and senescence are reduced or temporarily ceased, and therefore the larval, pupal, and adult stages are arrested. As metabolism is a defining characteristic of life, cryptobiosis is sometimes considered a third state between life and death (Clegg, 2001) or even "temporary death" or "potentially reversible death" (Neuman, 2006).

Cryptobiosis can be induced by several environmental factors, e.g. by lack of water (anhydrobiosis), low temperature (cryobiosis), high temperature (thermobiosis), lack of oxygen (anoxybiosis), high osmolarity (osmobiosis), starvation or a combination of above factors (Crowe, 1971; Walz, 1979; Clegg, 2001; Gutiérrez et al., 2001; Watanabe, 2006; Rebecchi et al., 2007). Desiccation induced anhydrobiosis and freeze-induced cryobiosis are the most extensively studied states. The anhydrobiosis and cryobiosis are, however, not equivalent states and likely involve different mechanisms for protecting the cells and tissues (Crowe et al., 1992).

Anhydrobiosis in tardigrades is connected to a special morphological adaptation that includes the formation of a barrel-shaped structure ("tun"). The animal transition to a tun by actively contracting its body anterior-posteriorly and by withdrawing its legs and head (Rebecchi et al., 2019). Successful maintenance of morphological tun state (**Figs. 1B, 2A, B, 5A**) in anhydrobiosis does not require interactions between the cells (Halberg et al., 2013). It produces a new spatial organization of some internal organs (**Paper III**). Therefore, mechanical injuries are tolerated in the state of cryptobiosis.

Only tissues and organisms in the cryptobiotic stage can tolerate very low and very high temperatures (over 100°C), or sojourn in liquid helium (Hinton, 1960; Neves et al., 2020). It is known that cryptobiosis evolved several times during evolution, because it is present in unicellular organisms (bacteria, protists; Potts, 2005), among mosses, lichens, liverworts, plants (Cannone et al., 2017) and their seeds (Alpert, 2000, 2005), but also in metazoans (nematodes, rotifers, tardigrades, insects, crustaceans; Alpert, 2005; Rebecchi et al., 2019). Still, only some species among invertebrates, and no vertebrates, have evolved this capability (Wright et al., 1992). Biochemistry and physiology of cryptobiosis allowing survival in a state of complete metabolic shut-down remain poorly understood (Watanabe et al., 2002; Schill et al., 2004) and our current knowledge is based on the research of few model systems, mainly *Artemia* cysts, corn embryos, and nematodes (Wright, 2001). Although some attention has been paid to anhydrobiosis in tardigrades (Wright et al., 1992; Rebecchi et al., 2007; Møbjerg et al., 2011), there is still not much known about the induction and reactivation phases of cryptobiosis (Westh et al., 1991; Westh and Kristensen, 1992; Halberg et al., 2009a; Hengherr et al., 2010).

Mechanisms underlying the ability to enter cryptobiosis have attracted considerable scientific interest for decades (Fry, 1966; Crowe, 1971). In addition, more extreme weather conditions around the globe including rising temperatures, severe droughts and generally higher weather variability put desiccation-tolerant organisms in a position of increased interest. Drought is currently among the main threats to the world´s food security. It may reduce maize yield by approximately 15%, soybean yield by approximately 40% (Clement et al., 2008). Singh et al. (2015) pointed out how an understanding of the principles of anhydrobiosis and programmed cell death may possibly help the development of drought-resistant crop plants that could solve the global food security problem. Recently, many countries and international organizations launched projects on exploring the drought tolerance and water-saving mechanisms of plants to identify key genes that could improve drought tolerance (Lawlor, 2013). Tardigrade cryptobiosis, therefore, might bring new insights and possible solutions to the above problems.

In general, cryptobiosis can be divided into three phases: (i) the induction phase detecting and reaching upon stress stimuli, (ii) the inactive dormant phase and (iii) the reactivation phase (Wright, 2001; Carlsson et al., 2008). For a successful survival, the whole process must involve production of bioprotectants that either prevent the cellular damage during the action of stress stimulus/stimuli or can repair the accumulated damage after a return to normal conditions and reactivation of metabolism (Crowe, 2002; Jönsson and Schill,

2007; Carlsson et al., 2008; Neumann et al., 2009; Neves et al., 2020). Of note, both pathways could act in parallel and are not mutually exclusive (Förster et al., 2009).

Organisms with cryptobiotic activities can be divided into two groups. The first group contains those that possess cryptobiotic ability only during their early stages of development. This group includes bacterial and fungal spores, pollen, seeds of vascular/seed plants, certain insect larvae, thick-shelled “winter eggs” of shrimp (*Artemia*) and species from the taxa Arthropoda, Crustacea, Brachiopoda and Insecta. The second category includes those organisms that may enter cryptobiosis over entire life cycle (also repeatedly during their life histories, **Paper II**) and includes a number of species from Bacteria, Protozoa, and three groups of invertebrates (the members of phyla Rotifera, Nematoda, and Tardigrada), various species of mosses, lichens and algae, as well as vascular/seed plants (Crowe, 1971; Crowe and Cooper, 1971; Wright et a., 1992; Jönsson and Järemo, 2003; Watanabe, 2006).

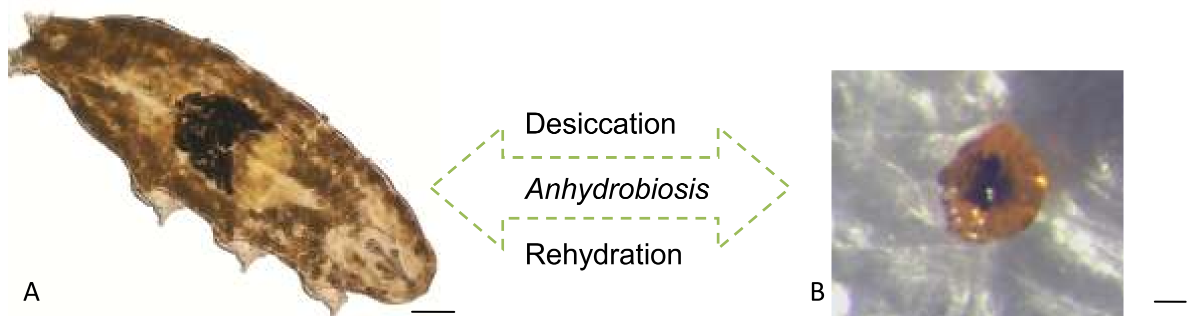

Figure 5. Anhydrobiosis of *R.* cf. *coronifer*. Reversible morphological changes between **(A)** hydrated and fully extended tardigrade and **(B)** anhydrobiotic, so called tun, formed in response to desiccation. Light microscopy, bar = 100 µm.

1.6 THE STORAGE CELLS

The body cavity of tardigrades is filled with body cavity lymph containing free body cavity cells, which are coelomocyte-type cells, also called the storage cells or bodies, coelomocytes, and Speicher cells (Węglarska, 1975; Dewel et al, 1993; Reuner, 2010a). In active animals, the cells move passively in the lymph and fill the empty spaces between organs such as gonad, gut and nerve chord, although they seem to temporarily adhere to the basement membrane of other tissues (Węglarska, 1975; Dewel et al., 1993).

The coelomocytes (Gr. koilos, hollow; kytos, container) are omnipresent in most of the coelomates. They play a key role in defence response in many invertebrates (Tahseen, 2009). Generally, they are classified based on differential staining, ultrastructure, behaviour and granule composition, but the classifications are inconsistent (Kauschke et al., 2001; Adamowicz, 2005). For instance, the older literature distinguishes mucocytes, vacuolocytes and lymphocytes as well as large and small granulocytes in earthworms (Kale and Krishnamoorthy, 1979). However, hyaline- and granular amoebocytes, both with phagocytic activity, and eleocytes (free-floating chloragocytes) without phagocytic activity, but producing bioactive molecules, were identified (Cooper et al., 1995). The latter types correspond to the three groups of coelomocytes observed using the flow cytometry (Mácsik et al., 2015). Other authors reported basophils with eccentrically located nuclei, acidophils with red-orange granules, neutrophils with large centrally located nuclei and modified peritoneal cells around intestines called chloragocytes and granulocytes (Joris, 2000; Kaushke et al., 2001; Calisi et al., 2009).

Coelomocyte-type cells, called hemocytes, produce humoral proteins and recognize the foreign particles. They are mediators of cell-mediated (innate) immunity in insects. Their basic functions are adhesion to foreign particles (nodulation and encapsulation), transportation of cuticular particles, and mainly phagocytosis of bacteria, yeast and apoptotic bodies. Also, they contain phenoloxidase precursors (Lavine and Strand, 2002). They are usually classified into prohemocytes, granulocytes, plasmocytes, spherule cells and oenocytes. However, this classification is not united as well (Lavine and Strand, 2002).

Nematodes, that are phylogenetically close to tardigrades, have highly specialized coelomocytes. They occur in fixed positions, adjacent to the gonads or the other internal organs in the body cavity. They vary in size, form (ovoid or with many branches) and number (Tahseen, 2009). The nematode coelomocytes should have a similar function as tardigrade storage cells. The nematode coelomocytes provide the turgor-hydrostatic pressure

for the animal, lubricate space among the tissues and are involved in intercellular signalling and nutrient transport. They also may store a vitamin B12, synthesize proteins and secrets (Basyoni and Rizk, 2016). Early workers identified two to six cells located in the anterior third of the nematode pseudocoelom (Bolla et al., 1972). In parasitic nematodes, coelomocytes become giant cells during the parasitic phase of life, whereas in free living species, these cells remain relatively small (Bolla et al., 1972). The cells were termed as coelomocytes, stellate cells, pseudocoelocytes (Bolla et al., 1972) and amoebocytes, elaeocytes, athrocytes and phagocytes (Tahseen, 2009). Due to their small number and size, these cells were largely ignored in nematodes (Tahseen, 2009). In tardigrades, the only known coelomocyte-type cells are storage cells.

Tardigrade storage cells are responsible for important physiological functions and are highly metabolically active (Ramazzotti and Maucci, 1983; Węglarska, 1957; Poprawa, 2006; Hyra et al., 2016a, b). Although their shape and content may vary, whether they may or may not be classified into specific groups has not been resolved yet. Our knowledge about these cells comes only from studies performed on individual specimens and therefore very little is known to classify these cells.

The main known function of storage cells is to synthesize, accumulate and transport nutrients and store reserve material (Węglarska, 1957, 1975; Rosati, 1968; Ramazzotti and Maucci, 1983; Szymańska, 1994; Poprawa, 2006; Reuner et al., 2010a; Hyra et al., 2016a; Jönsson et al., 2019). In some species, the cells are involved in specific functions and are considered to be a kind of a separate adipose tissue. They facilitate lipid and protein transport to developing oocytes and synthesize yolk precursors (e.g. in *Paramacrobiotus richtersi* Murray, 1911 and *Dactylobiotus dispar* Murray, 1907; Szymańska, 1994; Poprawa, 2006; Rost-Roszkowska et al., 2011; Hyra et al., 2016a) and vitellogenins (in *Macrobiotus polonicus* Pilato, Kaczmarek, Michalczyk and Lisi, 2003, *Isohypsibius granulifer granulifer* Thulin, 1928 and *Xerobiotus pseudohufelandi* Iharos, 1966*)*. Similarly to fat bodies in insects, the storage cells store nutrients, which are used (in some species) during periods of starvation (Reuner et al., 2010a) and transport them to the ovary where the nutrients are endocytosed (micropinocytosis; Poprawa, 2005; Hyra et al., 2016a, b). The storage cells are also active in phagocytosis (Kinchin, 1994) and have tyrosinase activity in the cytoplasm, perhaps related to immunological defense functions correlating to hemocytes in insects (Volkman and Greven, 1993; Greven, 1993). In species of *R.* cf*. coronifer* (Richters, 1903) and *Milnesium tardigradum* (Doyère, 1840), the cells serve as a source of energy for entering to and returning from cryptobiosis, especially anhydrobiosis (in species of *M. tardigradum*, in *R.*

cf. *coronifer* it is discussed below in section 3.2.1; Węglarska, 1975; Jönsson and Rebecchi, 2002; Reuner et al., 2010a). However, results presented in **Paper II** did not confirm this for storage cells of *R.* cf. *coronifer*. Nutrients can be released from storage cells during periods of tardigrade starvation and/or used as the precursors of vitellogenins in some species (e.g. *H. exemplaris*; Hyra et al., 2006b).

The storage cells have usually an amoeboid shape, but they differ in terms of morphology, shape, size and number across species (Szymańska, 1994, 1995; Poprawa, 2006; Hyra et al., 2016a, b). Nutrients availability, environmental conditions and especially synthesis, storage and transport of nutrients and yolk precursors to ovaries during oogenetic stages, mainly during time when animals do not intake food, have an impact on storage cell ultrastructure and/or numbers (Szymańska, 1994, 1995; Poprawa, 2006; Reuner et al., 2010a; Hyra et al., 2016a, b). In general, the size of storage cells is 10 to 15 µm (with a minimum of 5 and a maximum of 26 µm). During vitellogenesis, the cells are the largest and after the egg being laid, they are usually the smallest (Szymańska, 1994, 1995; Poprawa, 2006; Reuner et al., 2010a; Hyra et al., 2016a). Small tardigrade species have a higher number of smaller storage cells, whereas, the larger species have a lower number of storage cells that are bigger. The number of storage cells is discussed in the *Results and discussion* section (chapter 3.1, p34). The size of storage cells in some species, e.g *R.* cf. *coronifer,* affects the tardigrade survival after return from anhydrobiosis (Jönsson and Rebecchi, 2002), whereas in other species it seems to be storage cells independent (Reuner et al., 2010a).

1.6.1 General ultrastructure of storage cells

Storage cells have large irregular polyploid nuclei with the nucleolus, well developed rough endoplasmic reticulum (RER), Golgi complex, numerous mitochondria, ribosomes, lysosomes and spheres with reserve material. The main structure in storage cells is the large autolysosome (Węglarska, 1975) and spheres of various electron densities (Poprawa, 2006).

Histochemical analyses revealed that the main nutrients stored in storage cells could be lipid droplets (e.g. in *R.* cf. *coronifer*; **Paper III** and **IV**), polysaccharides and glycerol (*I. g. granulifer*; Hyra et al., 2016b), while in some other species (*M. richtersi, D. dispar*) the major nutrients are proteins and also granular glycogen occurring in the whole-cell cytoplasm or possibly in cytosomes (Szymańska, 1995; Poprawa, 2006; Hyra et al., 2016b). Animals utilize the reserve material (the polysaccharides being utilized first, followed by lipids) from

storage cells during encystment. The resorption of whole cells was observed (Węglarska, 1975). A particular combination of stored substances is species-dependent, and this variability is likely caused by tardigrade habitat (freshwater versus limno-terrestrial) and/or cryptobiotic ability of the species. For example, lipids are the main component of storage cells in *H. exemplaris* (freshwater and moss dwelling species, feeding on algae), *M. polonicus* (moss dwelling tardigrade, feeding on algae and/or rotifers) and *X. pseudohufelandi* (dry terrestrial, moss dwelling tardigrade, food not known), while they play only the minor role of the storage cells in *I. granulifer granulifer* (freshwater species, food not known) (Hyra et al., 2016b). The specimens of *X. pseudohufelandi* contain large amounts of proteins in storage cells in addition to lipids. Large amounts of lipids in storage cells are connected to species living in dry habitats and to the ability of cryptobiosis (Węglarska, 1975). For instance, *I. g. granulifer* has no lipids in storage cells and no cryptobiotic ability (Hyra et al., 2016b).

2 MATERIALS AND METHODS

2.1 Model species: *Richtersius* cf. *coronifer*

The model species of all studies *R.* cf. *coronifer* (Eutardigrada, Macrobiotidae) is a limno-terrestrial herbivorous tardigrade, with documented high ability to tolerate extensive desiccation (Jönsson and Rebecchi, 2002; Jönsson et al., 2005, 2008). It is one of the most extensively studied tardigrades with respect to anhydrobiosis (Westh and Ramløv, 1991; Jönsson and Guidetti, 2001; Jönsson et al., 2001, 2005; Ramløv and Westh, 2001; Ivarsson and Jönsson, 2004). Likewise, it has been subject to studies on tolerance to other environmental stress agents, including high temperatures (surviving up to +70°C for 1 h in the anhydrobiotic state; Ramløv and Westh, 2001), and low temperatures (survival of -196°C in the hydrated state; Ramløv and Westh, 1992).

Dehydrated specimens have a remarkable ability to tolerate low linear energy transfer (LET) radiation (gamma rays; Jönsson et al., 2005), high-LET irradiation with 2.55 MeV protons at doses up to 10 000 Gy (Nilsson et al., 2010), ultra-violet (UV) irradiation (280-400nm) and exposure to space vacuum and solar/galactic radiation (Jönsson et al., 2008). Moreover, its desiccated eggs can tolerate even the most extreme desiccation, at residual water levels well below one mass per cent (Jönsson et al., 2008). Specimens of *R.* cf. *coronifer* also withstand exposures to different external salinities (Halberg et al., 2009b).

Tardigrades generally contain relatively low amounts of trehalose when in an anhydrobiotic state (2.3% of the dry body mass; Westh and Ramløv, 1991) compared to other anhydrobiotic animals (e.g. cysts of desiccated brine shrimps *Artemia salina* = 13-20% of the dry body mass; Clegg, 1965; Watanabe et al., 2002; and larvae of *P. vanderplanki* = 20% of the dry body mass; Sakurai et al., 2008). Among the tardigrade species, specimens of our model species contain one of the highest amounts of trehalose in anhydrobiotic state (the highest trehalose levels were found in *Macrobiotus islandicus* = 2.9%; Jönsson and Persson, 2010). Besides the remarkable stress tolerance, tardigrades of this species also belong to the largest measuring sometimes up to 1000 µm.

2.2 Study population

The species of *R.* cf. *coronifer* mainly inhabit mosses of the Arctic and Alpine regions (found at altitudes of more than 500 to 1000 meters; Ramazzotti and Maucci, 1983; Westh

and Kristensen, 1992; Maucci, 1996). They are known in many localities, such as northern Norway (Trømso), Greenland (Disco) Island, Turkey, Svalbard Islands, Spitzbergen, Sweden (Öland island), the Himalayas, and in South America (Ramazzotti, 1956; Ramazzotti and Maucci, 1983; Westh & Ramløv, 1988).

Since no culturing method has been developed for *R.* cf. *coronifer*, specimens used in all presented studies were collected and extracted from a natural moss-living population in Alvar habitat of the Baltic Sea island Öland (South-Eastern Sweden). The specimens live in mosses (mainly *Orthotrichum cupulatum)* growing on limestone rock fences, directly exposed to winds and insolation, which leads to rapid temperature and humidity changes. The relative humidity (RH) may change dramatically over summer, from over 90% RH during the night, down to an average of 60% in the mid-day. However, the relative humidity can occasionally drop to 20% RH in the summer months (Jönsson et al., 2001). The species thus inhabits very exposed and rapidly desiccating substrates. It belongs to the xerophilic tardigrade species and can enter anhydrobiosis successfully in conditions with relatively low humidity (Jönsson et al., 2001). This Swedish population as well as the population from northern Italy are parthenogenetic and consist almost exclusively of females (Rebecchi et al., 2003). Development of unfertilized eggs seems to be the dominant mode of reproduction in this population. However, other populations from Greenland, Mongolia and central Italy include females and several males with spermatozoa within the gonad and are considered as gonochoristic-amphimictic (Guidetti et al., 2016). The genus *Richtersius* contains more than one evolutionary lineage and Guidetti et al. (2016) suggested distinguishing two evolutionary lineages. The first lineage consists of *R.* cf. *coronifer* from the neotype locality and the second consists of gonochoric-apomictic populations. Based on those data, our chosen Swedish population belongs to the first lineage.

2. 3 Data collection and analyses

The animals were extracted from a natural environment (desiccated moss). After the moss cushions were soaked in distilled water for about one hour, the sieve technique was used (**Paper I, II, III, IV**).

For observation of mitosis (**Paper I** and **II**), the specimens underwent the initial observations and species analysis, and then were fixed and stained *in toto* with acetic-lactic orcein, which is a standard staining method (Tonzetich, 2004). In animals containing mitotic

storage cells, the total number of storage cells was counted, and the mitotic index was calculated (**Paper I and II**). Furthermore, the predictive phenotypic traits of mitosis in storage cells were analyzed in relation to hydrated specimens (**Paper I**) as well as in connection to repeated anhydrobiotic cycles (**Paper II**). The collected data contained measurements of total body length, buccal tube length, gut content, egg developmental stage, the occurrence of moulting, and number, shape and size of oocytes (**Paper I** and **II**). The egg developmental stage was classified similarly as in Rebecchi and Bertolani (1994) based on a five-grade classification, containing a new category: “Stage O”. Likewise, the storage cells size and shape (regular-spherical or irregular-crescent with not straight borders) were examined to predict their connection to mitosis occurrence (**Paper I** and **II**).

Anhydrobiotic induction of individuals was used in **Paper II, III and IV** to: (A) analyze survival after repeated cycles of desiccation/rehydration (**Paper II**), (B) analyze tun formation and tissue and cell organisation in desiccated specimens (**Paper III** and **IV**). In **Paper II,** specimens were desiccated for 24 hours followed by 5-hour rehydration. The survival estimate was based on body movement (slowly moving, fully moving or fully active, and moulting) that occurred within 5 hours. Animals were classified as dead if they did not exhibit any body movement within 24 hours. Before each desiccation/rehydration cycle, the number of animals in a proper tun stage (full body contraction), semi-tun stage (body not fully contracted), and the extended stage were determined under a stereomicroscope. The presence or absence of gut content was recorded.

Tuns (anhydrobiotic stage; **Paper III** and **IV**) as well as rehydrated and heat stress treated specimens (**Paper IV**) were analysed using the light microscopy, scanning and transmission electron microscopy (based on semi- and ultra-thin sections of animal bodies). Polysaccharide, protein and lipid reserves in storage cells were detected using histochemical staining techniques (PAS and Bonhag´s method, Sudan black B staining and BODIPY 494/503; Litwin, 1985). Three-dimensional reconstruction of tuns was based on series of semi-thin sections (500 nm thick). The sections were stained, photographed and aligned into correct order and position (in detail described in **Paper III**). **Paper IV** consists of a descriptive and experimental part. The descriptive part is focused on the comparison of storage cells in desiccated and hydrated specimens. The experimental part focused on the effects of the combination of long-term desiccation and heating (50°C, 24h) on storage cells and specimen survival. In **paper IV,** confocal microscopy was used to visualize proliferating and dying cells using an anti-phosphohistone H3 antibody, and a TUNEL assay, respectively.

3 RESULTS AND DISCUSSION

3.1 Mitosis in storage cells and eutely in tardigrades and (Paper I, II)

The study presented in **Paper I** (Czernekova and Jönsson, 2016a) investigates the proliferation of tardigrade storage cells. The total number of storage cells was counted in those animals containing mitotic storage cells (**Paper I**, **II**; Czerneková and Jönsson, 2016b) and the mitotic index was calculated. Predictive phenotypic traits of storage cells mitosis were analyzed to find the correlation between the occurrence of mitotic storage cells and morphometric characteristics. We measured total body length, buccal tube length, gut content, egg developmental stage, the occurrence of moulting, and number, shape and size of oocytes (**Paper I** and **II**). In addition, the storage cells size was measured and correlation of their shape and occurrence of mitosis was analyzed (**Paper II**).

The number of storage cells varied among the individuals but also between the adults and juveniles. The juveniles had a higher frequency of mitosis in storage cells compared to adults. Moreover, the numerical growth of storage cells from juvenile to adult stage was documented. In both studies (**Paper I** and **II**), only about 20% of studied animals had mitotic storage cells. Those studies showed that tardigrade storage cells have a low mitotic index, 1.27% in **Paper I** and1.60% in **Paper II**, respectively. Mitotic cells occur also in other tissues, such as ganglia, pedal glands, and oesophagus, but those cells were not quantified since the storage cells were the main focus of our studies (**Paper I** and **II**). *R.* cf. *coronifer* specimens had on average 600±209 storage cells in adults, and 425±23 in juveniles (**Paper I**), but the total number of storage cells can vary from 300 to 1100 in adults and from 60 to 800 in juveniles (**Paper I** and **II**). Numbers of storage cells varies among tardigrade species and its high inter-species variability is observed in other species as well. The total number of storage cells was estimated to 395±136 in *Milnesium tardigradum*, and 1069±324 in *Paramacrobiotus tonollii* (Reuner et al., 2010a).

Since the mitotic index is low and is connected to moulting that usually corresponds to the late egg development stage, in adults, storage cells division seems to be closely related to animal growth. This is supported by our finding that mitosis in storage cells was more frequent in juveniles than in adults (**Paper I**), indicating build-up of the storage cells in juveniles. Mitotic cells also occur in other tissues (ganglia, pedal glands, oesophagus) (**Paper I, II**), which is in agreement with observations of mitotic cells in some other tardigrade species (Bertolani, 1970a, b; Poprawa et al., 2015).

Whilst in some tardigrade´s organs (nervous tissue, epidermis) the number of cells does not increase with animal growth, claw glands, storage cells and ventral ganglia have variable cell numbers (Bertolani, 1970a, b). However, a recent study (Gross et al., 2018) did not confirm mitosis in any other cells than midgut cells in the species of *Hypsibius exemplaris*. The mitotic cells were reported as midgut epithelium progenitors "crescent-shaped cells" (at the anterior) and "ring-shaped region" (at the posterior). Lack of cell divisions in other tissues may indicate that mitosis is triggered by specific physiological and/or environmental conditions (Bertolani, 1970a, b; Gross et al., 2018).

In juveniles, none of the phenotypic characters were significant predictors triggering the occurrence of mitosis, whereas, in adults, mitosis was significantly associated with the late egg developmental stage (late vitellogenesis and choriogenesis), that is connected to moulting process and empty gut content. (**Paper I**). Gut content was, however, a significant predictive trait for mitosis occurrence in storage cells only in the multivariate model I. including the egg developmental stage variable, but not in multivariate model II., including a moulting variable. This may indicate a stronger association of occurrence of mitosis to moulting process that is connected to late choriogenesis. During this stage, animals do not eat, and their large ovaries oppress the midgut lumen, therefore the gut content is excreted. This explanation is supported also by results presented in **Paper II**, that did not confirm any significant correlation between mitosis in storage cells and gut content.

Storage cells cytoplasm is filled by a number of vacuoles or vesicles with reserve material (**Paper III** and **IV**). While reserve material of storage cells increased in early vitellogenesis stage, no change was observed during late egg developmental stages when mitosis is more likely to take place. Decrements of reserve material after oviposition were observed (**Paper IV**), but since both studies (**Paper I** and **II**) did not find correlation between occurrence of mitosis and storage cells volume, the explanation that mitotic division in storage cells towards the end of egg developmental stage may function as restoration of cell number after their resorption has little support. By compiling data from **Paper I, II, III, IV** together it is possible to conclude, that physiological processes related to moulting might represent the strongest predictor for the occurrence of mitosis in storage cells.

Mitosis in storage cells does not seem to be triggered by energetic stress of desiccation cycles, since the occurrence of mitotic storage cells, as well as the storage cells number, tended to decline with a number of repeated desiccation cycles (**Paper II**). Because the storage cells are the major repository of energy resources (Węglarska, 1957; Rosati, 1968;

Węglarska, 1975; Szymańska, 1994), the loss of cells might indicate (A) energetic stress (and reabsorption of the cells), (B) cellular damage (and possible cell death), or (C) both mechanisms.

Our studies on mitosis are of interest for understanding tardigrade extraordinary tolerance to environmental and laboratory stress conditions. This tolerance is related to tun formation and metabolic arrest, however, several studies observed that tardigrade tolerance to irradiation is independent on whether the animals are desiccated or hydrated (Jönsson et al., 2005; Horikawa et al., 2006; Nilsson et al., 2010). Anhydrobiosis and irradiation are both processes causing DNA damage and cell divisions are not desirable when there are more genomic lesions (Hengherr et al., 2008a, b; Neumann et al., 2009; Beltran-Pardó et al., 2013b). These lesions cause loss of genomic integrity and might be lethal for the animal (Bolus, 2001; Stobbe et al., 2002; Watanabe et al., 2006; França et al., 2007; Neumann et al., 2009; Beltran-Pardó et al., 2013a, b). Based on a higher sensitivity to gamma-irradiation in early embryogenesis, one of the suggested explanations for radio-tolerance in tardigrades was their eutely and lack of mitotically active cells (Beltran-Pardó et al., 2013a, b, 2015; Jönsson et al., 2013), particularly higher frequency of mitosis in early developmental stage (Jönsson, 2019).

Since results presented in **Paper I and II** did not confirm eutely in tardigrades of *R.* cf. *coronifer* and reported mitotic storage cells in juveniles (37.9%) as well as in adults (18.3%), this occurrence of mitosis in tardigrade cells could be seen as consistent with the hypothesis of an efficient DNA repair system underlying the radiation tolerance of tardigrades (Jönsson et al., 2005; Beltran-Pardó et al., 2015). It is also supported by a recent study (Kuzmic et al., 2018) that reported increased accumulation of protein carbonylation (a common marker of oxidative stress) caused by higher doses of UVC irradiation in both the desiccated and the hydrated state of tardigrades. The recovery, therefore, requires an efficient DNA repair system. However, although the storage cells divide, the animals with observed mitotic cells have a very low mitotic index (the percentage of mitotic storage cells of all storage cells; 2.9 mitotic storage cells per juvenile and 8.8 mitotic storage cells per adult), and thus low cell turnover. Thus, most of the storage cells carry out their specialized function and do not divide. Such cells are considered to be in the G_0 phase. They are not dormant and can be very actively engaged in protein synthesis and secretion (Watanabe et al., 2006; França et al., 2007). **Paper IV** provides a distinction among storage cells by a description of two types of storage cells with different ultrastructure. The first type of storage cells is metabolically active, and its main function is energy storage and distribution. These cells probably

represent the cells with the cessation of cell division. The second cell type might represent young, undifferentiated, possibly stem-cell-like cells that divide after physiological and/or environmental triggers (**Paper I** and **IV**). The cell division cessation provides extra time for DNA repair (Watanabe et al., 2006; França et al., 2007). Since similar delays as seen after gamma irradiation were observed in the recovery of tardigrades of *R.* cf*. coronifer* after stress response (**Paper II**), it may indicate presence of cell damage, cell cycle arrest and activity of repair process (Beltran-Pardó et al., 2013a, b; Jönsson et al., 2016). The observed low mitotic index implies that the cell division is suppressed until specific mitotic trigger factors occur (O´Farrel, 2001). Such trigger factors for tardigrade storage cells remain to be studied.

Eutely, a term introduced in 1909 by Eric Martini, was traditionally attributed to miniaturized animals such as the nematode *C. elegans* (Cunha et al., 1999; Azevedo and Leroi, 2001), rotifers (Gross et al., 2018), and in earlier studies also to tardigrades. Such organisms were described as having tissues without regenerative capacity and zero rate of cell turnover in adults (Immelmann, 1959; Beltran-Pardó et al., 2015; Nelson et al., 2015; Milo and Philips, 2016). According to this theory, individuals of eutelic organisms have the same number of cell lineages and the same total numbers of cells (Rusin and Malakhov, 1998; Azevedo and Leroi, 2001).

Our data, therefore, indicate that tardigrades cannot be characterized as eutelic organisms. Up to now, the published literature data on mitosis occurrence in tardigrades is scarce. There is no evaluation of the mitotic index of different cell types and current data are not consistent. Some authors (Bertolani, 1970a, b; Dewel and Clark, 1973; Greven, 1976; Ząbczyk, 2000; Gross et al., 2019) did observe mitotic regenerative cells in midgut cells, whereas others (Rost-Roszkowska and Poprawa, 2008; Møbjerg et al., 2011; Rost-Roszkowska et al., 2011, 2013) did not confirm mitosis occurrence at all. The most recent research verified the existence of regenerating cells in the midgut (~40 cells) of adult tardigrade, but these cells exhibited very low cell turnover - 6 to 10 cell divisions per day (Gross et al., 2018; Gross et al., 2019). The precise cell numbers in tardigrade tissues are not known, but no cell number constancy was found among the muscle cells (from 40 up to 140; Ramazzotti and Maucci, 1983), pedal glands, either in nervous tissue, where each ganglion ranges between 20 to 75 neurons (Bullock and Horridge, 1965; Bertolani, 1970a, b; Węglarska, 1975; Zantke et al., 2008, Gross et al., 2019).

Marcus (1929) suggested partial cell constancy since he found a constant cell number in pharynx (comprising from 51 cells). The studies on tardigrade relatives, nematodes, also

suggest the existence of quasi-eutely, i.e. “almost constant number of cells”, instead of full “eutely” (Rusin and Malakhov, 1998). Cunha et al. (1999) support this hypothesis with the observation that most of the free-living terrestrial nematode species are not eutelic at least in some tissues and show variations in cell numbers. Results presented in **Paper I** and **II** support the view that tardigrades are quasi-eutelic. The state of quasi-eutely was also supported by the observation of regenerative capacity in midgut progenitor cells (Gross et al., 2018).

The term “eutely” itself should be used with caution, because of the criticism based on the cell number variability (CNV) in various tissues and organs of different, traditionally eutelic and non-eutelic taxa (Azevedo and Leroi, 2001). Organs, which were usually considered as eutelic, such as the epidermis of *Caenorhabditis elegans* (CNV=2%), yolk and gastric glands of the rotifer *Hydatina senta* (CNV=4.5%) and the segmental ganglia of the leech *Hirudo medicinalis* (CNV=1%) exhibited CNV ~ 5%. The recent estimations in the epidermis of 20 nematode species showed that nine of them had CNV in 5 - 15% range, which is higher than in traditionally accepted eutelic organism. Based on results from **Paper I** and **II,** CNV in tardigrade storage cells was 49% (n=8) in juveniles, and 34% (n=39) in adults, which is too high to be considered as eutely.

3.2 Anhydrobiosis in *R.* cf. *coronifer* (Paper II, III, IV)

Since the state of anhydrobiosis is ametabolic and is not connected with energy consumption, tardigrades may stay in this state for several years. The anhydrobiosis can be entered recurrently and at any stage of the life cycle of *R.* cf. *coronifer*. But entrance into and exit from anhydrobiosis relies on physiological processes that may be energetically costly but are poorly understood.

Therefore, the aim of the following work (**Paper II**) was to improve the understanding of these processes by determination how many consecutive periods of anhydrobiosis tardigrades can survive. The objective of this study was to analyze the factors constraining anhydrobiotic survival and patterns of survival under repeated cycles of desiccation: size, shape and number of storage cells, morphometric characteristic (such as gut content, body size) and frequency of mitosis (discussed above, *section 3.1*). Two other studies (**Paper III** and **IV**) aimed to understand the internal morphology of desiccated organisms. We analysed the body re-arrangement by 3D reconstruction of body organisation and ultrastructure

of tissues and cells in the desiccation state (**Paper III**). Further, we focused on the ultrastructure of tun and body wall, ovary, midgut and especially of the storage cells in long term desiccated and rehydrated specimens (**Paper III** and **IV**).

3.2.1 Experimentally-induced repeated anhydrobiosis

Very few previous studies have evaluated how many consecutive periods of anhydrobiosis tardigrades can survive. Survival of 8 – 14 desiccations was reported, but only on three eutardigrade specimens, and survival of 9 desiccations cycles, with 50% survival after fifth desiccation cycle (Lance, 1896; Baumann, 1922). Hengherr et al. (2008a) observed recovery rates of 88 -100% over 9 consecutive desiccations, however, the animals could feed and replenish their energy. We investigated patterns of survival in our model species under repeated cycles of desiccation/rehydration (24 h of desiccation and 5 hours of rehydration) when the animals were not fed (**Paper II**). Tardigrades of *R.* cf. *coronifer* can survive the maximum of 6 repeated desiccation cycles (non-cultured conditions) with the clear significant decline of survival rate after repeated desiccations (**Paper II**).

The fifth desiccation cycle seemed to be critical because there was a steeper survival decline. The animals had also significantly lowered the number of storage cells after the fifth desiccation. As mentioned above, it is assumed that storage cells serve as an energy store (Węglarska, 1975) and the re-absorption of storage cells with starvation and anhydrobiosis in some tardigrade species has been reported (Reuner et al., 2010a). Therefore, it seemed that animals reached an energetic constraint and did not have energy available to exit the fifth and enter the next cycle. On the other hand, in contrast to a previous study (Jönsson and Rebecchi, 2002), no reduction in cell size after several desiccation cycles, and also no depletion of gut content by repeated desiccation was observed (**Paper II**). In addition, the starvation of *R.* cf. *coronifer* specimens in continuously hydrated conditions did not result in a significant decline in the number of storage cells (Jönsson et al., 2005; Jönsson et al., 2008). Therefore, it seems that *R*. cf. *coronifer* have an energy budget different from other tardigrade species and/or the energy budget used for starvation stress and anhydrobiosis can also differ. This is consistent with studies performed by Reuner et al. (2010a) and Hyra et al., (2016a, b) on other eutardigrade species.

Midgut digestive cells have been lately shown to serve as another energy budget in tardigrades in the species *I. g. granulifer* (Hyra et al., 2016a). Storage cells of this species

contain mainly polysaccharides (Hyra et al., 2016b), whilst the reserve material in storage cells of *R.* cf. *coronifer* is composed mainly of lipids and polysaccharides (**Paper III, IV**), similarly to the storage cells of *H. exemplaris* and *M. polonicus* (Hyra et al., 2016a, b). No decrements of lipid or polysaccharide content in storage cells were observed in connection to anhydrobiosis (**Paper III, IV**). Instead, the protein spheres were diminished in desiccated tardigrades (**Paper IV**) which indicates protein degradation and/or utilisation during stress conditions. In eukaryotic cells two main pathways of protein degradation were described, ubiquitin-proteasome pathway and lysosomal proteolysis, an uptake of proteins by lysosomes (Cooper and Hausman, 2018).

Lysosomes were not observed in storage cells of *R.* cf. *coronifer*. Instead, high amounts of autophagosomes (double-membrane vesicles with heterogeneous cellular contents targeted to degradation) were present after 3-5 hours of rehydration from anhydrobiosis (**Paper IV**). Autophagy was also observed in the midgut digestive cells of *I. g. granulifer* (non-anhydrobiotic species with different reserve material in storage cells), but not in the midgut of species *H. exemplaris* and *M. polonicus* containing similar reserve material in storage cells to *R*. cf. *coronifer*. Autophagy is a physiological process involved in routine organelle turn-over (e.g. in metamorphosing insects), but it is likewise used for recycling of material during starvation. It is involved in starvation tolerance in any cell and in the maintenance of minimum metabolic rate (Gutiérrez et al., 2001; Lockshin and Zakeri, 2004). Autophagy contributes to mobilization of intracellular lipid stores and may be crucial for lipid metabolism (Kaushik et al., 2010). Furthermore, its protective mechanism in starved cells was confirmed (Lockshin and Zakeri, 2004). It is also the first step of cell degradation, which is followed by apoptosis (Regiorri and Klionski, 2005). In this context, autophagy may play a crucial role in the physiology of storage cells in *R*. cf. *coronifer* and anhydrobiosis of tardigrades in general. This is supported by the study on ciliates, where autophagy plays a crucial role in cyst formation during decrements of cellular volume (Duszenko et al., 2011).

After the fifth desiccation cycle, significantly more tardigrades were not able to contract properly during tun formation. They formed "semi-tuns" instead of "proper tun" formations. Thus, contraction started to be uncontrolled and incomplete. This result is compatible with the observation of Bauman (1922). In that study, tardigrades undergoing repeated desiccation were unable to produce a cuticular secretion, which resulted in uncontrolled contraction of the body. Such changes in the ultrastructure of epidermal cells in connection to anhydrobiosis are discussed in the next section (*3.2.2*).

Based on collected data, it seems that the declining survival after multiple anhydrobiotic cycles in *R.* cf. *coronifer* is not only caused by depletion of energy in storage cells but rather it is caused by the accumulation of cellular damage. This explanation is also supported by the current study of carbonylation accumulation during the anhydrobiotic state (Kuzmic et al., 2018). Autophagy also seems to be an important mechanism in tardigrade anhydrobiosis and survival. The precise role of intense autophagy and recycling mechanisms during nutritional deprivation and anhydrobiosis remain to be studied.

3.2.2 Tun formation in *R.* cf. *coronifer*

The main morphological changes after desiccation include extreme water loss connected with cuticle folding, and inner organs packing and relocation. The morphological transition into tun formation seems to be necessary for anhydrobiotic survival of limno-terrestrial tardigrades (Crowe, 1971; Sømme, 1996). Tun formations (**Fig. 5a**) were observed also among the Arthrotardigrades (e.g. *Styraconyx haploceros*; Jørgensen and Møbjerg, 2014), but also in *Echiniscoides sigismundi*, both of which are marine heterotardigrade species. The latter species survive short term desiccation, but the survival is less dependent on tun formation (Hygum et al., 2016). Therefore, our understanding of tun formation can bring important insight into the understanding of anhydrobiosis. Studies on *R.* cf. *coronifer* confirmed the necessity of tun formation mediated by musculature for survival in this species (Halberg et al., 2013a; **Paper II**). Results of these studies are in line with our observation of decreased survival connected with an inability to form proper tuns (**Paper II**) and with the observations on tun ultrastructure as well (**Paper III**). **Paper III** furthermore proved that moulting does not restrict tun formation.

During anhydrobiosis, the organisms lose most of their water by evaporation, which causes high structural stress (Halberg et al., 2013a). Reduction of body water in *R*. cf. *coronifer* was estimated to be up to 87 ± 5% in the course of transfer from the active to dehydrated tun stage. In bdelloid rotifers (Bdelloidea) a corresponding loss of water of 60% has been reported (Ricci et al., 2008). Tun formation was analyzed by 3D reconstruction of the tun with a focus on the inner organ packing (**Paper III**, **Fig. 1**). The inner organ relocation is dependent on the rigid buccal tube and the ovary size is limited by rigid eggshells (**Paper III**, **Fig. 2**). The storage cells enclose all the inner organs and fill up almost all the inner space between organs, but it is difficult to say, whether the storage cells play any role in the protection of those organs (**Paper III and IV**).

One part of tun formation is represented by cuticular folding and formation of creases made by this folding. The thick parts of the cuticle are in contact with air, whilst the thinner parts, which are more permeable to water, are out of contact with the air. Thus, the thick, less permeable parts of the cuticle could block aerial exposure, protect cells against reactive oxygen species (ROS; Almeida et al., 2005), and serve as mechanical protection of the tun. Based on the results presented in **Paper III**, although the cells were shrunk as a result of desiccation, morphological damage on the ultrastructural level of cells and tissues was not found. But changes of pigmentation in epidermal cells in desiccated animals were observed. The pigment granules may play a role in desiccation tolerance because they can either be utilized or denatured during entry or exit from anhydrobiosis (**Paper III**). Epidermal cells also contain lipid vacuoles, which were previously observed in other tardigrade species to discharge their content to the cuticular layers, which provide a barrier to water exchange (Baccetti and Rosati, 1971; Walz, 1982; Dewel et al., 1993). Therefore, it is possible, that cuticular lipids play an important role in anhydrobiosis (**Paper III).**

In conclusion, there seems to be no obvious damage to the overall ultrastructure from desiccation. Rather, the incorrectly formed tuns after fifth desiccation cycle (**Paper II**) in combination with the ultrastructure of storage cells (**Paper IV**) indicate cell damage at a molecular level.

3.2.3 The effect of long-term desiccation in combination with temperature stress

R. cf. *coronifer* can survive long periods of time in anhydrobiosis and results presented in **Paper IV** confirmed 100% survival (n=14) after 6 months in desiccated state and survival up to 6 repeated desiccation cycles (**Paper II**). Furthermore, it is known for extraordinary irradiation capacity and survival up to 19 days of starvation (unpublished data). In tardigrade storage cells, an increase in DNA fragmentation with time spent in the dry state (up to 10 months) was reported (Neumann et al., 2009). However, some species have been reported to survive in the dry state for 9 to 20 years (Guidetti and Jönsson, 2002; Jørgensen et al., 2007). Therefore, **Paper III** and **IV** focused on possible morphological and cellular changes linked to desiccation and heat stress. The studies compared the ultrastructure of active and desiccated animals after long-term anhydrobiotic state (**Paper III**) and ultrastructure of storage cells in desiccated, hydrated and dead animals (**Paper IV**). In tardigrades, desiccation leads to overall cellular shrinkage, change in cellular shape (in case of storage cells the shape changed from the circular to amoeboid). Due to high

electron density of cyto- and nucleoplasm in desiccated cells, organelles such as RER and Golgi complexes are barely visible. The average desiccated storage cells size is 11.8µm (**Paper III** and **IV**) and their whole inner space is filled with membrane-coated spheres of different electron density (**Paper IV**). In general, water evaporation causes drastic changes in inter- and intra-molecular interactions, such as hydrogen bonding between proteins and membranes that normally would not interact in bulk of water (Rebecchi et al., 2007; Wolkers et al., 2002). In normal cells, drying damages the cellular membranes and proteins leading to cell death, and consequently the death of the whole organism (Rebecchi et al., 2007). Based on the ultrastructural analyses from **Paper III** and **IV,** no cellular damage on ultrastructural level was observed.

The ultrastructure of normally highly metabolically active cells such as epithelial cells of ovary wall or storage cells does not indicate any secretory activity under desiccated conditions. Coated vesicles resulting from endocytosis in enterocytes were also not observed in a desiccated state (**Paper III**). These observations support the hypothesis of at least low (maybe arrested) metabolic activity in anhydrobiotic stage and are in line with other studies (e.g. Pigoń and Węglarska, 1955; Wright, 2001). Electron-microscopic comparison of desiccated and rehydrated cells did not reveal structural and morphological damage of membranes and organelles in dried cells of *R.* cf. *coronifer*. This observation is in line with the study on *M. hufelandi* (Walz, 1979). Generally, we can conclude that the reversible evaporative water loss accompanying 6 months long desiccation does not change the integrity on the cellular level of organisation in *R.* cf. *coronifer*.

Also, the combined effect of long-term desiccation and temperature stress on those animals was included in **Paper IV**. Hydrated as well as desiccated (6 months long) specimens of *R.* cf. *coronifer* were exposed to temperature stress of +50°C for 24 hours. While non-heated desiccated tardigrades were fully active after 3 hours of rehydration (survival 100%, n=14), the heat stress of tuns caused a decrease in survival rate (40% survival, n=14). Detrimental effects of long-term desiccation plus heating did not arise from general cell structure damages. But since the required recovery time was longer in heated specimens, the damages may be rather at the level of molecular components necessary for cell survival. Moreover, some differences such as heterochromatin increase and change in reserve material were observed between dried and hydrated storage cells. These changes support the prediction that heat likely caused damage to molecular components of repair mechanisms and/or caused such damage that was not possible to repair. Nevertheless, heat treatments might produce some sublethally injured cells. Such cells can grow and survive only

under appropriate environmental conditions that allow recovery of the cellular lesions (Marcén et al., 2018).

Thermo-tolerance has been studied very little in tardigrades (Rahm, 1921; Ramløv and Westh, 2001; Neves et al., 2020). In laboratory conditions, the optimal temperature for tardigrade culturing is usually at room temperature (18°C), but it varies among species (Altiero and Rebecchi, 2001; Gabriel et al., 2007; Altiero et al., 2018). Nevertheless, the hydrated tardigrades tolerate up to 36°C (LT_{max}=37°C; Rebecchi et al., 2009, LT_{max}=38.7°C; Li and Wang, 2005). Temperature exposure time is a significant variable for tardigrade survival (Neves et al., 2020). In the anhydrobiotic state, the short-term (1 h) heat tolerance up to approximately 100°C has been reported (Hengherr et al., 2008a, b), and *R.* cf. *coronifer* has been reported to tolerate exposure up to 70°C for 1 hour with no lethal effect while exposure to of 80°C resulted in survival of less than 20% (Ramløv and Westh, 2001). Long term tolerance to 37°C for 21 days was reported in *P. richtersi* (Rebecchi et al., 2009), and to 63.1°C (LT_{50}) in *R. varieornatus* (Neves et al., 2020). Thermo-tolerance seems to be associated with anhydrobiosis only indirectly (Rebecchi et al., 2009) which is supported by observed acclimation to higher temperatures (Li and Wang, 2005; Neves et al., 2020).

Since thermo-tolerance has been reported in anhydrobiotic stage, it is explained by vitrification hypothesis (Hengherr et al., 2009a). Other possible explanations include a crucial role of muscle protein filaments (Halberg et al., 2013), the protective role of heat shock proteins or LEA proteins (Neves et al., 2020). In general, our observations of the ultrastructure of storage cells support the vitrification hypothesis, that cells are "frozen" in a glassy state. Detailed ultrastructure of storage cells is described below (*section 3.3*). Heat stress, however, caused ultrastructural changes in the density of reserve spheres in desiccated storage cells. Spheres in non-heated specimens were homogenous, whereas cellular spheres in heated specimens were filled with granules of lower electron density indicating a change in the distribution of the stored material.

The large amounts of lipids were present in all storage cells (**Paper III, IV**). Lipids have been proposed to play a key role in heat stress management of the eukaryotic, especially mammalian, cells (Balogh et al., 2013). Likewise, their role in anhydrobiosis has been discussed (Womersley et al., 1982; Kinchin, 1993; West et al., 2001; Wharton et al., 2008). However, there is a disagreement in the literature whether lipids are involved in anhydrobiosis. Some studies claim lipids are not involved (Womersley et al., 1982), whereas others suggest a direct relationship between lipid reserve and anhydrobiotic survival

(Preston and Bird, 1987). Since lipids are not utilized during the desiccation/rehydration cycles in *R.* cf. *coronifer*, they may represent immediate energy for starved animals. Alternatively, lipids may be used for metabolic processes connected to preparation for anhydrobiosis and synthesis of protective molecules such as glycerol (**Paper III** and **IV**, Kinchin, 1993). Since lipids are present in dehydrated storage cells, they may also maintain the spatial distribution of cells in the absence of bulk water (Womersley et al., 1982; Kinchin, 1993). Large amounts of lipid droplets were also found in epidermal cells of *R.* cf. *coronifer* (**Paper III**). This is in line with observations of epidermal cells in other anhydrobiotic tardigrade species, *M. tardigradum* and *M. hufelandi* (Dewel et al., 1993; Walz, 1982). Because specimens of *R.* cf. *coronifer* do not have a cuticular wax layer, large lipid reserves might serve as lipid supply to the cuticle and thus helping to reduce evaporative water loss as shown in nematodes (Womersley et al., 1982).

It was revealed that desiccated animals of *P. richtersi* contain a higher percentage of polysaturated fatty acids and thiobarbituric acid reactive substances (Rizzo et al., 2010). In another tardigrade species, *P. areolatus*, glycogen is the first utilized material during dehydration (Crowe, 1975). In nematodes, lipid and glycogen content is decreased during dehydration (Crowe et al., 2005; Womersley et al., 1982). Hyra et al. (2016a) found no lipid reserve material in storage cells of tardigrade of *I. g. Granulifer* that has no anhydrobiotic ability. In this context, more studies on the role of lipids in anhydrobiosis (and cryptobiosis in general) of tardigrades would be valuable.

3.3 Ultrastructure of storage cells (Paper III, IV)

Storage cells are clearly visible as they passively move in the body cavity by currents of the body cavity fluid caused by the movement of animals (Kinchin, 1993; **Paper I**, **II**). The storage cells are coelomocyte-type cells. Their general characteristic, ultrastructure and function are described in *section 1.6*.

3.3.1 The storage cells of *R.* cf. *coronifer*

Like the other tardigrade species (e.g. *Macrobiotus hufelandi*, Rosati, 1968), the shape of storage cells of *R.* cf. *coronifer* is quite variable in active animals - from circular, amoeboid, oval or spherical. In desiccated animals, the storage cells have an amoeboid shape

(**Paper I, II, III**, **IV**). The size of storage cells in adult animals varies from 6 up to 20 µm. The average size of adult storage cells in the active state is 13.7µm (±2.3, n=295), and 10.53µm (±2.4, n=32) in juveniles (**Paper I, III**). In the light microscope, SC appear as circular globules with granular structure. Occasionally, oval SC with orange fibrous material were observed (unpublished data). Storage cells with irregular edges were previously seen to be attached to various organs, such as ovaries (Węglarska, 1957), epidermis and gut (Rosatti, 1968). Similarly, storage cells of *R.* cf. *coronifer* were sometimes attached to epidermis or gut. The cells absorb material from the coelomic liquid near the intestine by pinocytosis and phagocytosis. The absorbed material is subsequently transported to the epidermis (Węglarska, 1975).

In general, the storage cells in active tardigrades have an electron-lucent cytoplasm and a large circular nucleus with a distinct nucleolus. They contain ribosomes, mitochondria, RER, Golgi apparatus, large autophagosome. The fine structure of the first cell type is similar to other Parachela species (**Paper IV**), but *R.* cf. *coronifer* storage cells differ in the stored reserve material. The main compartments of the cells are spheres filled with reserve material of different electron density, containing large amount of lipids and polysaccharides, and low amount of proteins (**Paper III**, **IV**). In other species (e.g. *Dactylobiotus dispar, M. richtersi*) their size varies with the oogenetic stage (Poprawa, 2006; Węglarska, 1957, 1975). In *R*. cf. *coronifer*, the amount of reserve materials stored in storage cells decrease after oviposition and thus vary with regard to moulting stage and starvation (**Paper IV**). In contrast to studies on other species (Szymańska, 1994; Poprawa, 2006; Hyra et al., 2016b), the observations presented in **Paper IV** did not confirm the production of vitellogenins in storage cells of *R.* cf. *coronifer*.

Two types of storage cells fill the body cavity fluid in *R.* cf. *coronifer* (**Paper IV**). Ultrastructure of type I cells changes during oogenesis. They contain nucleolus with nucleolar vacuoles (also called a nucleolar cavity) with low electron density. The presence of irregular vacuoles varies with respect to the oogenetic stage. Nucleolar vacuoles were observed during previtellogenesis, but not during and after vitellogenesis. Vacuoles are generally rare in animal cells (Stępiński, 2014). In plants, vacuoles of irregular shape imply on dispersion and activation of chromatin (Stępiński, 2014), and it is, therefore, possible that in tardigrades, it relates to high nucleoli activity during previtellogenesis (**Paper IV**). Type I cells are also characterized by the presence of many mitochondria, cisterns of RER and specific spheres of reserve material. Large amounts of mitochondria present in these cells indicate high metabolic activity.

The storage cells of type II were found in a smaller amount and only in females. Their ultrastructure is similar during all oogenetic stages. These cells have fewer organelles and no nucleolar vacuoles. They may represent young undifferentiated cells or even stem cells. Cell lineage origin of storage cells is not known. Interestingly, the cell developmental origin of coelomocytes in other species is also unclear. In holothurians, they originate from a common stock of stem cells (Hetzel, 1963). They are of mesodermal origin in nematodes (Tahseen, 2009) and echinoderms (Chia and Xing, 1996). As storage cells are coelomocyte-type cells it is possible that tardigrade storage cells develop from these undifferentiated cells of type II, which might represent some kind of stem cells. The study presented by **Paper IV** is the first to ultrastructurally verify two storage cells types in tardigrades.

3.3.2 Storage cell ultrastructure with regard to survival of stress conditions: desiccation and heat stress

In normal cells, the main types of damage related to drying are denaturation and aggregation proteins, leakage and fusion of membranes, destabilisation of RNA, DNA and chromatin (Tunnaclife et al., 2010). As described above, desiccated storage cells have increased proportions of heterochromatin and they partially lose their nucleoli (**Paper III**). Heterochromatin reflects a functionally inactive state of the genome. It is associated with gene repression but also determines the spatial organisation of the genome (Nikolov and Taddei, 2015). This observation, therefore, supports hypothesized cessation of the cell cycle and mitosis in storage cells during anhydrobiosis. Moreover, heterochromatin protects DNA against radiation damage and can diminish the accessibility of radicals to the DNA (Falk et al., 2008) and may thus also enhance radiotolerance of desiccated *R*. cf. *coronifer*.

Although we detected few differences in cell structures between hydrated and desiccated animals, our observations are in line with the prediction of vitrification hypothesis not only for storage cells but also for epidermal cells, ovary and midgut cells. However, a comparison of dead and dried animals revealed intact organelles and membranes in both groups. This finding supports the idea that desiccation injury is caused by changes proceeding at the biochemical level, while structures are protected from deformations.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

1. Mitotic division of tardigrade storage cells correlates with the growth phase of the animal. Mitotic storage cells occurred with higher frequency in examined juveniles (38%) than in adults (18%). The mitotic index was higher in adults. Mitosis is more frequent in moulting tardigrades.

2. Even though the mitotic index is low, the number of storage cells in single animal varies among individuals and within individuals over time. Tardigrades thus cannot be classified as eutelic at least for storage cells.

3. Tardigrades of *R.* cf. *coronifer* can survive the maximum of 6 repeated desiccation cycles (non-cultured conditions). Their survival rate, mitosis in storage cells, and ability to form "tun" declined with repeated desiccations.

4. Desiccation stress leads to cellular shrinkage and changes the cellular shape but causes no ultrastructural change of organelles and membranes in cells in *R.* cf. *coronifer*. After desiccation, the epidermal cells reduced their pigmentation granules and the lipid vacuole content was also diminished.

5. Although the combining effect of desiccation and heat stress affected tardigrade survival, it did not cause cellular damage at the ultrastructural level. The cause of reduced survival may instead depend on damage at a molecular level - heterochromatin amounts increased, the nucleolus was partially lost, and the amount and content of reserve material changed after desiccation and heat stress.

6. The content of stored material in storage cells is species-dependent, and *R.* cf. *coronifer* storage cells differ from those of other tardigrade species. The main reserve materials are lipids and polysaccharides.

7. We identified two storage cells types based on their ultrastructure. The first cellular type includes metabolically active cells, exhibiting their specific function. The second cellular type is represented by young undifferentiated cells.

In conclusion, the obtained results are in line with the vitrification hypothesis, but since detrimental effects of stress conditions did not arise from observable damage to general cell structures, the hypothesis alone seems to be insufficient to explain all

mechanisms involved in the protection of *R.* cf. *coronifer* under desiccation and other stress conditions. Since we found large amounts of lipids in all storage cells of *R.* cf. *coronifer* in all studies presented here, lipid composition and metabolism are next to be studied to reveal the mechanisms involved in desiccation survival of this organism.

The research of anhydrobiosis focused mainly on sugar metabolism and stress proteins, but the knowledge of regulatory mechanisms, stabilisation of cellular architecture during stress conditions is still fragmentary. The role of lipids has not been studied yet and metabolic studies of storage cells are largely lacking. Such studies have the potential to bring a deeper insight into cryptobiosis and metabolism of tardigrades. They could be of interest for the medical and food industry related to cellular preservation, stabilisation and storage of biological materials, organ and tissue preservation for transportation, and storage of blood cells or cell lines (Jönsson, 2019). Few promising approaches were recently published (Chen et al., 2012; Tanaka et al., 2015; Hashimoto et al., 2016; Boothby et al., 2017; Hashimoto and Kunieda, 2017; Chavez et al., 2019). Some molecules (glucose, paraformaldehyde, dimethylsulfoxide) have already been used as additives for preservation but most of those are toxic, causing cellular death and have, therefore, weak results (Schill et al., 2009). The non-toxic disaccharide trehalose has been shown to preserve stored platelets as cell cryoprotectant (Wolkers et al., 2001, 2002). Trehalose has also been microinjected into human oocytes providing protection against freeze stress (Eroglu et al., 2002). Application of anhydrobiosis might serve as an inspiration for dry vaccines and bring new technology that could be more available over the world and used without a need for the refrigerator (Schill et al., 2009).

Acknowledgements

First, I would like to thank Ingemar who introduced international science to me and was my great mentor who trusted in me and my work. I am truly grateful for coming to your department, for your expertise you shared with me, for space for creativity and discussions about tardigrades, but also for openness and trust, so I could always honestly tell you about any professional or personal struggles. I was very lucky I could visit your department and I enjoyed working with you. I am also very grateful for the financial support I got from Kristianstad University (HKR) so I could be present at the HKR department, attend valuable courses and take part in conferences.

I would also like to thank my principal supervisor, doc. Petr Svoboda, who gave me the opportunity to work on tardigrades. I am grateful for getting support for studies I was interested in.

Next thanks belong to my colleagues from the University of Silesia in Katowice. Big thanks belong to Izabela for our nice collaboration on tardigrades at the Department of Animal Histology and Embryology. Special thanks also belong to Lukasz who was always there for a talk and who showed me more of Polish culture and cuisine. In addition, I am also grateful for all advices I got from Roberto Guidetti at the beginning of my studies.

My stay in Sweden was very special for many reasons. I am deeply thankful for visiting house 20. Some of the meetings will always remain very special to me and shaped my personal development in many ways. Many people could be listed here, but I would like here just thank you all for introducing me to Swedish culture and showing me your own homes and families, different ways to live and new philosophies and music. It gave me a bit more fun and joy over my very isolated times and I felt a bit more like being home. Your invitations meant much more to me than you have ever thought. Special thanks belong to Tom and Johan who were around for inspiring discussions, and many trips.

I also need to thank my current colleagues at Jan Evangelista Purkyně for giving me a gentle push, motivation and support to get this work done. Special thanks belong to doc. Jaromír Hajer who introduced tardigrades to me and believed in me from my bachelor studies.

Last but definitely not least I wish to thank my mum and especially to my brother for always being present and supporting me. Many friendships and relationships suffered due to my studies, nevertheless, the special thanks belong to those who always were present and supported me in many ways. Petra T., Adéla M., Karel N. and Monika Ch. – cordially thank you.

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ZOOLOGICAL Journal of the Linnean Society

Zoological Journal of the Linnean Society, 2016, **178**, 888–896. With 1 figure

Mitosis in storage cells of the eutardigrade *Richtersius coronifer*

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Received 31 August 2015; revised 13 March 2016; accepted for publication 24 March 2016

Although tardigrades are sometimes reported as eutelic animals, mitosis has been reported in several somatic tissues of adult eutardigrades. The occurrence of cell division in storage cells is particularly interesting in light of the important role that these cells play in the physiology of tardigrades. We present data on the occurrence of mitosis in storage cells of the eutardigrade *Richtersius coronifer* (Richters, 1903), and analyse mitotic cells in relation to different body characteristics, including egg development stage, moulting, gut content, body length, number and size of oocytes, and shape and size of the storage cells. Mitosis was present in ~20% of all animals, and was more frequent in juveniles than in adults. The proportion of cells with mitosis ('mitotic index') was low: 0.76% in juveniles and 1.47% in adults. In juveniles, none of the measured phenotypic characters had significant predictive power for mitosis, whereas in adult animals in moult or in late egg developmental or post-laying stage were more likely to have mitotic storage cells. The association with the later part of the moulting process was particularly strong. The low mitotic index and the strong association with moulting suggests that mitosis in storage cells may be connected with somatic growth rather than cell renewal, and that the purpose of cell division may relate to a need of more cells to support the enlarged body after moulting. However, the specific life cycle of tardigrades, where energy intake and depletion, egg development, and moulting is highly intertwined and synchronized, make conclusions about the functional role of mitosis in storage cells difficult, however, and more studies are needed to reveal the mechanisms inducing mitosis in these interesting cells.

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doi: 10.1111/zoj.12440

ADDITIONAL KEYWORDS: cell division – coelomocytes – development – mitotic index – moulting – tardigrada.

INTRODUCTION

Tardigrades are sometimes characterized as eutelic animals, with a constant number of cells, in which the capacity to multiply is lost before differentiation, implying that the body organs are composed of permanent tissue without regenerative capacity (e.g. Nelson, Guidetti & Rebecchi, 2015; Milo & Philips, 2016). As shown previously by Marcus (1929, cited in Bertolani, 1970a,b), however, somatic mitosis occurs in many, perhaps all, organs of eutardigrades, including, e.g., storage cells, transition cells of the midgut, epidermis, and ganglia, and therefore tardigrades cannot be considered as truly eutelic animals. Bertolani (1970a) reported that mitotic cells were particularly frequent in animals that were in the simplex stage (during moulting), and there also seemed to be a seasonal effect with more somatic mitosis in animals collected during spring. In a second paper, Bertolani (1970b) verified the variability between individuals in cell number for the third ganglion, Malpighian tubules, and storage cells, and also showed that variation was not related to the size of the animal. He therefore argued that somatic mitosis in tardigrades was related to regeneration of tissues rather than to the body growth of the animal, and that cell constancy of an organ (when observed) should be considered a secondary characteristic. The question of somatic mitosis and regeneration of cells

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in tardigrades is of interest not only related to a general understanding of the development and life cycle of these animals, but also to the reported high tolerance to radiation (e.g. May, Maria & Guimard, 1964; Jönsson, Harms-Ringdahl & Torudd, 2005; Horikawa *et al.*, 2006). Dividing cells are generally more sensitive to radiation because cell division is highly dependent on correct DNA information in order to give rise to viable new cells.

Storage cells were one of the cell types in which Bertolani (1970a) found a high frequency of mitosis. These cells belong to the cell category 'coelomocytes' (Tahseen, 2009), and have the role of storage and distribution of energy and nutrition in the body (Kinchin, 1994). They are single, freely floating cells in the body cavity fluid, with a few hundred up to more than a thousand cells per individual, and with large intraspecific and interspecific variation in numbers (Reuner *et al.*, 2010). Storage cells in tardigrades are known to contain stores of lipids and glycogen (May, 1946/1947; Węglarska, 1975; Szymanska, 1994), and the presence of tyrosinase activity, reported by Volkmann & Greven (1993), has indicated immunological functions; however, in general our understanding of the physiological functions of storage cells is still very poor.

The aim of the current study was to provide more detailed information on the occurrence of mitosis in storage cells of tardigrades, and to analyse possible connections between the occurrence of mitosis and different phenotypic characteristics of the animal.

MATERIAL AND METHODS

Origin and extraction of specimens

We used the limno-terrestrial species *Richtersius coronifer* (Richters, 1903), a moss-dwelling herbivorous species that has been subject to numerous studies on environmental tolerance, including desiccation, freezing, and radiation (e.g. Westh & Ramløv, 1991; Jönsson, Borsari & Rebecchi, 2001; Jönsson & Rebecchi, 2002; Jönsson *et al.*, 2005, 2013). No laboratory culturing method has been developed for this species and specimens were therefore collected from a natural population at Öland in south-eastern Sweden (for a description of the area, see Jönsson *et al.*, 2001). This population consists of mainly females with parthenogenetic reproduction, but males have occasionally been observed at very low frequency (Rebecchi *et al.*, 2003). All populations of this species studied so far have had a diploid karyotype with $2n = 12$ chromosomes and no differentiation in chromosome size (Rebecchi *et al.*, 2003).

Specimens of *R. coronifer* were extracted from desiccated moss (*Orthotrichum cupulatum*, Hoffman ex Bridel, 1801) by hydrating moss cushions in distilled water (to avoid the possible negative effect of tap water quality) for 5 h, followed by extraction with sieves (250 and 40 μm mesh size) under running tap water. Extracted tardigrades were stored in distilled water at room temperature (c. 22°C) for 1–2 days until preparation and observation.

Preparation and analysis of mitosis and animal characteristics

Initial observations and preparation for analysis

Specimens were first transferred with an Eppendorf micropippette to a glass slide and covered with a cover slip. Under observation with a light microscope, the excess water was carefully absorbed with small strips of filter paper, until the pressure prevented animals from moving. Images of the whole animal, buccal tube, and storage cells were taken for later measurement using image-analysis software. The time taken for the collection of all images for a single specimen was about 10 min. Images for the measurement of storage cells (size and shape) were taken at 40× objective magnification, images of oocytes were taken at 10× objective magnification, and images of body length were taken at 4× objective magnification.

After this initial observation, specimens were fixed in Carnoys' solution (methanol : acetic acid, 3 : 1) for 3 h and stained *in toto* in a drop of acetic lactic orcein, then covered with a cover slip and gently smashed, according to the method used by Rebecchi (1991). This staining method reveals chromosomes and allows the identification of cells undergoing mitosis. All slides were examined the following day (after ~20 h) with a light microscope (Olympus BX60) fitted with a digital camera (INFINITY 1; Lumenera Corp.) and image analysis software (INFINITY ANALYZE 6.0; Lumenera Corp.).

Analysis of mitosis and morphometric characteristics

The following morphometric variables were measured: total body length, buccal tube length, gut content, egg developmental stage, occurrence of moulting, number of oocytes, shape and size of oocytes, and occurrence of storage cells with mitosis. Measurement of total body length was taken from the anterior to the posterior part of the body, excluding the fourth pair of legs, and buccal tube length was measured according to Pilato (1981). In the analysis we only used body length as a measure of overall body size, as the buccal tube could not be measured in specimens in the simplex moulting

stage; however, body length was significantly correlated with buccal tube length ($r = 0.50$, $P < 0.001$, $N = 175$), and should therefore give a reliable estimate of the overall size of the animal. Juveniles were recognized by the presence of cuticular pores (see Guidetti *et al.*, 2016). Gut content was analysed at 20× magnification and classified as empty, medium (part of gut filled with material of orange to brownish coloration), or full (gut filled with black content).

The stage of egg development was determined with a five-grade classification. Stage 0: post-laying stage, with no oocytes visible inside the ovary, and without buccopharyngeal apparatus. Stage I: small gonad, and ovary filled with undifferentiated cells of similar size and appearance; buccal apparatus always present. Stage II: female gonad with oocytes of different sizes, nurse cells visible, and buccal tube present. Stage III: larger and longer gonad with oocytes of similar size and significant volume of yolk. In some of the females, oocytes were large and almost ready to lay, and the processes were visible. In one case egg-laying was observed during this stage. Females in stages I–III were never found to be moulting. Stage IV: gonad filled with large oocytes ready to lay and animal in process of moulting, absence of sclerified parts of the buccopharyngeal apparatus (simplex stage), or with double cuticle and new sets of claws and sclerified parts of the buccopharyngeal apparatus visible. This classification differs from that of Rebecchi & Bertolani (1994) by the new category 'Stage 0', which represents animals in simplex stage but without any signs of oocytes. These animals may have laid their eggs before entering moulting, or have refrained from developing eggs in between two moulting events. Also, our stages III and IV differ from those of Rebecchi & Bertolani (1994), mainly with respect to the presence of moulting, which in our study was connected to stage IV but not to stage III. In Rebecchi & Bertolani (1994) stage–III specimens were moulting (simplex stage) whereas stage–IV specimens were classified as post-moulting animals that had not yet laid their eggs. In our study we were not able to distinguish the latter category, but possibly some individuals in our category III may represent specimens that had already completed moulting but not yet laid their eggs. In order to distinguish between the egg developmental process and the moulting process, we categorized the egg developmental stage variable from the state of the oocytes only, and the moulting variable from the state of moulting only. The moulting variable included the categories non-moulting, simplex (buccal apparatus absent), and post-simplex (buccal apparatus and double cuticle present).

Storage cell size and shape was estimated from 20 randomly selected cells per individual. Cell size was calculated as the mean of two diagonal diameter measurements for each cell. The shape of the cells was classified as 'regular' (spherical) or 'irregular' (crescent, borders not straight, or cells filled with orange filamentous material). The filamentous material observed in some storage cells was noticed also in the gut of some specimens. In animals in which mitotic cells were found, the total number of storage cells was estimated at 100× magnification by manually counting all distinguishable cells in the body, in order to calculate the proportion of mitotic storage cells in individual specimens.

The presence of mitotic storage cells was estimated by investigating all storage cells in each specimen for the presence of condensed chromosomes (Fig. 1). In about 40% of the animals with mitotic cells, the total number of storage cells could be counted, and the proportion of cells in mitosis was then calculated. In the other specimens the single storage cells were not distinguishable to the same extent, and were often overlapping, therefore no reliable counting could be done.

Statistics

Phenotypic predictors of the occurrence of mitotic storage cells were analysed with logistic regression (IBM SPSS© STATISTICS 23.0). We followed the model-building strategy suggested by Hosmer & Lemeshow (1989), with the initial selection of variables based on univariate logistic regressions and $P < 0.25$ as the screening criterion for selecting variables for the multivariate model. Within multivariate regression models, significant effects were evaluated by likelihood ratio analysis, with $P < 0.05$ as the criterion for significance. Variables with significant

Figure 1. Storage cell of *Richtersius coronifer* with chromosomes in metaphase of the mitotic cycle. Light microscopy. Scale bar: 10 μm.

effects in the multivariate models were incorporated into the final models.

RESULTS

Occurence of mitosis in storage cells

In total, 242 specimens were examined: 29 juveniles and 213 adults. In our observations only one male was found, determined by the presence of spermatozoa. Storage cells undergoing mitosis were found in 20.7% (50/242) of all examined specimens. The frequency of mitosis was significantly higher in juveniles (37.9%) than in adults (18.3%; $\chi^2 = 6.0$, $P = 0.014$). The average percentage of mitotic storage cells among all cells ('mitotic index') in animals in which mitosis was found, and in which the total storage cell number was quantified, was 1.27% (SD = 0.83%, $N = 24$), and differed marginally significantly between juveniles and adults ($F_{1,22} = 4.13$, $P = 0.055$), with a tendency towards lower numbers in juveniles (0.76%, SD = 0.66%, 0.19–1.85%, $N = 7$; adults, 1.47%, SD = 0.82%, 0.17–3.0%, $N = 17$). This corresponds to an average of 2.9 mitotic storage cells per animal in juveniles, and 8.8 cells in adults. The mean number of storage cells in these specimens was 375 (SD = 185, range 65–650) in juveniles and 598 (SD = 239, range 290–1166) in adults, a statistically significant difference ($F_{1,22} = 4.86$, $P = 0.038$).

Mitotic cells were also observed in other tissues, such as ganglia, pedal glands, and the oesophagus, but were not quantified because the focus of this study was on storage cells.

Predictive traits of storage cell mitosis

Phenotypic traits predicting the occurrence of mitotic storage cells were analysed separately for juveniles and adults, and characteristics of tardigrades with respect to the presence of mitotic storage cells are provided in Tables 1–3 for continuous morphometric (Table 1) and categorical traits (Tables 2 and 3).

The results of the univariate logistic regressions are presented in Table 4. In juveniles, only gut content satisfied the screening criterion, but the *P*-value was too high for considering this trait to have a significant effect on the occurrence of mitosis. Thus, none of the predictor variables could explain the variation in storage cell mitosis in juveniles. In adults, egg developmental stage, gut content, moulting, storage cell size, storage cell shape, and oocyte number passed the screening criteria, and were used in the multivariate models. Due to collinearity between the egg developmental stage and moulting, and the resulting redundancy in the multivariate model (as moulting animals were restricted to specific stages of egg development), these variables were analysed in separate models together with the other selected variables.

In the multivariate model including egg developmental stage (model I), egg developmental stage and gut content showed significant effects (Table 5), and these effects remained in the final model in which non-significant variables were removed (Table 6). For egg developmental stage, animals in stages I and III were significantly less likely (12.7 and 5.9 times, respectively, calculated as the inverse of the odds ratio) to have mitotic storage cells compared

Table 1. Mean (SD/*N*) values of morphometric traits in juvenile and adult *Richtersius coronifer*, with and without mitotic storage cells

	Body length*	Storage cell size†	Oocyte number	Oocyte size‡
Adults				
With mitotic cells	644.87 (105.80/39)	12.66 (1.62/39)	1.92 (1.78/39)	120.81 (26.83/19)
Without mitotic cells	645.94 (118.67/174)	11.91 (2.38/174)	1.51 (1.87/173)	125.67 (40.71/65)
Juveniles				
With mitotic cells	388.43 (67.44/11)	10.46 (1.49/11)	–	–
Without mitotic cells	393.68 (94.25/18)	10.85 (2.93/18)	–	–

Metric measurements are given in micrometers (μm).

*Body length measured from anterior to posterior ends, excluding the fourth pair of legs.

†Mean size of storage cells calculated from measurements of 20 cells per animal, based on the mean of two measurements for each cell.

‡Two measurements (crossed diameters) were taken for each oocyte and an average oocyte size was calculated for each animal.

One-way ANOVA analyses between animals with and without mitotic cells for each trait. Adults: body length, $F_{1,211} = 0.003$, $P = 0.96$; storage cell size, $F_{1,211} = 3.454$, $P = 0.064$; oocyte number, $F_{1,210} = 1.594$, $P = 0.21$; oocyte size, $F_{1,82} = 0.239$, $P = 0.63$. Juveniles: body length, $F_{1,27} = 0.026$, $P = 0.87$; Storage cell size, $F_{1,27} = 0.170$, $P = 0.68$.

Table 2. Number and percentage (in brackets) of animals with and without mitotic storage cells in *Richtersius coronifer*, with respect to different categories related to gut content, storage cell shape, and moulting

	Gut content			Storage cell shape		Moulting		
	Empty	Medium	Full	Regular	Irregular	Non-moulting	Simplex	Post-simplex*
Adults								
Mitotic cells	16 (37.2)	18 (15.8)	5 (8.9)	30 (21.4)	9 (12.3)	6 (5.8)	10 (19.6)	23 (39.0)
No mitotic cells	27 (62.8)	96 (84.2)	51 (91.1)	110 (78.6)	64 (87.7)	97 (94.2)	41 (80.4)	36 (61.0)
Juveniles								
Mitotic cells	4 (66.7)	7 (33.3)	0	8 (40.0)	3 (33.3)	6 (40.0)	1 (75.0)	4 (40.0)
No mitotic cells	2 (33.3)	14 (66.7)	2 (100.0)	12 (60.0)	6 (66.7)	9 (60.0)	3 (25.0)	6 (60.0)

*Post-simplex: animals with buccal apparatus and double cuticle.
Pearson chi-square analyses between animals with and without mitotic cells for each trait. Adults: gut content, $\chi^2 = 14.05$, d.f. = 2, $P < 0.001$; storage cell shape, $\chi^2 = 2.66$, d.f. = 1, $P = 0.10$; moulting, $\chi^2 = 27.65$, d.f. = 2, $P < 0.001$. Juveniles: gut content, $\chi^2 = 3.52$, d.f. = 2, $P = 0.17$; storage cell shape, $\chi^2 = 0.12$, d.f. = 1, $P = 0.73$; moulting, $\chi^2 = 0.33$, d.f. = 2, $P = 0.85$.

Table 3. Number and percentage (in brackets) of adult *Richtersius coronifer* with and without mitotic storage cells in different stages of egg development

	Egg developmental stage				
	0	I	II	III	IV
Mitotic cells	8 (25.0)	1 (2.6)	3 (9.7)	2 (6.1)	25 (32.1)
No mitotic cells	24 (75.0)	38 (97.4)	28 (90.3)	31 (93.9)	53 (67.9)

For an explanation of egg developmental categories, see main text.
Pearson chi-square analysis between animals with and without mitotic cells: $\chi^2 = 22.12$, d.f. = 4, $P < 0.001$.

with the reference stage 0 (no visible oocytes), whereas animals in stage IV (last stage, with eggs ready for laying) had a similar likelihood (odds ratio near 1) of having mitotic storage cells as the reference stage. For gut content, animals with a full gut were four times less likely to have storage cells in mitosis compared with animals with an empty gut.

In the multivariate model including moulting (model II), moulting and oocyte number variables showed significant effects on the occurrence of mitosis (Table 5), but in the final model only the effect of moulting remained significant (Table 6). The predictive effect of moulting was very strong, however, particularly the post-simplex category characterized by the double cuticle. The adjusted odds ratio suggests that animals in this category were 15 times more likely to have mitotic cells compared with non-moulting animals, and about 11 times more likely than those in the simplex stage. The reason that gut content showed up as a significant effect in model I but not in model II is probably that the moulting variable absorbed more of the gut content effect than the egg developmental stage variable, because of a strong association between gut content and moulting.

DISCUSSION

Our study confirms the report of Bertolani (1970a) that storage cells of tardigrades clearly divide, and that the number of cells differs among individuals. The average proportion of animals with mitotic storage cells was about 20%, which may be compared with the estimates reported by Bertolani (1970a) for *Macrobiotus hufelandi* (Schultze, 1833) (16.9%), *Macrobiotus areolatus* (Murray, 1907) (12.2%), and *Paramacrobiotus richtersi* (Murray, 1911) (48.1%). The estimates by Bertolani (1970a), however, also included mitotic cells observed in organs other than storage cells. A higher proportion of juveniles had storage cells undergoing mitosis compared with adults, which may be related to a build-up of storage cell number during the juvenile stage, an explanation supported by the lower total number of storage cells recorded in juveniles. Among the individuals in which mitotic storage cells were found, the

Table 4. Univariate logistic regression models for occurrence of storage cell mitosis (0, no mitosis; 1, mitosis found) in *Richtersius coronifer*, in which potential predictive variables were evaluated individually

Predictor variable	LL	LR	d.f.	*P*
Juveniles				
Body length	19.23	0.028	1	0.87
Gut content	17.19	4.12	2	0.13
Storage cell size	19.16	0.18	1	0.67
Storage cell shape	19.19	0.12	1	0.73
Moulting	19.08	0.35	2	0.84
Adults				
Body length	−101.40	0.003	1	0.96
Gut content	−114.85	12.89	2	0.002
Storage cell size	−99.78	3.25	1	0.071
Storage cell shape	−100.00	2.80	1	0.094
Moulting	−87.57	27.66	2	< 0.001
Egg developmental stage	−88.97	24.86	4	< 0.001
Oocyte number	−100.42	1.55	1	0.21
Oocyte size	−44.78	0.25	1	0.62

LL, log-likelihood; LR, likelihood ratio.

Table 5. Main effects logistic regression models for prediction of mitosis occurrence in storage cells of *Richtersius coronifer*

Predictor variable	LL	LR	d.f.	*P*
Model I: with egg developmental stage	−81.93	38.54	10	< 0.001
Egg developmental stage	−91.59	14.23	4	0.0066
Gut content	−88.17	7.39	2	0.025
Storage cell shape	−84.65	0.34	1	0.56
Storage cell size	−84.77	0.58	1	0.45
Oocyte number	−85.58	2.20	1	0.14
Model II: with moulting	−83.40	35.60	7	< 0.001
Moulting	−91.59	14.23	2	0.0066
Gut content	−85.39	3.98	2	0.14
Storage cell shape	−83.59	0.39	1	0.53
Storage cell size	−84.13	1.46	1	0.23
Oocyte number	−85.50	4.19	1	0.041

Predictor variables explained 24–25% of the variation in mitosis occurrence (Nagelkerke $R^2 = 0.24$ and 0.25, for models I and II, respectively). The goodness of fit of the model was high (Hosmer and Lemeshow test: $P = 0.80$ and 0.78 for models I and II, respectively).

proportion of dividing cells was always very low, amounting to a few percentages, and not far from the estimates of Bertolani (1970a) in *M. hufelandi* (1.84%), *M. areolatus* (2.20%), and *P. richtersi* (5.08%). The slightly higher values in the study by Bertolani may well be a result of measuring mitosis also in other organs in that study. Bertolani (1970a) also showed that tardigrades collected during spring had significantly higher proportions of mitotic cells compared with animals collected during winter (2.0% in spring compared with 1.7% in winter in *M. hufelandi*). Animals for the current analyses were collected during the winter period (November–February), and our estimates for adult *R. coronifer* therefore correspond very well to those of *M. hufelandi*. The reason for the seasonal variation in mitosis occurrence is unclear, but is of great interest in order to understand the factors influencing the induction of mitosis in storage cells.

The estimated mitotic index of 0.76% in juveniles and 1.47% in adults indicates a relatively low cell turnover rate. Referring to studies in rats, Leblond & Walker (1956) considered a mitotic index below 0.8–1.5% as infrequent mitosis, in which cell division contributes to growth alone, whereas cell populations with a mitotic index exceeding 1.5% were seen as producing more cells than are needed for growth, thereby representing the renewal of cells. Given that these conclusions can be transferred to cell proliferation in invertebrates, our results would indicate that the low mitotic index estimated in tardigrade storage cells reflects cell division connected with the growth of the animal, rather than to cell renewal. This interpretation is also supported by the close connection between mitosis and moulting in adult *R. coronifer*, as the increase in body size after each moult may require more storage cells for sufficient energy supply and other possible functions. It should be emphasized, however, that moulting is not always connected with an increase in body size (Walz, 1982), and that the rate of body growth is not linear over the lifespan, but is more rapid early in life and diminishes as the animal gets older (Nelson, 1982). In contrast to this suggestion that mitosis in storage cells is related to the growth of the animal, Bertolani (1970b) proposed that mitosis in somatic tardigrade cells is mainly connected to the renewal of cells. His main argument was that the cell number does not seem to change over the course of the life cycle. Although this may be true for other tissues, we documented a higher number of storage cells in adult tardigrades compared with juveniles, suggesting that numerical growth of these cells takes place.

The analyses of traits predicting the occurrence of mitotic storage cells showed a striking difference between juveniles and adults. None of the included phenotypic traits could explain the occurrence of mitosis in juveniles, whereas three traits showed significant effects in adults: moulting, stage of egg development, and gut content. We currently have no plausible explanation for this difference between

Table 6. Final logistic regression models for prediction of mitosis occurrence in storage cells of *Richtersius coronifer*

Predictor variable	B	SE(B)	Wald	AOR* (95% CI)	LL	LR	d.f.	P
Model I								
Intercept	−1.495	0.177	71.25				1	< 0.001
Egg developmental stage					−91.59	14.23	4	0.0066
Egg developmental stage I	−2.534	1.095	5.35	0.079 (0.0090–0.68)			1	0.021
Egg developmental stage II	−0.870	0.766	1.29	0.42 (0.093–1.88)			1	0.26
Egg developmental stage III	−1.771	0.843	4.42	0.17 (0.033–0.89)			1	0.036
Egg developmental stage IV	0.074	0.518	0.020	1.076 (0.39–2.97)			1	0.89
Gut content					−88.17	7.39	2	0.025
Gut content (medium)	−0.654	0.461	2.012	0.52 (0.21–1.28)			1	0.16
Gut content (full)	−1.396	0.616	5.15	0.25 (0.074–0.83)			1	0.023
Model II								
Intercept	−1.490	0.177	70.63				1	< 0.001
Moulting					−100.42	28.41	2	< 0.0001
Moulting (simplex)	1.358	0.550	6.088	3.89 (1.32–11.44)			1	0.014
Moulting (double cuticle)	2.724	0.587	21.50	15.24 (4.82–48.19)			1	< 0.001
Oocyte number	−0.173	0.137	1.59	0.84 (0.64–1.10)	−85.50	2.71	1	0.10

Coding for mitosis: 0, no mitosis; 1, mitosis found. Models I and II refer to separate logistic regression models in which egg developmental stage and gut content (model I) or moulting and oocyte number (model II) were used as independent predictor variables. Reference categories for categorical variables: egg developmental stage, stage I; gut content, empty; moulting, non-moulting. Log-likelihood (LL) represents the log-likelihood of the model without inclusion of the variable in question. Likelihood ratio (LR) was calculated as $-2(LL_{reduced\ model}-LL_{full\ model})$, where $LL_{reduced\ model}$ is the model without inclusion of the evaluated predictor variable. Predictor variables explained 21–22% of the variation in mitosis occurrence (Nagelkerke $R^2 = 0.218$ and 0.214, for models I and II, respectively). The goodness of fit of the model was high (Hosmer and Lemeshow test: $P = 0.99$ and 0.94 for models I and II, respectively). Model I: $LL_{full\ model} = 86.12$, LR = 30.56, d.f. = 6, $P < 0.001$. Model II: $LL_{full\ model} = 86.22$, LR = 29.96, d.f. = 3, $P < 0.001$. *AOR: adjusted odds ratios, which are not identical to the odds ratios calculated for variables singularly. Non-adjusted odds ratios for the categorical variables may be calculated directly from the data in Tables 2 and 3.

juveniles and adults, and the complete lack of association between mitosis and moulting in juveniles is puzzling, as moulting was a strong predictor in adults. A high occurrence of mitosis connected with phase of moulting was also reported by Bertolani (1970a), and there is a striking similarity in the proportion of animals with mitotic cells among moulting animals in these two studies [43.9% in Bertolani (1970a) versus 42.9% in the current study, when our two categories of moulting are pooled]. The strong association between storage cell mitosis and moulting in adults may or may not represent a functional relationship between these two processes; however, we find it difficult to believe that the production of new storage cells could be directly involved in the process of moulting. Rather, we think it is more likely that the strong association is a result of developmental timing, in which the cell proliferation is initiated when the animal has reached the moulting stage. This does not exclude the possibility that the moulting process may physiologically trigger the initiation of mitosis in storage cells, and ecdysteroid hormone signals that activate key cell cycle genes and regulate cell division in moulting animals have been reported (Quinn *et al.*, 2012).

One of the main functions of storage cells is to store and distribute energy within the body, and storage cell size has previously been shown to change over the egg developmental cycle, reaching its smallest size towards the end of the cycle or after egg laying (Szymanska, 1994; Jönsson & Rebecchi, 2002). This change in cell size is apparently connected with dynamics in energy uptake and demand, representing a build-up of stores in the early part of the egg developmental cycle and a depletion of these reserves towards the end of the cycle. The eggs then reach their largest size, and feeding is prevented by the expulsion of mouthparts during moulting and by the body cavity being full of mature eggs. Szymanska (1994) noted that the number of storage cells in some individuals of *P. richtersi* was very low after egg laying, suggesting that cells were resorbed completely in connection with a depletion of energy. Restoration of the cell number would then require cell division. However, Reuner *et al.* (2010) did not find a

significant decline in storage cell number after starvation for 7 days in *Milnesium tardigradum* (Doyère, 1849), *Macrobiotus sapiens* (Binda & Pilato, 1984), and *Paramacrobiotus tonollii* (Ramazzotti, 1958), and in our own data there is also no tendency towards differences in cell number at different stages of egg development. Therefore, the possibility that mitotic activities in storage cells towards the end of the egg developmental cycle (when moulting also occurs) may have the function of restoring the number of cells after resorption currently has little support. However, the intertwined patterns of energy dynamics, egg development and moulting in tardigrades, well documented and reflected also in the results of this study, suggest that more investigations on the role of energetic status and other potential factors influencing the initiation of mitosis in storage cells are clearly needed. In this context, experimental studies in which the influence of specific agents is tested would be of particular interest.

ACKNOWLEDGEMENTS

This work was supported by the Academy of Sciences of the Czech Republic (RVO: 67985823), the Mobility Fund, Charles University, Prague, The Swedish National Space Board (grant 87/11), and Kristianstad University, Sweden.

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II

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RESEARCH ARTICLE

Experimentally Induced Repeated Anhydrobiosis in the Eutardigrade *Richtersius coronifer*

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OPEN ACCESS

Citation: Czernekova M, Jönsson KI (2016) Experimentally Induced Repeated Anhydrobiosis in the Eutardigrade *Richtersius coronifer*. PLoS ONE 11(11): e0164062. doi:10.1371/journal.pone.0164062

Editor: Bob Goldstein, University of North Carolina at Chapel Hill, UNITED STATES

Received: June 22, 2016

Accepted: September 19, 2016

Published: November 9, 2016

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Data Availability Statement: Data used in the analyses are available from the Dryad data depository, URL: http://dx.doi.org/10.5061/dryad.78vd3.

Funding: MC received funding from the Academy of Sciences of the Czech Republic (RVO: 67985823) and the Mobility Fund, Charles University in Prague (http://www.cuni.cz/ukeng-49-version1.txt). MC and KIJ received funding from Kristianstad University Sweden (www.hkr.se).

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Tardigrades represent one of the main animal groups with anhydrobiotic capacity at any stage of their life cycle. The ability of tardigrades to survive repeated cycles of anhydrobiosis has rarely been studied but is of interest to understand the factors constraining anhydrobiotic survival. The main objective of this study was to investigate the patterns of survival of the eutardigrade *Richtersius coronifer* under repeated cycles of desiccation, and the potential effect of repeated desiccation on size, shape and number of storage cells. We also analyzed potential change in body size, gut content and frequency of mitotic storage cells. Specimens were kept under non-cultured conditions and desiccated under controlled relative humidity. After each desiccation cycle 10 specimens were selected for analysis of morphometric characteristics and mitosis. The study demonstrates that tardigrades may survive up to 6 repeated desiccations, with declining survival rates with increased number of desiccations. We found a significantly higher proportion of animals that were unable to contract properly into a tun stage during the desiccation process at the 5th and 6th desiccations. Also total number of storage cells declined at the 5th and 6th desiccations, while no effect on storage cell size was observed. The frequency of mitotic storage cells tended to decline with higher number of desiccation cycles. Our study shows that the number of consecutive cycles of anhydrobiosis that *R. coronifer* may undergo is limited, with increased inability for tun formation and energetic constraints as possible causal factors.

Introduction

Water availability is one of the most important ecological factors and evolutionary pressures on terrestrial life. Despite the fact that water is crucial for all life forms, numerous organisms (including prokaryotes, protozoa, fungi, plants and animals) survive temporary drying to equilibrium with the air humidity by entering a highly stable and reversible state called anhydrobiosis, a special form of the ametabolic life state known as cryptobiosis [1,2,3,4,5]. During anhydrobiosis the organism loses most of its water by evaporation and has to protect cell

structures from damage caused by water loss [6]. The biochemical and physiological nature of such protectant systems in anhydrobiotic organisms are not well understood, but are of considerable interest both from a general biological perspective and from the applied sciences where dry biological systems play an important role (e.g., medicine and food storage) [7,8].

Among animals, tardigrades represent one of the main groups in which a capacity for anhydrobiosis is widespread. Tardigrades are microscopic aquatic animals found in a variety of habitats worldwide [9], and they are particularly common in semi-terrestrial microhabitats such as mosses, lichens and leaf litter. In these environments they are exposed to periods of desiccation that varies in frequency and length, and the anhydrobiotic capacity of semi-terrestrial tardigrades is an evolutionary adaptation to survive under such conditions. Their ability to enter anhydrobiosis is well documented [5,10,11], and the anhydrobiotic state may be entered recurrently and at any stage of their life cycle (so-called "holo-anhydrobiosis"; [12]).

The state of anhydrobiosis, characterized as an ametabolic condition, is not connected with any energy consumption, and this explains why tardigrades may stay in this state for many years, even decades, and still be able to revive [13,14]. However, the entrance into and exit of anhydrobiosis relies on physiological processes that are likely to be energetically costly, and evidence of energy-depletion in storage cells (coelomocytes with circulatory and energy storage functions) of tardigrades over a single cycle of anhydrobiosis has been reported [15,16]. This suggests that multiple cycles of anhydrobiosis may eventually deplete the energy stores of the animal and represent a potential constraint on how many times in a row a tardigrade may successfully enter anhydrobiosis, given that energy stores cannot be replenished by feeding. Apart from energy depletion, desiccation may also give rise to damage to cell components, including DNA [17], and multiple anhydrobiotic periods may therefore also challenge the maintenance of cell structure integrity.

Very few previous studies have evaluated how many consecutive periods of anhydrobiosis that tardigrades are able to survive. According to Baumann [18], Lance [19] was the first to examine repeated desiccation in tardigrades, reporting survival of 8–14 desiccations, but only three eutardigrade specimens were used. Baumann [18] reported the first and so far the only more extensive study on this subject, including 15 animals of the genus *Macrobiotus* (species not given). The results showed that single specimens were able to survive up to 9 repeated desiccations, but already after 5 desiccations about 50% of the animals had died. The only other study where tardigrades have been exposed to several sequential cycles of anhydrobiosis is that by Hengherr et al. [20], in which specimens of the eutardigrade *Milnesium tardigradum* were repeatedly desiccated with intermediate 7-day periods of hydration under cultured conditions allowing feeding. The study found no decline in anhydrobiotic performance over 9 consecutive desiccations.

The purpose of the current study was to investigate the patterns of survival in a tardigrade under repeated cycles of desiccation/rehydration and the potential effect of repeated desiccation on size, shape and number of storage cells. We also analyzed if body size, gut content and frequency of mitosis in storage cells change over the course of repeated desiccation.

Materials and Methods

Study organism

In our desiccation experiment we used medium sized (average body length of 653 μm, n = 80) specimens of the eutardigrade *Richtersius coronifer* (Richters, 1903). The population consists almost exclusively of females [21]. No culturing method has been developed for this species, and specimens were extracted from a natural moss-living population in Alvar habitat of the island Öland (see [22] for description of the Alvar area). The study did not involve endangered

or protected species, and moss samples were not collected within an area where permission was required. Moss (*Orthotrichum cupulatum*) containing tardigrades were collected dry and stored at room temperature for 3 days until use. Moss cushions were hydrated for about 2 hours in tap water and within the following 3 hours 400 active specimens were extracted with sieves (mesh size 250 and 40 μm) under running tap water. Extracted animals were washed thoroughly with distilled water to remove adherent particles.

Anhydrobiotic induction and recovery

The general procedure of our study consisted of repeated 24 hour periods of desiccation at 95% relative humidity (RH) followed by 5 hours of rehydration. These times were assumed to provide enough time for the animal to enter anhydrobiosis and to rehydrate and adjust physiologically for the hydrated state, respectively. After each desiccation cycle, 10 revived specimens were selected for analysis of mitosis in storage cells and morphometric characteristics, and the remaining animals that were alive were mixed and prepared for a new period of desiccation. This procedure was repeated until there were too few alive animals to continue.

At the start of the experiment, 400 randomly chosen specimens were put individually on ten replicate filter papers (5 x 2 cm) in petri dishes and transferred into a desiccator with a saturated potassium nitrate (KNO3) salt solution providing a relative humidity of 95%. The 24h desiccation allowed the animals to enter anhydrobiosis and equilibrate with the surrounding humidity condition. Since the total number of animals available for each new desiccation cycle steadily declined (due to removal of specimens for analysis + mortality of some specimens at each cycle), the mean number of animals in each replicate was a decreasing function of the number of desiccation cycles (1st cycle = 40 specimens/replicate, 2nd = 38.4, 3rd = 31.5, 4th = 22.9, 5th = 14.9, 6th = 3.5). In all desiccation cycles the number of specimens in the 10 replicates differed by maximum one animal.

Before each rehydration all specimens were examined under stereomicroscope and the number of animals in tun stage (S1A Fig), semi-tun stage (body not fully contracted, S1B Fig) and extended (non-tun) stage (S1C Fig) in each sample were recorded. Gut content was also recorded and specimens were classified as having gut content (dark gut) or no gut content (yellow gut). Twenty hydrated animals not included in the desiccation experiment were initially selected and used as controls for comparison with the repeatedly dehydrated animals.

Survival of the rehydrated animals was evaluated after 3 and 5 hours, and the latter estimate was used for most animals in the survival analysis. However, in the survival estimate we also included the 10 specimens selected for mitosis and morphometric analysis, and these were collected at the 3 hour survival estimate. All specimens alive at the 3 hour evaluation were also alive after 5 hours. No specimens inactive during all 5 hours of rehydration recovered after this time (dead specimens were examined for 24 hours). Animals were assumed to be dead if there was no visible body movement. After each rehydration period, alive specimens were put together in a single dish and transferred individually with an Irwin loop to 10 new filter papers, followed by a new period of desiccation. For each replicate sample within a desiccation-rehydration cycle the proportion of surviving animals was calculated and used in the analysis.

Initial observations and preparation for analysis

The 10 animals randomly selected after each desiccation/rehydration cycle were transferred in a drop of water to an object slide, covered with a cover slip, and observed under light microscope (Olympus BX60) fitted with a digital camera (INFINITY 1, Lumenera Corp.). Images of the whole animal, buccal tube and storage cells were taken for later measurements using the image analysis software (INFINITY ANALYZE 6.0, Lumenera Corp.). The time for collection

of all images for a single specimen was about 10 minutes. Images for measurement of buccal tube and storage cells were taken at 40X magnification.

After initial observation specimens were transferred into Carnoys´ solution for 3 hours and stained *in toto* in a drop of acetic-lactic orcein according to the method used by Rebecchi [23]. Slides were examined the following day under the light microscope.

Analysis of morphometric characteristics and mitosis

Measured morphometric variables were body length, buccal tube length, gut content, size and shape of storage cells and presence of mitotic storage cells. Measurement of total body length was taken from anterior to posterior part of the body excluding the fourth pair of legs, and buccal tube length was measured according to the description by Pilato [24]. The measurements of body size and buccal tube length were significantly correlated (Pearson correlation; $r = 0.27$, $N = 62$, $P = 0.036$), and in order to include also specimens that were in a simplex stage without buccal tube (about 22% of the data) we only used body length in our analyses. Gut content was analyzed at 20X magnification and classified as empty, medium (part of gut filled with brownish coloration material) or full (gut filled with black material). Note that these analyses of gut content represent a different estimate than that made on all animals prior to rehydration (dark gut/yellow gut).

Storage cell shape and size were estimated from 20 randomly selected cells per individual (and from different parts of the bodies). Cell size was calculated as the mean of two diagonal right angle diameter measurements for each cell. From the diameter measurement cell volume was calculated (assuming a spherical shape) and used in the analysis. Shape of the cells was characterized for each examined individual as regular (spherical) or irregular (crescent or borders not straight). There were no specimens with mixed cell shapes; all cells in an animal were either regular or irregular.

In order to calculate the mitotic index (proportion of all storage cells that were in mitosis) at the individual level, the total number of clearly recognizable storage cells was estimated manually at 100X magnification with phase contrast and oil, using image analysis software (see above). Smashed cells, without clear borders, were not counted. Mitotic storage cells (S2 Fig) were identified from the presence of condensed chromosomes.

In total 420 specimens were used in the experiment, 400 specimens in the repeated desiccation sequence and 20 as controls. Mitosis and morphometric analyses were performed on 80 specimens.

Statistical analyses

Statistical effects on survival of repeated desiccations were tested using ANOVA and linear regression analysis (IBM SPSS Statistics v.23). Proportion data were arcsin-transformed before statistical analyses. In tests where parametric assumptions were not met, non-parametric statistics were used. Associations between mitosis occurrence and desiccation cycles/phenotypic traits were analyzed with logistic regression. Statistical tests were considered significant when $P < 0.05$, and marginally significant when $P = 0.05$.

Results

Survival rates after repeated desiccations

Survival rates differed significantly among the repeated desiccations ($F_{5,54} = 20.96$, $P < 0.001$; Fig 1 and Table A in S1 File) and the trend was a clear and significant decline in survival rate with increased number of desiccation cycles (linear regression; $F_{1,58} = 87.62$, $r = -0.78$,

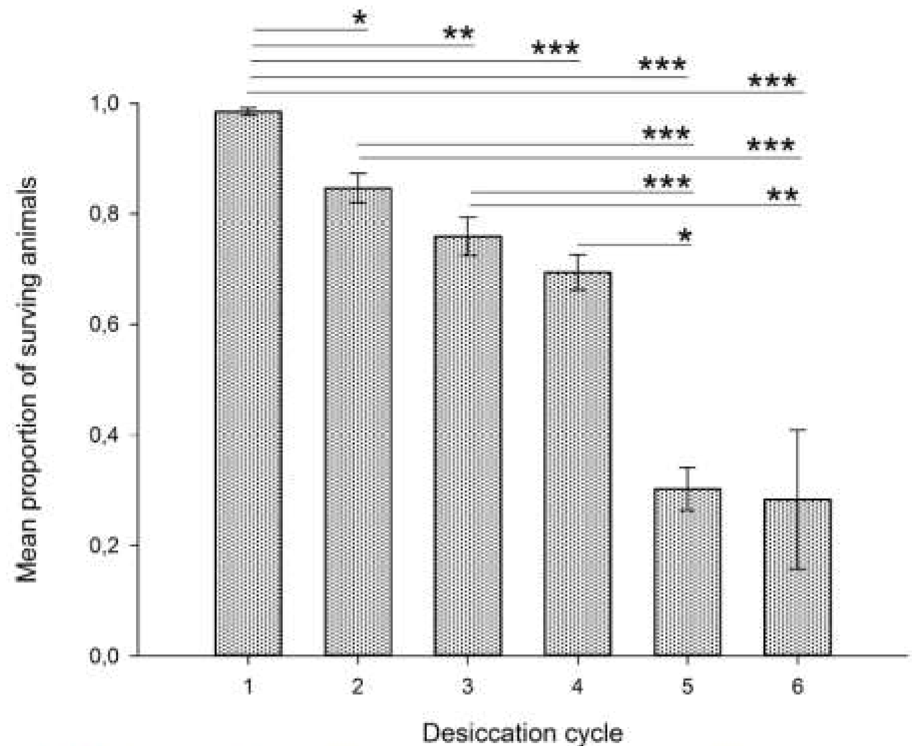

Fig 1. Mean survival rate after different number of repeated desiccations. Error bars indicate standard error of the mean. Significant differences at P<0.05 (*), P<0.01 (**) and P<0.001 (***) are indicated.

doi:10.1371/journal.pone.0164062.g001

$P < 0.001$). The highest survival rate was recorded after the first desiccation (98.5%) and the lowest non-zero survival rate was recorded after the sixth desiccation (28.6%). After the 6th desiccation only 10 specimens were alive and all of these were used for morphometric and mitosis analyses.

Associations between repeated desiccations and phenotypic traits based on examination of all specimens after each desiccation cycle

The proportion of animals that were in a semi-tun stage after the 24h desiccation differed significantly among desiccation cycle groups ($F_{5,54} = 8.96$, $P = 0.000$), with desiccation groups 5 and 6 having significantly more semi-tuns than most of the other groups (Fig 2 and Table A in S1 File). A similar pattern was observed for animals that were in an extended state after desiccation but within-group variation was high and there were no significant differences among desiccation groups ($F_{5,54} = 1.57$, $P = 0.19$; Fig 2 and Table A in S1 File). The proportion of animals with dark gut content after desiccation differed significantly among desiccation cycle groups (Kruskal-Wallis; $\chi^2 = 15.0$, df = 5, $P = 0.010$; Fig 3 and Table A in S1 File), with the 1st and 2nd desiccation group having a significantly lower proportion of specimens with dark guts compared to groups 4–6.

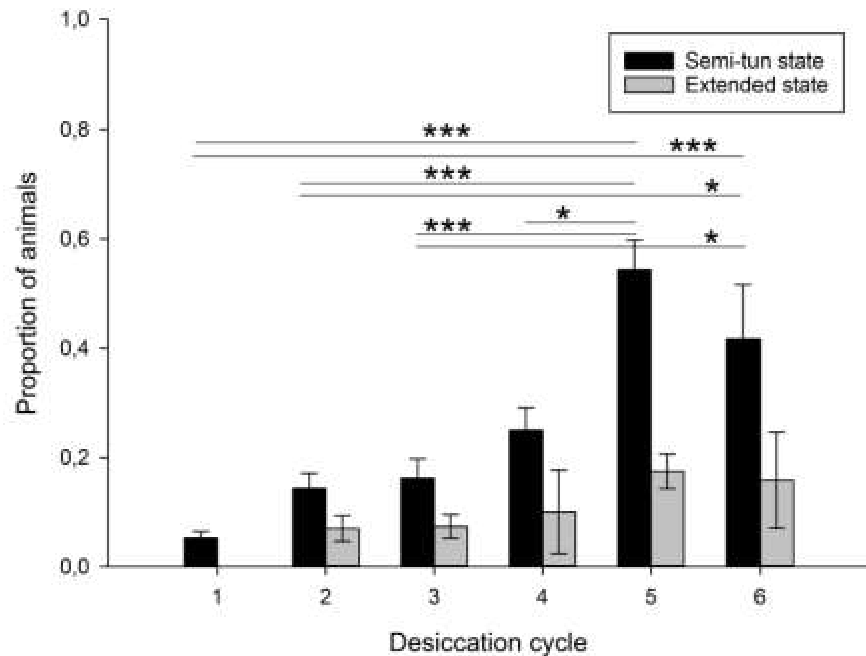

Fig 2. Mean proportion of animals that were in semi-tun and extended states after each respective desiccation cycle. Error bars indicate standard error of the mean. Horizontal bars indicating significances refer to the semi-tun data. Significance levels of P<0.05 (*) and P<0.001 (***) are indicated.

doi:10.1371/journal.pone.0164062.g002

Associations between repeated desiccations and phenotypic traits based on 10 selected specimens after each desiccation cycle

There was an overall significant difference in the mean number of storage cells among desiccation cycle groups, including also the control group ($F_{6,72}$ = 3.91, P = 0.002; Fig 4 and Table B in S1 File). This difference was exclusively due to significantly lower number of storage cells in animals exposed to five (P = 0.015) and six (P = 0.004) desiccation cycles, compared to the control animals. No significant differences were found among desiccation cycle groups (including controls) with respect to body length ($F_{6,73}$ = 2.07, P = 0.067; Table B in S1 File) or storage cell volume ($F_{6,69}$ = 0.99, P = 0.44; Fig 4 and Table B in S1 File). The storage cells had regular shape in almost all of the specimens, and only one control specimen and two specimens of the first desiccation group were characterized as having irregular cells. The proportion of animals in different gut content categories did not differ significantly among desiccation cycle groups (χ^2 = 18.4, df = 12, P = 0.10), and there was no trend in gut content in relation to number of desiccation cycles (Spearman´s rank Analysis; r_s = 0.10, N = 80, P = 0.37).

Frequency of mitosis in storage cells

Storage cells undergoing mitosis were found in 17.5% (14/80) of all examined specimens. The average percentage of mitotic storage cells among all cells ("mitotic index") was 0.28% (SD = 1.25, n = 79) when all analyzed animals (also zero-values) were included, and 1.60% (SD = 2.65, 0.11–10.19%, n = 14) when only animals where mitosis was found were included.

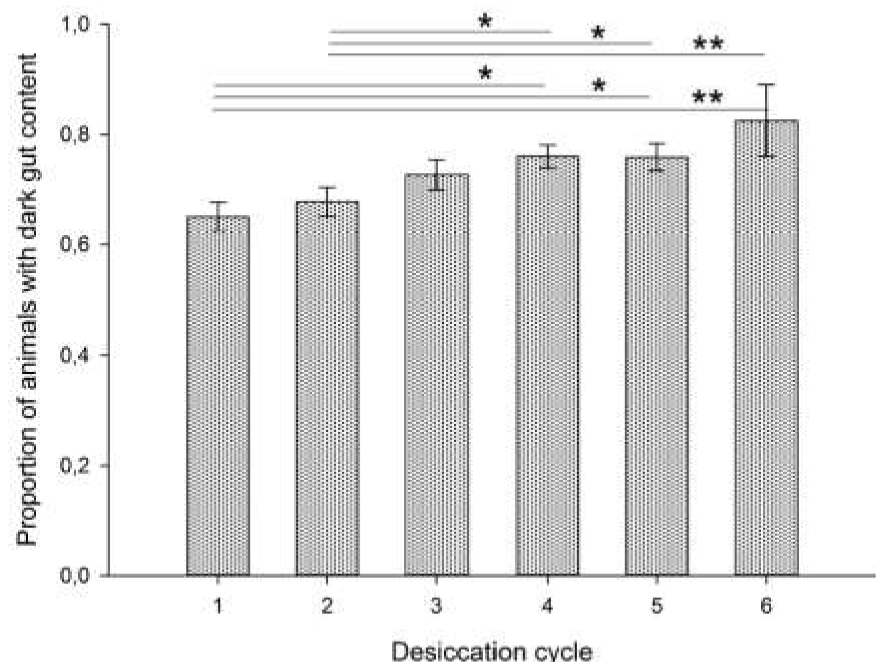

Fig 3. Mean proportion of animals with dark gut content after each respective desiccation cycle. Error bars indicate standard error of the mean. Significant differences at P<0.05 (*) and P<0.01 (**) are indicated.

doi:10.1371/journal.pone.0164062.g003

The proportion of animals with mitotic storage cells in each desiccation cycle varied between 0 and 0.4 (Table 1). Occurrence of mitotic cells in an individual specimen was not related to storage cell volume (Wald = 0.39, df = 1, P = 0.53), gut content (Wald = 3.02, df = 2, P = 0.22), or body length (Wald = 0.040, df = 1, P = 0.84), but was significantly negatively associated with number of desiccation cycles (ß = –0.32, Wald = 3.89, df = 1, P = 0.049) and positively associated with storage cell number (ß = 0.003, Wald = 4.064, df = 1, P = 0.044). Animals with no mitotic cells had on average 699 (SD = 221) storage cells, while animals with mitotic cells had 832 (SD = 155) storage cells. There was a significant negative correlation between the mitotic index for an animal and the number of repeated desiccations that it had experienced (r_s = –0.23, P = 0.041, n = 79, Table 1). Thus, the frequency of mitosis tended to decline with the number of desiccation cycles and increase with number of storage cells. Since both mitosis occurrence and storage cell number tended to decline with number of repeated desiccations (see previous section), it is difficult to disentangle the causal relationships between mitosis, storage cell number and number of repeated desiccations.

Discussion

Our study shows that tardigrades of the species *R. coronifer* are able to survive a maximum of 6 repeated desiccations under non-cultured conditions, with declining survival rates as the number of desiccations increased. This result is slightly lower than in the study by Baumann [18] who reported animals surviving up to 9 desiccation cycles. Fig 5 compares these two studies with respect to the proportion of the *initial population* of animals surviving after different

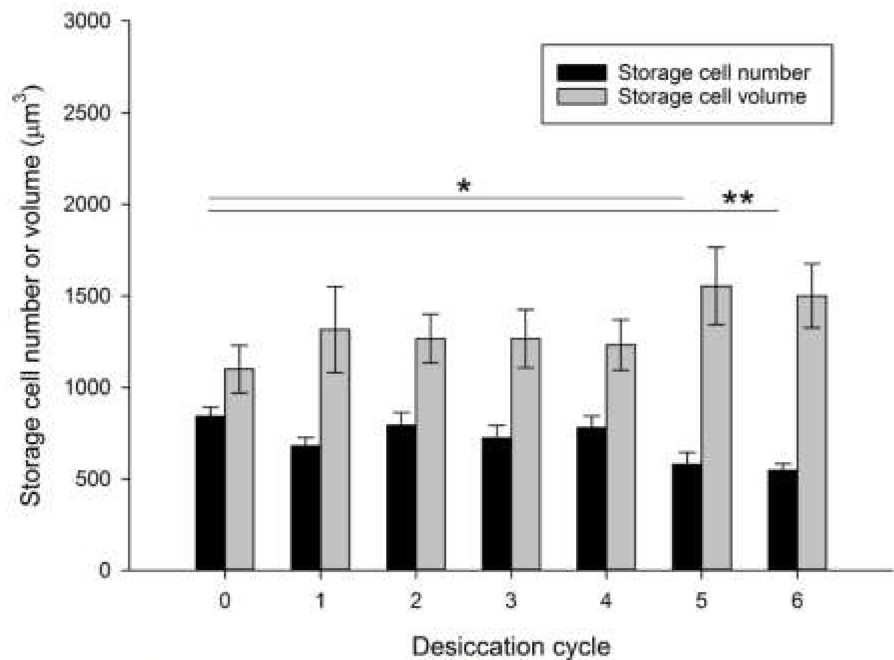

Fig 4. Mean number of storage cells and storage cell volume (μm^3) in tardigrades exposed to different number of desiccation cycles. Error bars indicate standard error of the mean. Horizontal bars indicating significances refer to the storage cell number data. Significant differences at P<0.05 (*) and P<0.01 (**) are indicated.

doi:10.1371/journal.pone.0164062.g004

numbers of desiccation cycles. Since we removed 10 specimens after each desiccation cycle, the proportion of survivals in relation to the initial population were calculated based on the specific desiccation cycle survival rate (given in Table A in S1 File) and the number of animals entering each cycle had we not removed those 10 surviving specimens. Both figures suggest that *R. coronifer* has a steeper decline in survival rate with increased number of desiccation cycles.

Table 1. Proportion of specimens with mitotic storage cells and calculated mitotic index (no. mitotic cells/total number of cells per individual) for different desiccation cycle groups.

Number of desiccations	Proportion of specimens with mitotic storage cells	Mean mitotic index(SD)
0	0.32	0.21 (0.60)
1	0.40	0.57 (1.05)
2	0	0
3	0	0
4	0.20	1.16 (3.21)
5	0.20	0.12 (0.35)
6	0	0

The estimates of mitotic index include also individuals with no mitotic cells (mitotic index = 0). Controls (0 desiccations) include estimates from 19 specimens for proportion specimens with mitotic cells and 20 specimens for mitotic index, while the 1–6 desiccations include 10 specimens.

doi:10.1371/journal.pone.0164062.t001

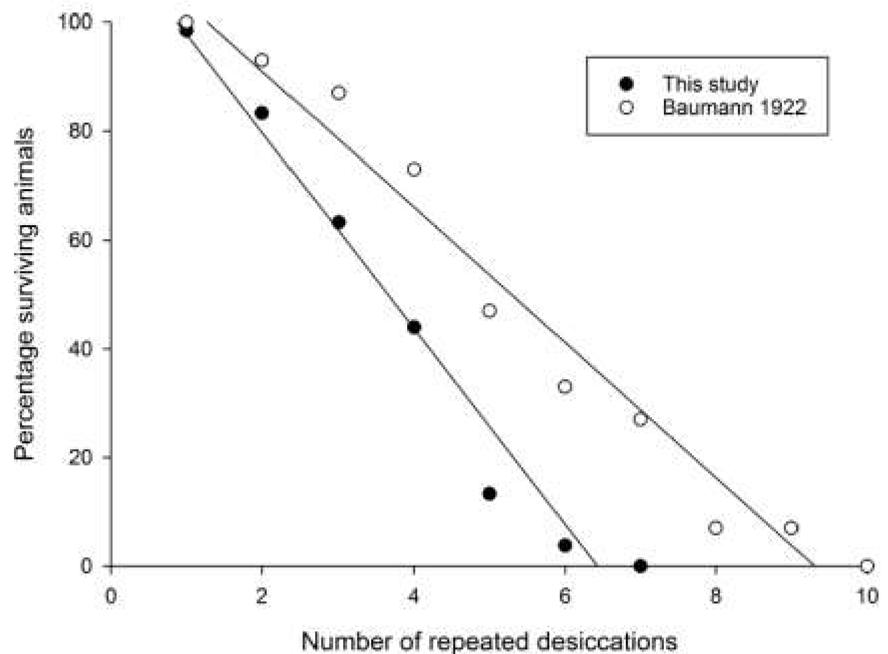

Fig 5. Percentage surviving tardigrades after repeated number of desiccations estimated in the current study and in that reported by Baumann [18]. Note that survival data from the current study were calculated based on estimated rates of survival for each desiccation cycle, not on the original number of animals, since 10 surviving animals were removed for analysis after each desiccation cycle. Initial number of animals: 400 in this study, 15 in Baumann´s study [18]. See main text for more detailed explanation.

doi:10.1371/journal.pone.0164062.g005

compared to Baumann [18]. However, Baumann [18] only used 15 animals in total, and the last desiccation cycles included very few animals (the 9th only one animal, which survived!). He also used a different desiccation-hydration schedule, with 24 hour desiccations (at 35–45% relative humidity), and 15 minute rehydrations. His results must therefore be taken with some caution. In contrast to Baumann [18] and the current study, Hengherr et al. [20] did not observe a decline in the probability to survive a cycle of anhydrobiosis over 9 consecutive desiccations (recovery rates were 88–100%), but the animals were allowed to feed and replenish their energy stores for 7 days between the desiccations, which naturally could have influenced the results.

The decline in the probability of a tardigrade to survive a desiccation cycle with number of previous desiccations suggests a progressive deterioration in the functions underlying anhydrobiosis. One plausible explanation for this limitation on multiple desiccations is that the animals eventually reach an energetic constraint, where the physiological processes necessary for the transition to and from the anhydrobiotic state is not supported by available energy. In support of this explanation our analyses showed a significant reduction in the number of storage cells after the fifth desiccation, indicating reabsorption of cells. Reabsorption of storage cells was also reported by Węglarska [25] as an effect of starvation in specimens of the eutardigrade *Dactylobiotus dispar* Murray, 1907 (formerly: *Macrobiotus dispar*). Howeverowever, data from another experiment in *R. coronifer* in which animals were starved for several days under

continuously hydrated conditions at room temperature did not show a significant decline in number of storage cells (Czernekova & Jönsson, unpublished data). It is therefore not likely that the reduced storage cell number in this study was related to starvation during the short (5 hour) repeated periods of hydration.

Energy depletion connected to anhydrobiosis is also expected to lead to reduced size of the storage cells, as previously shown by Jönsson and Rebecchi [15]. In that study, storage cell area decreased by 14% over a single desiccation cycle in *R. coronifer*, corresponding to a decrease in cell volume by 22%. Decreased storage cell size has also been documented in the late phase of oogenesis, when energy investment into the eggs are high [15,26]. At this phase, food intake is prevented partly due to body cavity constraints, and partly because the feeding apparatus is expelled in connection with the onset of moulting (a stage called "simplex"). Reuner et al. [16] also reported significantly reduced storage cell size in three eutardigrade species (*M. tardigradum*, *Paramacrobiotus tonollii* and *Macrobiotus sapiens)* in response to 7 days of starvation, and in *M. tardigradum* in response to one cycle of anhydrobiosis. However, the number of storage cells in that study [16] was not affected neither by starvation nor by a period of anhydrobiosis. In our study, no significant reduction in cell size was found, even after several desiccation cycles. We also found no evidence that the energy depositions represented by gut content were depleted by repeated desiccation, a finding that was also reported by Baumann [18] in his study on repeated desiccation. Several previous studies on *R. coronifer* show that this species is able to survive continuous starvation at room temperature for many days and even weeks [27,28]. The declining survival rates after multiple desiccations therefore cannot be due to depletion of energy from the hydrated periods. The energy demands and role of storage cells in anhydrobiotic survival of *R. coronifer* therefore remains unclear.

The decline in survival with more desiccation cycles, particularly clear in the 5th and 6th cycle, corresponded with a higher proportion of animals that were unable to contract properly and create tuns when they were desiccated. This was also observed by Baumann [18], who suggested that the animals after a number (4 to 7) of repeated desiccations were unable to produce a cuticular secretion that prevented too rapid desiccation, resulting in uncontrolled contraction of the body and its organs and therefore incomplete tun formation. Our results on changes in body morphology over repeated desiccations are thus fully compatible with those of Baumann [18], but we did not evaluate any characteristics of the cuticle. Whether the failed tun formation after repeated desiccations is related to incomplete cuticular secretion remains to be studied, and ultrastructure analyses of cuticle and epidermis in animals exposed to different number of repeated desiccations would be highly interesting in this context.

The overall frequency of mitosis found in this study was similar to that reported in Czernekova and Jönsson [29], who found mitosis in 18.3% of the adult individuals of *R. coronifer*, and a mitotic index of 1.47% (based on individuals where mitotic cells were found). In that study it was also found that a higher frequency of mitosis was connected with the period of moulting which usually corresponds to the late phase of egg development. As mentioned above, that period is also characterized by smaller storage cells [15,26], but neither in the study by Czernekova and Jönsson [29] nor in the current study was cell size found to be associated with frequency of mitosis. Instead, the frequency of mitosis in storage cells tended to decline with the number of repeated desiccations and the total number of storage cells. Since the latter variable also declined with the number of repeated desiccations it is difficult to know if storage cell number directly affected mitosis frequency, or if both mitosis frequency and storage cell number were influenced by some other common factor related to repeated desiccation. However, the possibility that frequency of mitosis in storage cell is stimulated by energetic stress receives no support from our study, since this would predict an increase in mitosis with repeated desiccations, rather than the observed decrease.

In conclusion, our study clearly shows that the ability of *Richtersius coronifer* to enter and successfully leave the anhydrobiotic state declines with the number of previous anhydrobiotic cycles experienced, thus verifying a limit of how many times this tardigrade can enter anhydrobiosis in a row. The causal explanation behind this decline in anhydrobiotic performance is unclear, but increased inability to morphologically rearrange the body into a proper tun seems to be involved. The ultimate physiological or energetic reason for this remains to be documented.

Compliance with Ethical Standards

Ethical approval: This chapter does not contain any studies with human participants performed by any of the authors.

Supporting Information

S1 Fig. a) Two desiccated specimens of *Richtersius coronifer* after proper tun formation. b) A desiccated specimens of *Richtersius coronifer* after semi-tun formation. c) A desiccated specimens of *Richtersius coronifer* in an extended (non-tun) state.
(JPG)

S2 Fig. Mitotic storage cell of *Richtersius coronifer*. Scale bar: 10μm.
(TIF)

S1 File. Table A. Survival estimates for different number of repeated desiccation cycles, and the number and percentage of specimens that were in semi-tun or extended state. [a] The total number of specimens were divided into 10 replicate samples. [b] Survival was evaluated after 3 and 5 hours of rehydration, except for specimens that were chosen for morphometry and mitosis analyses, which were evaluated 3 hours after rehydration. [c] Specimens with irregular tun were characterized as "semi-tun stage". [d] Specimens that did not contract during desiccation. See Method section for more information. **Table B. Mean number and size (diameter) of storage cells after repeated cycles of desiccations.** Estimates for group 0 (controls) were based on 19–20 specimens, while estimates for the other groups were based on 10 specimens. Four outliers with cell diameters > 20 um were removed from the data; two from group 0 (5440 and 8662 um^3), one from group 1 (4571 um^3), and one from group 6 (10409 um^3).
(DOC)

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Formal analysis: MC IJ.

Funding acquisition: MC IJ.

Investigation: MC IJ.

Methodology: MC IJ.

Project administration: MC IJ.

Resources: MC IJ.

Software: MC IJ.

Supervision: MC IJ.

Validation: MC IJ.

Visualization: MC IJ.

Writing – original draft: MC IJ.

Writing – review & editing: MC IJ.

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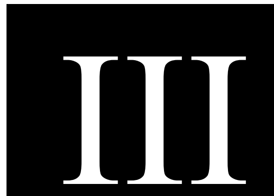
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Protoplasma (2017) 254:1367–1377
DOI 10.1007/s00709-016-1027-2

ORIGINAL ARTICLE

The structure of the desiccated *Richtersius coronifer* (Richters, 1903)

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Received: 26 May 2016 / Accepted: 12 September 2016 / Published online: 27 September 2016
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Abstract Tun formation is an essential morphological adaptation for entering the anhydrobiotic state in tardigrades, but its internal structure has rarely been investigated. We present the structure and ultrastructure of organs and cells in desiccated *Richtersius coronifer* by transmission and scanning electron microscopy, confocal microscopy, and histochemical methods. A 3D reconstruction of the body organization of the tun stage is also presented. The tun formation during anhydrobiosis of tardigrades is a process of anterior-posterior body contraction, which relocates some organs such as the pharyngeal bulb. The cuticle is composed of epicuticle, intracuticle and procuticle; flocculent coat; and trilaminate layer. Moulting does not seem to restrict the tun formation, as evidenced from tardigrade tuns that were in the process of moulting. The storage cells of desiccated specimens filled up the free inner space and surrounded internal organs, such as the ovary and digestive system, which were contracted. All cells (epidermal cells, storage cells, ovary cells, cells of the digestive system) underwent shrinkage, and their cytoplasm was electron dense. Lipids and polysaccharides dominated among reserve material of storage cells, while the amount of protein was small. The basic morphology of specific cell types and organelles did not differ between active and anhydrobiotic *R. coronifer*.

Keywords Anhydrobiosis · Cryptobiosis · Tardigrades · Tun · Ultrastructure

Handling Editor: Douglas Chandler

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Introduction

Among many adaptive survival strategies that organisms have evolved in response to harsh environmental conditions, the ability to survive in a state of complete metabolic arrest must be considered one of the most extreme. This state is called cryptobiosis and has fascinated biologists from the time when such organisms were first discovered in the early eighteenth century (for review, see Keilin 1959; Crowe 1975). Four types of cryptobiosis are traditionally recognized—desiccation (anhydrobiosis), freezing (cryobiosis), lack of oxygen (anoxybiosis) and high salt concentration (osmobiosis) (Keilin 1959)—of which anhydrobiosis is considered as the most common (Crowe 1975; Jönsson 2001). While most animals have limited ability to survive water loss (Watanabe 2006), species in some invertebrate taxa are able to survive complete dehydration.

These include micro-metazoans like bdelloid rotifers, nematodes and tardigrades, which have the ability to repeatedly enter anhydrobiosis at any developmental stage (Keilin 1959; Crowe 1975) and some dipteran larvae such as those of the chironomid *Polypedilum vanderplanki* (e.g. Kikawada et al. 2005). When exposed to desiccation, nematodes coil into a tight spiral (Wharton and Lemmon 1998), while tardigrades and rotifers form a "tun" in order to reduce the body surface area and, thereby, the rate of

evaporation (Baumann 1922; Kinchin 1994; Marotta et al. 2010). These morphological changes connected with dehydration represent an important aspect of the adaptation to dry conditions and are essential for successfully entering the anhydrobiotic state.

Several studies on the ultrastructure of the body wall of hydrated tardigrades have been reported (Baccetti and Rosati 1969, 1971; Crowe et al. 1971; Walz 1979, 1982; Guidetti et al. 2000; Greven et al. 2005), while studies on the tardigrade cuticle in the tun stage are scarcer (but see; Wright 1988; Szymańska 1995). A recent study by Halberg et al. (2013) described the tun morphology of the eutardigrade *Richtersius coronifer* (Richters, 1903), with special emphasis on muscular organization. Also, a few cytological studies have been reported on anhydrobiotic tardigrade tissues and cells, particularly storage cells (May 1949; Węglarska 1957; Crowe 1975).

In order to improve our understanding of the morphological characteristics of the desiccated state in tardigrades, we have investigated the internal morphology of desiccated organs, tissues and cells of a eutardigrade with well-documented ability to enter anhydrobiosis. We examined epidermal, ovarian, gut and storage cell structures with transmission electron microscopy and histochemical techniques. The study includes the first analysis of cuticle organization in moulting and non-moulting tardigrades in the tun stage. We also present a 3D reconstruction of the body organization of a tardigrade in the tun stage.

Material and methods

Material

In this study, we used the eutardigrade *R. coronifer* (Richters, 1903), a species with well-documented anhydrobiotic ability (Westh and Ramløv 1991; Ramløv and Westh 1992). This species can sometimes measure up to 1000 µm and has a cosmopolitan distribution, mainly limited to mosses in alpine regions (Ramazotti and Maucci 1983; Westh and Kristensen 1992). However, a relict population is also found in Alvar habitat of the Baltic Sea island Öland (Sweden), from which specimens for the current studies were collected. This population consists almost exclusively of females (Rebecchi et al. 2003).

Mosses (*Orthotrichum cupulatum*) containing tardigrades were collected in a dry condition and were kept in plastic bags in a freezer (−20 °C) until used. The mosses were hydrated for about 2 h in tap water and tardigrades then extracted with sieves (mesh size 250 and 40 µm) under running tap water. Extracted animals were washed thoroughly with distilled water to remove adherent particles.

Methods

Anhydrobiotic induction

Extracted specimens were dehydrated individually on small squares (5 cm^2) of a filter paper at 95 % relative humidity (RH) using a saturated salt solution (KNO_3) in a closed container. This procedure has been used in several previous studies (e.g. Jönsson et al. 2008) and ensures that the animals enter the anhydrobiotic tun state under controlled conditions with slow dehydration. After 24 h, the filter papers with dehydrated specimens (tuns) were enclosed in small plastic bags and kept in these conditions for 0.5 up to 2 months until preparation for microscopy.

Light and transmission electron microscopy

Twenty tuns were fixed with 2.5 % glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.4) (overnight at 4 °C) and postfixed with 2 % OsO_4 in 0.1 M phosphate buffer (2 h at room temperature). Dehydration and embedding were performed as described earlier (Rost-Roszkowska et al. 2013a, b; Poprawa et al. 2015c). Semi-thin and ultrathin sections were cut on a Leica ultracut UCT25 ultramicrotome. Semi-thin sections (700 nm thick) were stained with 1 % methylene blue in 0.5 % borax (Dykstra 1992) and examined with an Olympus BX60 microscope. Ultrathin sections (50 nm thick) mounted on the Formvar-covered grids (50 mesh) were stained with uranyl acetate and lead citrate (Reynolds 1963) and examined with the transmission electron microscope (Hitachi H500 at 75 kV).

Scanning electron microscopy

Ten tuns, fixed with glutaraldehyde as described above, were dehydrated in a graded concentration series of ethanol (10, 20, 30, 40, 50, 60, 70, 80, 90, 96, 100 % each for 2 min) and acetone (25, 50, 75, 100 % each for 2 min), dried at critical point in a Pelco CPD2, mounted on an aluminium stub and coated with gold in a Pelco SC-6 duster. Tuns were then examined with a Hitachi UHR FE-SEM SU 8010 scanning electron microscope.

Histochemical staining

Detection of polysaccharides (PAS method) Semi-thin sections were treated with a 2 % solution of periodic acid to remove osmium (10 min at room temperature) and stained with Schiff's reagent (24 h, 37 °C) (Litwin 1985). Slides were washed in tap water and observed with an Olympus BX60 microscope.

Detection of proteins (Bonhag's method) Semi-thin sections were treated with a 1 % solution of periodic acid to remove osmium (10 min at room temperature) and stained with bromophenol blue (BPB) (24 h, 37 °C) (Litwin 1985). Slides were washed in tap water and observed with an Olympus BX60 microscope.

Detection of lipids Semi-thin sections were stained with Sudan black B (15 min at room temperature) and washed with 50 % ethanol and distilled water (Litwin 1985). Slides were observed with an Olympus BX60 microscope.

BODIPY 494/503 detection of lipids The cuticles of ten desiccated specimens were punctured with a thin wolfram needle for better penetration of reagent inside the body. Samples were fixed with 2.5 % paraformaldehyde in Tris-buffered saline (TBS) (45 min, room temperature). After washing in TBS, staining with 20 μg/ml BODIPY 493/503 (Molecular Probes) (30 min in darkness/room temperature) was performed. After washing in TBS, specimens were counterstained with Hoechst 33342 staining (1 μg/ml, 20 min, room temperature), washed in TBS and whole-mounted on microscopic slides. Specimens were analyzed with an Olympus FluoView FV 1000 confocal microscope. Excitation at 493 nm was provided by an argon/krypton laser.

3D reconstruction Three-dimensional reconstruction of a tun was based on series of semi-thin sections. For these reconstructions, one tun fixed as described above (see "Methods" section: Light and transmission electron microscopy) was cut into series of semi-thin sections (500 nm thick). Sections were stained with 1 % methylene blue in 0.5 % borax, sequentially photographed with an Olympus XC50 digital camera mounted on an Olympus BX60 light microscope and archived as TIFF files using CellSens Standard software. The TrakEM2 plugin of ImageJ software (NIH, USA) was used to align the images in image stacks (correct order and correct position). Next IMARIS 8.2 software (Bitplane) was used for preparing the three-dimensional reconstruction. The surfaces of the tun and its internal organs were obtained using the surface creation tool of this program, which calculates the contour lines that are defined manually. The contour line for each visualized organ was placed independently using different colours.

Results

Gross morphology

Tun formation is a process of anterior-posterior contraction and longitudinal and intersegmental cuticle infolding (Fig. 1a, b). The head and limbs are also contracted and form foldings in some cuticular areas, but claws remain visible (Fig. 1a, arrows). The dorsal part of the tun is convex, while the ventral part is flattened (Fig. 1a, c, d) due to substrate attachment on the filter paper, where the specimens were dried. The 3D reconstruction revealed organ contraction and relocation. Figure 1c shows cluster of storage cells enclosing the inner organs, and Fig. 1d shows internal organs of the same specimen. In the following, we describe tissue and cell organization in more details.

The tissue organization of tun

Tun and body wall morphology

During the tun formation, loss of body fluid moves the internal organs towards the body centre, which becomes surrounded by storage cells (Fig. 1b–d). The contraction in the tun stage showed some individual morphological variability, influenced by the animal size and stage of oogenesis (Fig. 2a, b). Analysis of fine tissue structures showed contraction limitation caused by the length of the rigid stylet (Fig. 2a, b). The pharyngeal bulb is relocated in the ventromedial body plane, but the degree of relocation depends on the ovary size (Figs. 1d and 2a, b).

The tun wall was composed of cuticle and epidermis (Fig. 2c–e). The epidermis was formed by a simple squamous epithelium suspended by the basal lamina (Fig. 2c, d, black arrows). The epidermal cells were shrunken. The electron-dense cytoplasm contained ribosomes, mitochondria and large spheres of medium electron density (in some cases, the spheres were visible as electron-transparent vacuoles because lipids were partly removed during fixation) (Fig. 2c–e, asterisks). The cuticle of desiccated specimens was composed of epicuticle (ect) with discontinuous flocculent coat, intracuticle (ict) and procuticle (pct) (Fig. 2c, e). The thick pct had medium electron density, while the thinner ict was electron dense (Fig. 2c, e). The ect was composed of two layers: an inner ect of lower electron density (1) and a medium electron-dense outer ect (2) (Fig. 2e). The inner trilaminate layer separating ict from ect was hardly distinguishable (Fig. 2e, arrow). In two of the examined tuns, we observed a double cuticle (Fig. 2d), indicating an ongoing moulting process. Moulting bodies of different sizes and electron densities (electron-transparent and medium electron dense) were present in the cytoplasm of the epidermal cells (Fig. 2d, white arrows). Between the old pct and new ect, a layer with electron-dense granules was observed (Fig. 2d). A similar layer was present between the old pct and the old ict (Fig. 2d).

The ovary

During the tun formation, the single, sac-like ovary undergoes contraction (Figs. 1d and 2a, b). The size of the contracted ovary depended on the stage of oogenesis (Fig. 2a, b). The

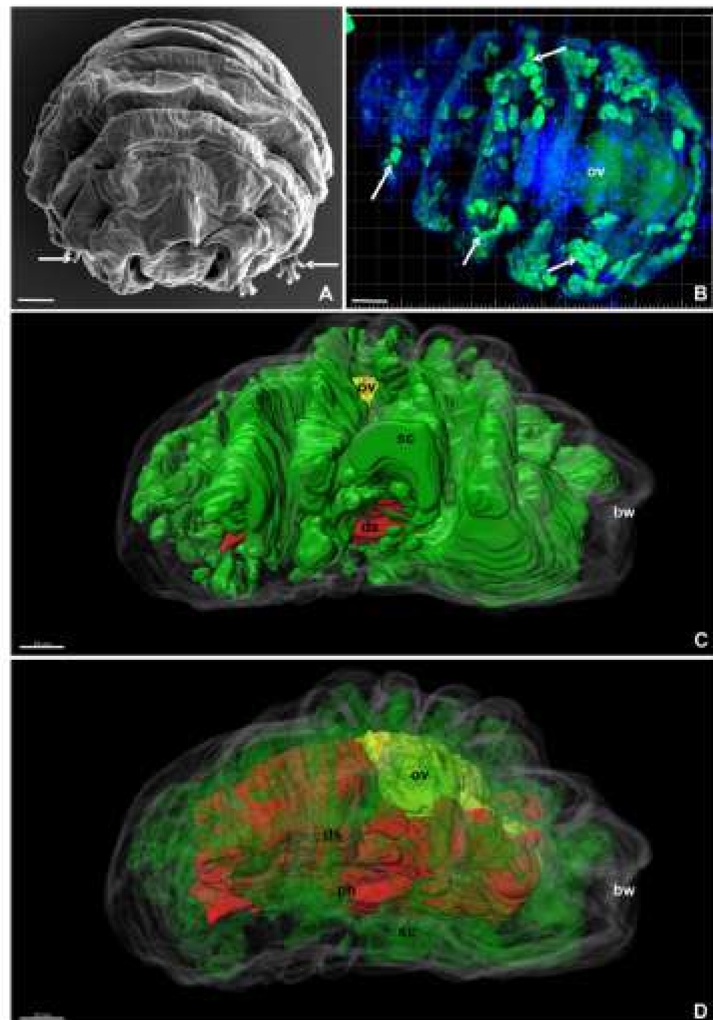

Fig. 1 Structure of the desiccated *Richtersius coronifer*. Morphology of the tun. **a** Tun of *R. coronifer*—anterior view: (*arrow*) claws. SEM, *bar* = 20 μm. **b** Tun stained with BODIPY 493/503 and DAPI: lipids in the storage cells are stained with green (*arrows*), *ov* ovary. Confocal microscope, *bar* = 20 μm. **c** 3D reconstruction of the tun: *bw* body wall, *ds* digestive system, *ov* ovary, *sc* storage cells, *bar* = 20 μm. **d** 3D reconstruction of the tun (the same specimen as in Fig. 2c): *bw* body wall, *ds* digestive system, *ov* ovary, *ph* pharynx, *sc* storage cells, *bar* = 20 μm

ovary wall was composed of the simple squamous epithelium situated on the basal lamina (Fig. 3a, b, black arrows). The cells of the gonad (somatic cells of the gonad wall, oocytes and trophocytes) were shrunken, except for oocytes surrounded by completely developed egg shells (Fig. 3a, b). The cytoplasm of the germ cells and the cells of the ovary wall was electron dense. Only nucleus (n), mitochondria (m) and reserve material (yolk) (rm, y) were clearly visible in the cytoplasm (Fig. 3a, b). Cisterns of rough endoplasmic reticulum and Golgi complexes were difficult to distinguish (Fig. 3b, white arrow).

The midgut

The digestive system was contracted, and the buccopharyngeal apparatus was relocated (Figs. 1d and 2a, b). The midgut lumen was narrow, and the midgut wall did not form folds (Fig. 3a). The shrunken cells of the midgut epithelium were located on the basal lamina. The basal membrane formed small folds, while the apical membrane formed microvilli (Fig. 3a). A peritrophic membrane was not observed. The cytoplasm of the midgut epithelium cells was electron dense. Mitochondria, nucleus, autophagosomes (au) and medium electron-dense spheres of the reserve material (rm) were visible in the cytoplasm of these cells, while the cisterns of rough endoplasmic reticulum (RER) and Golgi complexes were difficult to distinguish (Fig. 3a).

The storage cells

The storage cells of desiccated specimens filled up almost all the free inner space and, thus, surrounded the inner organs

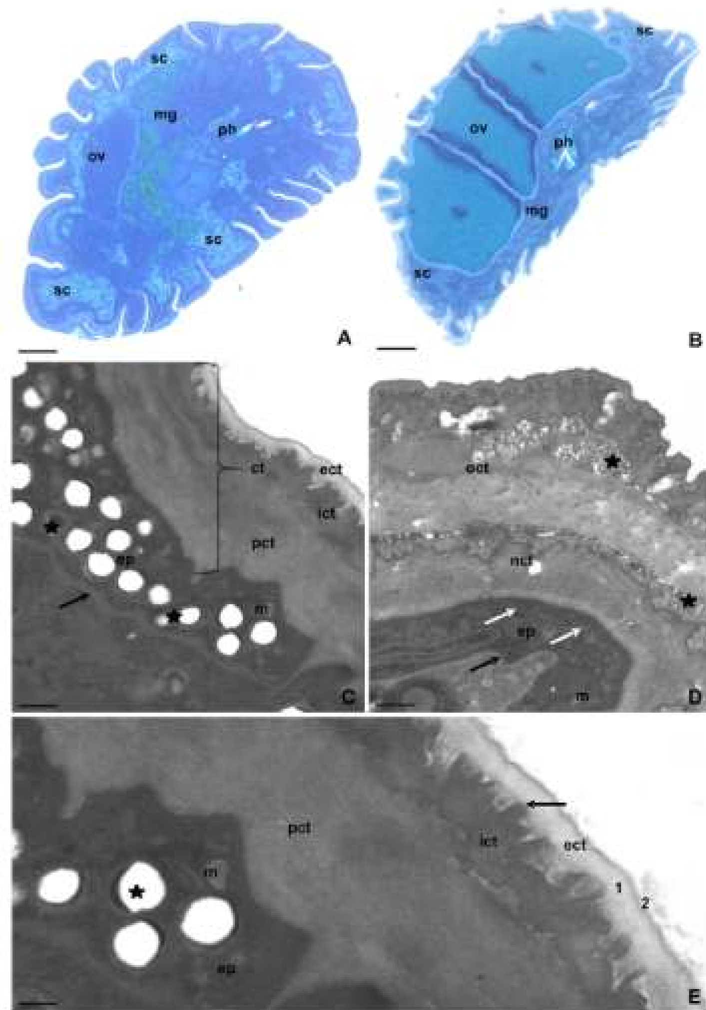

Fig. 2 Structure and ultrastructure of desiccated *Richtersius coronifer*. Morphology of the tun–body wall ultrastructure. **a**, **b** A longitudinal section through the tun: *mg* midgut, *ov* ovary, *ph* pharynx, *sc* storage cell. Methylene blue staining, LM. **a** Early oogenesis, *bar* = 23 μm. **b** Late oogenesis, *bar* = 27 μm. **c** Body wall of the tun composed of *ep* epidermis and *ct* cuticle: *ect* epicuticle, *ict* intracuticle, *m* mitochondrion, *pct* procuticle, *arrow* basal lamina, *stars* medium electron-dense spheres. TEM, *bar* = 0.6 μm. **d** Body wall of the tun with double cuticle (tun formation during moulting): *ep* epidermis, *m* mitochondrion, *nct* "new" cuticle, *oct* "old cuticle," *stars* electron-dense granules, *white arrows* moulting bodies, *black arrow* basal lamina. TEM, *bar* = 0.8 μm. **e** Higher magnification of the Fig. 3c: *ect* epicuticle, *ep* epidermis, *ict* intracuticle, *m* mitochondrium, *pct* procuticle, *1* inner epicuticle, *2* outer epicuticle, *arrow* trilaminate layer, *asterisk* medium electron-dense spheres. TEM, *bar* = 0.3 μm

(Figs. 1b–d and 2a, b). These cells had an amoeboid shape (Figs. 1b and 4a). The centre of each cell was occupied by a large irregular nucleus (Fig. 4a, n). The dark heterochromatin masses and nucleolus (nu) were embedded in a low electron-dense nucleoplasmic matrix. The electron-dense cytoplasm contained organelles, such as free ribosomes, mitochondria, cisterns of RER and Golgi complexes; however, the endoplasmic reticulum and Golgi complexes were difficult to distinguish (Fig. 4a). Some autophagosomes au were observed (Fig. 4a). Moreover, spheres of reserve material rm of different sizes and electron densities were present in the whole cytoplasm of the storage cells (Fig. 4a). Four types of spheres were distinguished: small (arrow) and large (rm1) homogenous spheres of high electron density and small (rm2) and large (rm3) non-homogenous spheres of medium electron density (Fig. 4a).

Histochemical analysis of the rm revealed the presence of large amount of lipid (Figs. 1b and 4b) and polysaccharide (Fig. 4c), while the amount of protein was small (Fig. 4d).

Discussion

Tun and body wall morphology

Our observations of semi-thin sections of tuns showed that organs such as the pharyngeal bulb were relocated as body fluid evaporated during desiccation. This is in line with observations by Halberg et al. (2013), who showed that the degree of longitudinal contraction during tun formation is individually variable but ultimately limited by the length of the rigid stylet as

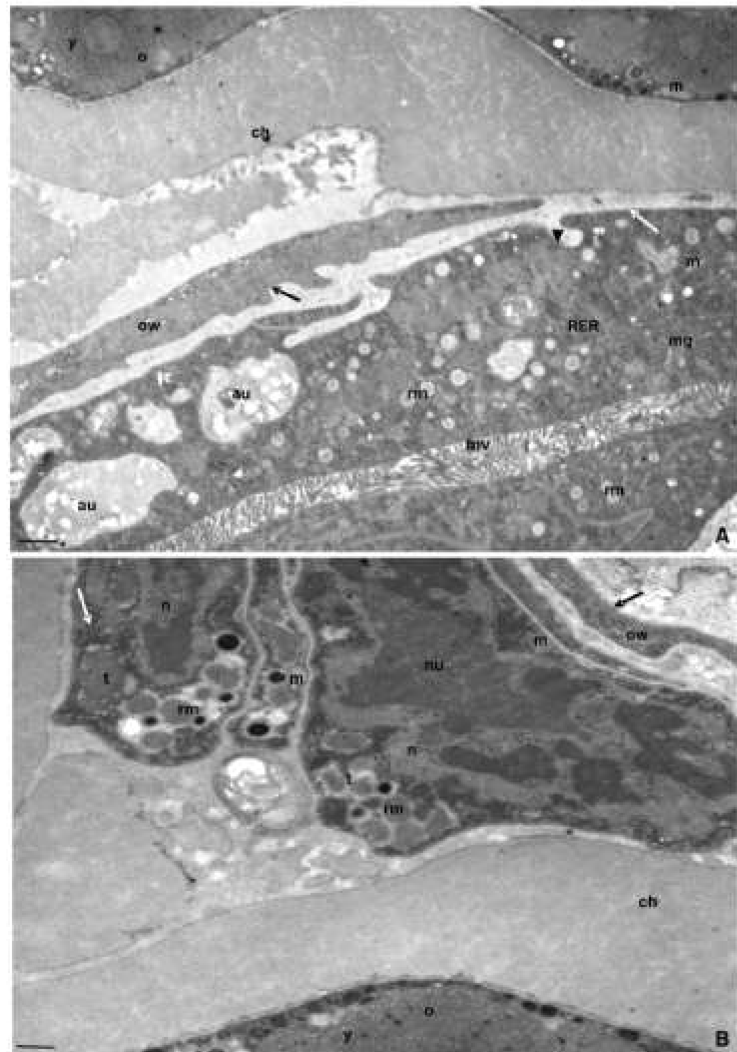

Fig. 3 Ultrastructure of the desiccated *Richtersius coronifer*. Ultrastructure of the ovary and midgut. **a** A longitudinal section through the middle region of tardigrade body: *au* autophagosome, *ch* chorion, *l* midgut lumen, *mv* microvilli, *mg* midgut, *m* mitochondrion, *o* oocyte, *ow* ovary wall, *RER* cisterns of rough endoplasmic reticulum, *rm* reserve material, *y* yolk inside the oocyte, *white arrow* basal lamina of the midgut epithelium, *black arrow* basal lamina of the ovary wall, *arrowhead* folds of the basal membrane of the digestive cells, TEM, *bar* = 1 μm. **b** A longitudinal section through the ovary: *ch* chorion, *m* mitochondrion, *nu* nucleolus, *n* nucleus, *o* oocyte, *ow* ovary wall, *rm* reserve material, *t* trophocyte, *y* yolk inside the oocyte, *white arrow* cisterns of rough endoplasmic reticulum, *black arrow* basal lamina. TEM, *bar* = 0.8 μm

pharyngeal bulb is repositioned. It is likely that tun formation could be limited also by stage of oogenesis, especially when the large oocytes are covered with chorion. To what extent organ relocation during tun formation affects anhydrobiotic survival remains to be studied; however, it seems that controlled rigorous packing of internal structures is crucial (Ricci et al. 2003; Halberg et al. 2013).

We observed cuticular foldings as flexion zones with SEM and also on semi- and ultrathin sections. The foldings might be a natural consequence of contraction in order to expand effectively the tardigrade's body after rehydration, as observed in new cuticles of moulting *Paramacrobiotus areolatus* (Murray, 1907) (Walz et al. 1971) and *Hypsibius convergens* (Urbanowicz, 1925) (Baumann 1961), where foldings allow expansion of the specimen's body after ecdysis. An alternative functional explanation might be derived from the observation of thick and thin cuticular areas in specimens of *P. areolatus* (Crowe et al. 1971; Walz et al. 1971; Walz 1982). The fine structure of both thin and thick parts is identical, but the thicker parts are in contact with the air, while the thinner and higher permeable cuticular parts are drawn into the body, out of contact with the surrounding air; therefore, the functional role of different thicknesses is related to water loss (Crowe and Madin 1974; 1975; Crowe et al. 1971; Crowe 1975). Similar differences in cuticular water permeability have been suggested in rotifers (Ricci et al. 2003). Both explanations could apply for dehydrated *R. coronifer*; however, the absolute thickness of the cuticular layers is difficult to assess accurately as sectioning angles are difficult to determine (Wright 1988; Kinchin 1994).

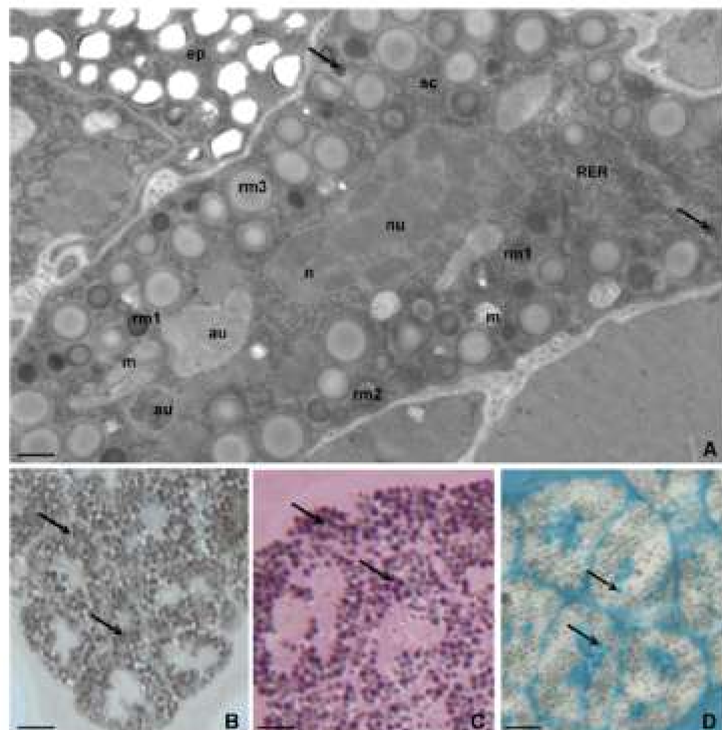

Fig. 4 Srtucture and ultrastructure of desiccated *Richtersius coronifer*. Storage cells of *R. coronifer*. **a** Ultrastructure of the storage cell: *au* autophagosome; *ep* epidermis; *m* mitochondrion; *nu* nucleolus; *n* nucleus; *RER* cisterns of rough endoplasmic reticulum; *rm1*, *rm2*, *rm3*, *arrows* reserve material; *sc* storage cell. TEM, *bar* = 0.7 μm. **b–d** Histochemical staining of the storage cells of *R. coronifer*; *arrows* indicate a positive reaction. LM. **b** Sudan black B staining, *bar* = 6 μm. **c** PAS method, *bar* = 4 μm. **d** BPB staining, *bar* = 5.5 μm

We report double cuticles and, thus, ongoing moulting in tuns, verifying that the old cuticle is retained during anhydrobiosis. Moulted cuticles have been shown to improve desiccation survival in nematodes (e.g. Gaur and Perry 1991) by slowing the rate of drying of the enclosed animals. Studies evaluating the impact of moulting on anhydrobiotic survival are currently lacking.

Generally, all epidermal cells of most tardigrades possess pigment granules, except the regions of muscle attachment (Greven 1980; Greven et al. 2005). In contrast to active specimens of *R. coronifer*, pigment granules were not observed in desiccated ones. Possibly, pigments are utilized in water barrier layers during anhydrobiosis. Alternatively, they might be denatured in process of dehydration. In bacterial cells, pigment oxidation and colour change during desiccation have been observed, and in cyanobacteria, even short-term drying leads to structural changes in the pigment antenna complexes and long-term drying leads to destruction of phycobiliproteins (Potts 1994).

Lipid droplets in epidermal cells of *R. coronifer* were observed also in *Milnesium tardigradum* (Doyère, 1840) (Dewel et al. 1993) and *Macrobiotus hufelandi* (Baccetti and Rosati 1971; Walz 1982). The function of these vacuoles has been connected with discharging of their content to the cuticle and lipid supply to the ict and wax layer, which provide a barrier to water exchange (Baccetti and Rosati 1971). In active and fully hydrated *R. coronifer*, no wax layer was observed (Guidetti et al. 2000). However, we documented interstitial cuticular layers in desiccated specimens undergoing moulting (see Fig. 2d) similar to the wax layer observed in active and non-moulting *M. hufelandi* (Baccetti and Rosati 1971). Moreover, a discontinuous layer of flocculent material and variable number of thin laminae beneath the ect were present in tuns; however, we have not found this in hydrated specimens of *R. coronifer* (unpublished data). Flocculent coat and the lipid granular layer are not expected to play any important mechanical function (Wright 1988). We did not observe a flocculent coat in moulting tuns with interstitial cuticular layers, but Greven (1974) observed a discontinuous layer of the flocculent or globular coat in active *M. hufelandi*, *Ramazzottius oberhaeuseri* (Doyère, 1840) and *P. areolatus*. The precise function and importance of flocculent coat in anhydrobiosis of tardigrades remain to be studied.

Ovary

The single, sack-like ovary (or hermaphroditic gonad) of tardigrades is located on the dorsal part of the body over the gut (Węglarska 1979, 1987; Poprawa 2005a; Poprawa et al. 2015a, b, c). Its size depends on the animal's age and the stage of oogenesis. At the beginning of oogenesis, the gonad is small and its volume starts to grow significantly during vitellogenesis and

reach the largest size at the end of the chorion formation (Węglarska 1979, 1987; Poprawa 2005a, b; Poprawa et al. 2015b, c). Our analysis of desiccated *R. coronifer* showed that the ovary does not undergo relocation during tun formation. Because of the body contraction during desiccation, the ovary closely adhered to the gut and was surrounded by storage cells, but its organization remained unchanged.

Previous studies have shown that the tardigrade ovary wall is composed of simple squamous epithelium suspended by the basal lamina (Węglarska 1979, 1987; Poprawa 2005a; Poprawa et al. 2015a, b, c). The cytoplasm of the epithelial cells is rich in organelles indicating high metabolic and secretory activity. These cells are responsible for synthesis and secretion of the chorion precursors (Węglarska 1982; Poprawa 2005b; Poprawa et al. 2015b, c). The cells of the ovary wall in desiccated *R. coronifer* were shrunken, and their cytoplasm was electron dense. Since metabolism is arrested during anhydrobiosis, we did not observe any secretory activity in cells of the ovary wall. The apical membrane of the cells of the ovary wall in many species forms protrusions that penetrate into the ovary lumen among the germ cells (Węglarska 1979; Poprawa 2005a, Poprawa et al. 2015b, c). We did not observe such protrusions in the gonad of analyzed specimens. It is difficult to say if this difference was caused by cell shrinkage during desiccation because nothing is known about the ultrastructure of the ovary wall in an active *R. coronifer*.

The tardigrade ovary type is meroistic, which means that, during oogenesis, germ cell clusters are formed (Węglarska 1979; Poprawa 2005a; Suzuki 2006, Poprawa et al. (2015b, c). In each cluster, one of the cells (or several cells in *M. tardigradum*, Suzuki 2006) develops into an oocyte, whereas the remaining cells become trophocytes, which support oocytes. In active animals, the cytoplasm of oocytes and trophocytes is rich in organelles responsible for yolk precursor synthesis and in spheres of yolk material (Węglarska 1979, 1987; Poprawa 2005a; Poprawa et al. 2015a, b, c). In desiccated *R. coronifer*, the contraction of the trophocytes was large, whereas the oocyte's contraction depended on the stage of oogenesis. Young (previtellogenic, early and middle vitellogenic stage) oocytes shrank similarly to the trophocytes. The contraction of older, fully developed chorion-covered oocytes was limited by this egg shell.

Midgut

The midgut of active eutardigrades has a tube-like shape (Greven 1976; Ząbczyk 2000; Avdonina et al. 2007; Rost-Roszkowska et al. 2010, 2013a, b; Biserova and Mustafina 2015; Hyra et al. 2016a). Generally, distinct anterior and posterior regions can be distinguished in this organ; however, in some species, for example, in *Dactylobiotus dispar* (Ząbczyk, 2000), the midgut is divided in three parts: promesenteron, mesomesenteron and metamesenteron. The midgut ultrastructure can vary according to animal nutritional conditions (Greven 1976). Biserova and Mustafina (2015) described structure and ultrastructure of the midgut in active *R. coronifer* 48 h after rehydration, however without note on specimen's nutritional conditions. They did not distinguish distinct regions in the analyzed organ, but they did observe a lack of midgut wall folding. The folding of the midgut wall is very common among tardigrades (Avdonina et al. 2007; Rost-Roszkowska et al. 2013a, b; Biserova and Mustafina 2015). Nevertheless, in line with a study by Biserova and Mustafina (2015), we also did not observe any foldings of the midgut wall in desiccated *R. coronifer*.

The midgut of many invertebrates possesses a peritrophic membrane (Loeb et al. 2001; Martin et al. 2006; Rost-Roszkowska and Undrul 2008; Grigoryeva 2010; Rost-Roszkowska et al. 2010). This anatomical structure can have a fluid (peritrophic gel) or membrane (peritrophic membrane) form which surrounds the food mass (Terra 2001; Grigorieva and Amosova 2004; Martin et al. 2006; Rost-Roszkowska and Undrul 2008; Grigoryeva 2010; Rost-Roszkowska et al. 2010) and protects the midgut epithelium against microorganisms and food abrasion. A peritrophic membrane was described in some species of tardigrades: *Halobiotus stenostomus* (Biserova and Mustafina 2015) and *Ramazzottius tribulosus* (Avdonina et al. 2007) and also in *R. coronifer* (Biserova and Mustafina 2015). However, in our analysis of desiccated specimens of *R. coronifer*, we did not observe a peritrophic membrane. This can be explained in two ways. The peritrophic membrane might be removed during tun formation; since animals do not eat and metabolism is arrested, the structure is therefore useless and might be synthesized de novo after rehydration. Alternatively, the membrane may be present only during certain periods of the animal's lifetime. This issue requires further research.

According to Biserova and Mustafina (2015), the cytoplasm of the digestive cells (enterocytes) is lucent and contains many different-sized coated vesicles, small electron-dense granules, rare cisterns of RER, a few ribosomes and large phagosomes with heterogeneous material. Enterocyte cytoplasm of desiccated *R. coronifer* appeared completely different. First of all, cell contraction during tun formation resulted in increased electron density of the cytoplasm. Moreover, we did not observe coated vesicles, the result of endocytosis, in the cytoplasm of enterocytes. During tun stage, the metabolism is arrested; therefore, the process of endocytosis is also arrested. Organelles such as cisterns of RER and Golgi complexes were barely visible as a result of increased cytoplasmic density of the digestive cells in tuns.

The ultrastructure of storage cells

Our study on desiccated specimens shows that storage cells occupy almost all of the free inner space and thus obscure the inner organs (compare confocal micrograph of storage cells of hydrated specimens shown in Hyra et al. 2016b).

Generally, dried storage cells were shrunken but preserved their membranes and organelles. We also observed an increase in nuclear heterochromatin together with partial loss of nucleoli in desiccated cells. This may imply reduction of nuclear activity at transcriptional and translational levels. As heterochromatin protects DNA against radiation damage, it can diminish the accessibility of radicals to the DNA (Falk et al. 2008). This was ascribed for *M. hufelandi* (Crowe 1975) and also bdelloid rotifers (May 1946; Walz 1979; Marotta et al. 2010). In contrast, Crowe (1975) and Walz (1979) observed distinct nucleoli in desiccated tardigrades of the species *P. areolatus*. Moreover, cell organelles appeared intact in dead as well as dried animals (Crowe 1975), suggesting that desiccation injury is connected with changes at biochemical level rather than physical disruption of membranes and organelles.

Our study showed an amoeboid shape of desiccated cells, while hydrated cells were more circular. The main function of storage cells is to store rm, and previous studies have shown that storage cells accumulate polysaccharides, lipids and proteins (Rosati 1968; Węglarska 1975; Dewel et al. 1993; Kinchin 1993, 1994; Szymańska 1994; Poprawa 2006; Reuner et al. 2010; Hyra et al. 2016b). This was confirmed in active specimens of *Paramacrobiotus richtersi* (Murray, 1911) (Szymańska 1994), *D. dispar* (Poprawa 2006), *Hypsibius dujardini* (Doyère, 1840), *Isohypsibius granulifer granulifer* (Thulin, 1928), *Macrobiotus polonicus* (Pilato, Kaczmarek, Michalczyk & Lisi, 2003) and *Xerobiotus pseudohufelandi* (Iharos, 1966) (Hyra et al. 2016b), and our study confirms this for desiccated *R. coronifer*. We observed spheres of rm of different sizes and electron densities throughout the cytoplasm of "dried" storage cells. Lipids and polysaccharides dominated in all observed material. Protein spheres, although present in both desiccated and hydrated specimens, were considerably reduced in numbers in desiccated specimens. Węglarska (1957) reported lipid and glycogen content in body cavity cells of *D. dispar* (Murray, 1907), and Crowe (1975) reported that storage cells of active well-fed *P. areolatus* contained both lipids and glycogen, while cells of anhydrobiotic specimens did not contain glycogen, indicating that this is utilized during dehydration (Crowe 1975). Also, in the nematode *Aphelenchus avenae* (Batian, 1865), accumulation of trehalose and glycerol, components which role in desiccation-tolerant animals has been much discussed but without a final conclusion (e.g. Crowe et al., 2005), during anhydrobiotic induction, was associated with lipid and glycogen decrease.

In conclusion, our study confirms that (a) midgut of the desiccated specimens of *R. coronifer* has no peritrophic membrane; (b) the desiccated epidermal cells have no pigment granules; (c) basic morphologies of specific cell types and organelles in *R. coronifer* do not generally differ between cells of active and anhydrobiotic tardigrades; (d) instead, cell cytoplasms appear with increased electron density due to water loss; and (e) no physical disruption of cell membranes and organelles due to anhydrobiosis, but the cell organelle morphology (lack of smooth and coated vesicles, narrow lumen of cisterns of RER) reflects the metabolic inactivity of the desiccated state.

Acknowledgments We would like to express our gratitude to Dr Danuta Urbańska-Jasik (University of Silesia, Katowice, Poland) and Dr Jagna Karcz (University of Silesia, Katowice, Poland) for their technical assistance.

Funding This study was funded by RVO: 67985823, the Mobility Fund, Charles University in Prague, and by the POIG.02.01.00-00-166/08 and POIG.02.03.01-24-099/13 grants.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors. All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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IV

RESEARCH ARTICLE

A comparative ultrastructure study of storage cells in the eutardigrade *Richtersius coronifer* in the hydrated state and after desiccation and heating stress

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OPEN ACCESS

Citation: Czerneková M, Janelt K, Student S, Jönsson KI, Poprawa I (2018) A comparative ultrastructure study of storage cells in the eutardigrade *Richtersius coronifer* in the hydrated state and after desiccation and heating stress. PLoS ONE 13(8): e0201430. https://doi.org/10.1371/journal.pone.0201430

Editor: Michael Klymkowsky, University of Colorado Boulder, UNITED STATES

Received: February 27, 2018

Accepted: July 16, 2018

Published: August 10, 2018

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Data Availability Statement: All relevant data are within the manuscript and Supporting Information files.

Funding: This work was supported by the Academy of Sciences of the Czech Republic (RVO: 67985823) (to MC), the Mobility Fund of Charles University in Prague (Czech Republic) (to MC), and the Kristianstad University (Sweden) (to KIJ). Microscopic analysis (confocal microscopy) was performed using the infrastructure that is

Abstract

Tardigrades represent an invertebrate phylum with no circulatory or respiratory system. Their body cavity is filled with free storage cells of the coelomocyte-type, which are responsible for important physiological functions. We report a study comparing the ultrastructure of storage cells in anhydrobiotic and hydrated specimens of the eutardigrade *Richtersius coronifer*. We also analysed the effect of temperature stress on storage cell structure. Firstly, we verified two types of ultrastructurally different storage cells, which differ in cellular organelle complexity, amount and content of reserve material and connection to oogenetic stage. Type I cells were found to differ ultrastructurally depending on the oogenetic stage of the animal. The main function of these cells is energy storage. Storage cells of Type I were also observed in the single male that was found among the analysed specimens. The second cell type, Type II, found only in females, represents young undifferentiated cells, possibly stem cells. The two types of cells also differ with respect to the presence of nucleolar vacuoles, which are related to oogenetic stages and to changes in nucleolic activity during oogenesis. Secondly, this study revealed that storage cells are not ultrastructurally affected by six months of desiccation or by heating following this desiccation period. However, heating of the desiccated animals (tuns) tended to reduce animal survival, indicating that long-term desiccation makes these animals more vulnerable to heat stress. We confirmed the degradative pathways during the rehydration process after desiccation and heat stress. Our study is the first to document two ultrastructurally different types of storage cells in tardigrades and reveals new perspectives for further studies of tardigrade storage cells.

supported by the POIG.02.01.00-00-166/08 (to IP) and POIG.02.03.01-24-099/13 grant (to IP).

Competing interests: The authors have declared that no competing interests exist.

Introduction

Tardigrades represent an invertebrate phylum with many species that have evolved adaptations to survive extreme levels of dehydration and freezing [1, 2, 3, 4, 5, 6]. This has allowed them to inhabit some of the harshest environments on Earth (e.g., continental Antarctica), as well as equally extreme microhabitats in other areas (e.g., sun-exposed lichens and moss on rocks) [7, 8]. Tardigrades do not possess circulatory or respiratory systems, but their body cavity is filled with storage (or body cavity) cells, which float freely in the body cavity lymph [7, 8] or sometimes adhere to the basement membrane of other tissues [7]. These storage cells are responsible for important physiological functions, primarily nutrient transport and storage of mainly lipids but also polysaccharides and pigments such as carotenes [9, 10]. They also produce protein substances, which are gathered inside with lipid globules [10], and in some tardigrade species, vitellogenins are developed in the storage cells [11, 12]. Their energy storage function is well illustrated by the change in cell size over the oocyte maturation cycle, during which the cells grow in size from the early to the middle part of the cycle and decrease in size towards the end of the cycle as the energy demand of the developing eggs increases [11, 12, 13]. A similar pattern has been shown for the amount of energy reserve material in the cells [14]. Declines in storage cell size connected with a period of anhydrobiosis have been reported (*Richtersius coronifer* (Richters, 1903) [13]; *Milnesium tardigradum* (Doyère, 1840) [15]). However Czerneková and Jönsson [16] did not observe such changes after repeated periods of anhydrobiosis in *R. coronifer*.

Storage cells have also been used to study of DNA damage induced by desiccation. Neumann et al. [17] documented DNA fragmentation in storage cells of *M. tardigradum* after periods in the anhydrobiotic state and showed that fragmentation increased with time spent in the dry state (from 2 days to 10 months). Since many limnoterrestrial tardigrades are able to revive successfully after years of anhydrobiosis [18, 19] these animals seem to have an extraordinary capacity to repair the damage that arises and is accumulated during the dry state. However, the extent to which storage cells are damaged ultrastructurally after long-term anhydrobiosis or exposure to other stressors remains to be documented.

High temperature is an agent that may disrupt cell structures such as membranes, DNA and proteins. Relatively few studies have evaluated thermotolerance in tardigrades. In the hydrated state an upper tolerance level of 36°C and 38°C after 24 h exposure was reported in *Borealibius zetlandicus* (Murray, 1907) [20] and in *Macrobiotus harmsworthi* (Murray, 1907), respectively [21]. In the anhydrobiotic state short-term (1 h) heat tolerance is considerably higher, and tolerances up to approximately 100°C have been reported [22], but variations in tolerance among tardigrade species are considerable [22, 23]. Older studies have reported even higher tolerances (up to 151°C for 30 min. exposure [24]). In *R. coronifer*, the tardigrade used in the present study, 1 h exposure of temperatures up to 70°C did not affect survival, but at 80°C, survival was below 20%, and at 85°C, it was near zero [25]. Most studies on heat tolerance in desiccated tardigrades have used short exposure times (1 h), but Rebecchi et al. [26] exposed anhydrobiotic tardigrades of the species *Paramacrobiotus richtersi* (Murray, 1911) to 37°C at 30–40% RH for up to 21 days, with no effect on survival. However, a separate experiment showed that the survival of dry animals over a 21 day period was inversely related to the relative humidity at which the animals were kept [26]. There were also indications of DNA damage (single-strand breaks) in animals exposed to the highest relative humidities. Analyses of how exposure to heat affects the cell ultrastructure of tardigrades have not been reported.

In this study, we compared the ultrastructure of storage cells in active and anhydrobiotic specimens of the eutardigrade *R. coronifer*. We also examined if storage cell structure was affected by heat stress.

Materials and methods

We used the eutardigrade *R. coronifer* (Fig 1A and 1B), a species belonging to the order Parachela, family Macrobiotidae. This species has well-documented anhydrobiotic ability (e.g., [23, 27, 28, 29]). The specimens were obtained from mosses at the Alvar habitat of the Swedish Baltic Sea island Öland [30]. Previous studies have shown that the population consists almost exclusively of females [30]. More than one tardigrade extraction method was used. Tardigrades were extracted from the sample by soaking dry mosses for 2 up to 4 h in distilled water, followed by mixing and shaking them off. The sediment/water mixture containing tardigrades was poured into cylinders and put aside for half an hour for decantation [31], additionally tardigrades were extracted with sieves (mesh size 250 and 40 μm) under running tap water. Only medium–large size (ca. 0.5–1.0 mm body length) specimens were used. Specimens analysed in the tun stage were desiccated individually on filter paper under 95% relative humidity (RH) using a saturated salt solution (KNO_3) in a closed container at room temperature (see, e.g., [32]). In specimens analysed in the hydrated state, the stage of oogenesis (see, e.g., [12]) was recorded in order to evaluate if storage cell structure differed between oogenesis stages.

I. Non-experimental analyses of storage cells in desiccated and hydrated specimens

Light and transmission electron microscopy. Forty-five active animals and fifteen tuns were fixed with 2.5% glutaraldehyde in a 0.1 M sodium phosphate buffer (pH 7.4, 4°C, 2 h). The material was post-fixed with 2% osmium tetroxide in a 0.1 M phosphate buffer (4°C, 2 h) and washed in a 0.1 M phosphate buffer. After dehydration in increasing concentrations of ethanol (30, 50, 70, 90, 95 and 100%, each for 15 min), a mixture of 100% ethanol and acetone (1:1, 15 min), and acetone (2 x 15 min), the material was embedded in epoxy resin (Epoxy Embedding Medium Kit; Sigma). Semi- (800 nm thick) and ultra-thin (50 nm thick) sections were cut on a Leica Ultracut UCT25 ultramicrotome. Semi-thin sections were stained with 1% methylene blue in 0.5% borax and observed with an Olympus BX60 light microscope. Some of the semi-thin sections (without staining with 1% methylene blue in 0.5% borax) were used for the histochemical methods (see below). Ultra-thin sections were put on formvar-covered copper grids and stained with uranyl acetate and lead citrate. The material was analysed with a Hitachi H500 transmission electron microscope at 75 kV.

Additionally, ultrathin sections from ten hydrated and five desiccated specimens of *R. coronifer* were used in order to evaluate the presence of structurally different storage cells. In each section, 100 randomly selected cells were analysed.

Ultrathin sections of the *R. coronifer* bodies (five active animals and five tuns) were also used to estimate the diameters of storage cells in active animal and in tun. Fifty storage cells in each of five active animals and fifty storage cells in each of five tuns were measured. The active animals and the tuns were at the same stage of oogenesis (late vitellogenesis).

Scanning electron microscopy. Five active animals and five tuns were fixed in 10% ethanol (2 min) and dehydrated in a graded concentration series of ethanol (20, 30, 40, 50, 60, 70, 80, 90, 4 x 100% each for 2 min), followed by a hexamethyldisilazane (HMDS) chemical drying series (ethanol:HMDS at 2:1, 1:1, 1:2 each for 10 min) and 100% HMDS (then allowed to air dry). Dried specimens were mounted on SEM stubs and coated with gold in a Pelco SC-6 duster. The material was examined using a Hitachi UHR FE-SEM SU 8010 scanning electron microscope.

Histochemistry and immunohistochemistry. Detection of polysaccharides (PAS method). Semi-thin sections (from 5 active specimens and 3 tuns) were treated with 2% periodic acid (10 min, room temperature) in order to remove the osmium tetroxide from the

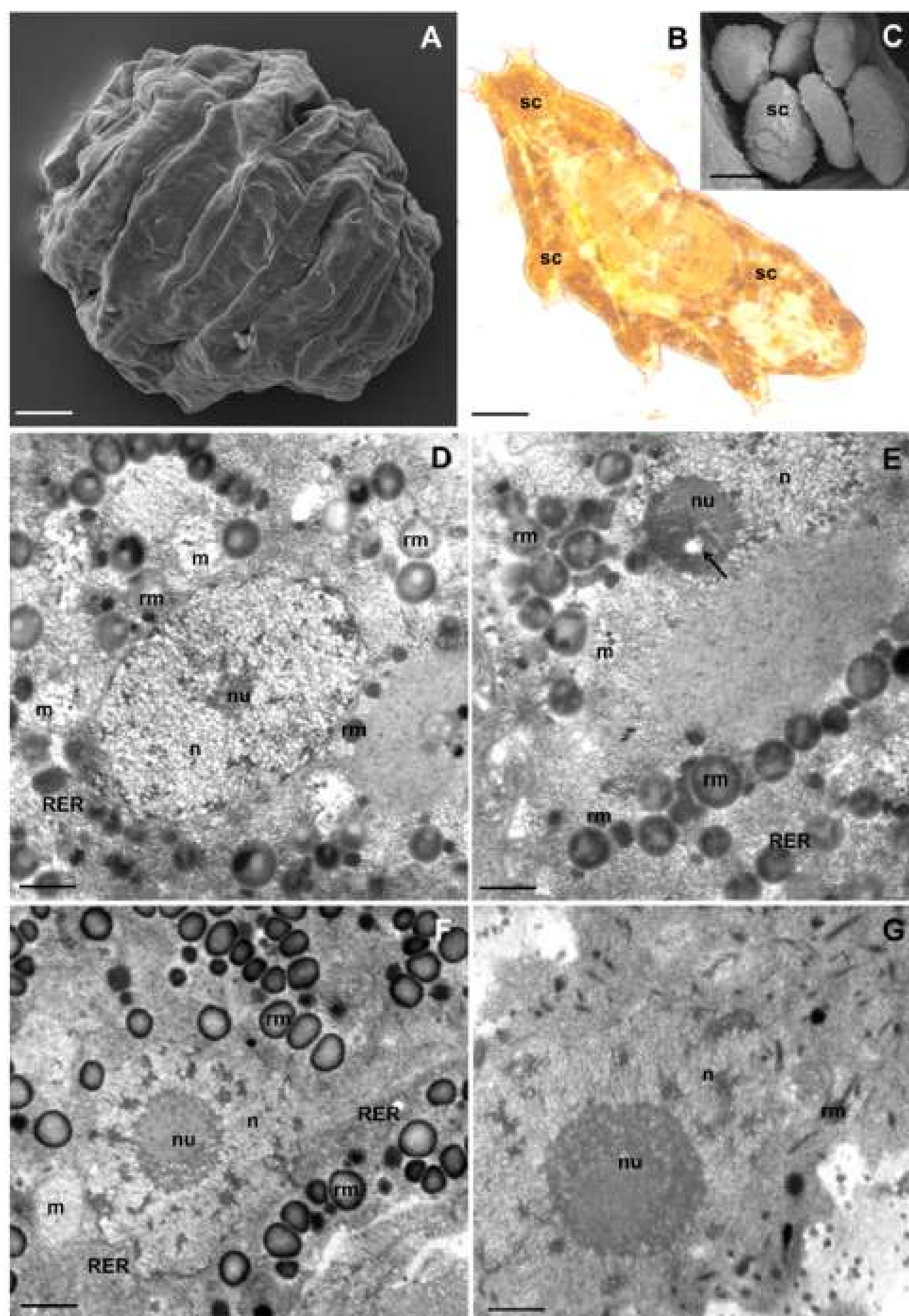
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Fig 1. Storage cells (SC) of *R. coronifer*. (A) Tun, SEM. Bar = 30 μm. (B) Active animal, LM. Bar = 20 μm. (C) Storage cells, SEM. Bar = 4 μm. (D-G) Ultrastructure of SC of non-experimental specimens, TEM: nucleus (n), nucleolus (nu), mitochondria (m), rough endoplasmic reticulum (RER), spheres of reserve material (rm). (D-E). SC of male specimens. (D) Bar = 0.58 μm. (E) Bar = 0.5 μm. (F-G) SC of female specimens. (F) SC of the first type during vitellogenesis, nucleolus vacuole (arrow). Bar = 0.8 μm. (G) SC of the second type. Bar = 0.5 μm.

https://doi.org/10.1371/journal.pone.0201430.g001

tissue, stained with Schiff's reagent for 24 h at 37°C [33] (Litwin, 1985), washed in tap water (15 min) and observed with an Olympus BX60 light microscope.

Detection of proteins (Bonhag's method). Semi-thin sections (from 5 active specimens and 3 tuns) were treated with a 2% solution of periodic acid as in the PAS method, stained with bromophenol blue (BPB) (24 h at 37°C) [33] (Litwin, 1985), washed in tap water (15 min) and observed with an Olympus BX60 light microscope.

Detection of lipids. To detect lipids, semi-thin sections (from 5 active specimens and 3 tuns) were stained with Sudan black B [33] at room temperature for 20 min, washed quickly in 50% ethanol then in distilled water and observed with an Olympus BX60 light microscope.

BODIPY 493/503 –detection of lipids. Ten hydrated specimens and five tuns of *R. coronifer* were punctured with a thin wolfram needle for better penetration of reagents inside the body and fixed with 2.5% paraformaldehyde in TBS (45 min, room temperature). The specimens were then washed in TBS and stained with 20 μg/ml BODIPY 493/503 (Molecular Probes) (30 min in darkness/room temperature). The material was then washed in TBS, stained with Hoechst 33342 (1 μg/ml, 20 min, room temperature), washed in TBS and whole-mounted on microscopic slides. The material was analysed with an Olympus FluoView FV 1000 confocal microscope. Excitation at 493 nm was provided by a multi-line argon laser.

Immunolabelling with anti-phosphohistone H3—a mitotic-specific antibody (for detection of cell proliferation). Ten hydrated specimens of *R. coronifer* were punctured with a thin wolfram needle for better penetration of the chemical reagents. The material was washed with TBS (5 min), 0.1% Triton X-100 in TBS (5 min) and incubated in 1% BSA in TBS (1 h, room temperature) without fixation. The material was then incubated overnight (16 h) in a 1:100 dilution of anti-phosphohistone H3 antibodies (Millipore) in 1% BSA in TBS. After incubation, the specimens were washed twice with TBS (5 min) and then incubated in a 1:200 dilution of goat anti-rabbit IgG Alexa-Fluor 488 conjugated secondary antibody diluted in 1% BSA in TBS (2 h, room temperature in darkness). Afterwards, the specimens were stained with DAPI (1 mg/ml, 20 min, room temperature in darkness). The material was mounted onto slides and analysed with an Olympus FluoView FV1000 confocal microscope. Excitation at 488 nm was provided by an argon/krypton laser.

TUNEL assay (detection of cell death). Ten hydrated specimens of *R. coronifer* were punctured with a thin wolfram needle, incubated in a permeabilization solution (0.1% sodium citrate) (2 min on ice in 4°C) and washed in TBS (3×5 min). The specimens were then stained with a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) reaction mixture (In Situ Cell Death Detection Kit, TMR red, Roche; 60 min at 37°C in the dark). A negative control was prepared according to the labelling protocol. The material was analysed with an Olympus FluoView FV 1000 confocal microscope. Excitation at 594 nm was provided by a multi-line argon laser.

II. Effects of long-term desiccation and heating on storage cell structures

Experimental design. We evaluated ultrastructural changes in storage cells after (i) desiccation of tardigrade specimens for six months and (ii) desiccation of tardigrade specimens for six months + heating at 50°C for 24 h. For both groups, analyses of storage cells were performed both before (i.e., still desiccated specimens) and after rehydration (three and five hours

post-rehydration). Three specimens in each of the four categories (heated desiccated, non-heated desiccated, heated rehydrated, and non-heated rehydrated) were used. In addition, 14 specimens each from category (i) and (ii) were prepared for analysis of survival. To analyse storage cell ultrastructure, we used transmission electron microscope and histochemical methods for detection of lipid, proteins and polysaccharides.

Anhydrobiotic induction, heating and rehydration. Extracted animals were washed thoroughly with distilled water to remove adherent particles. Five hours later the hydrated specimens were dehydrated individually on small squares (5 cm^2) of filter paper at 95% relative humidity (RH) using a saturated salt solution (KNO_3) in a closed container at room temperature. After 24 h, the filter papers with dehydrated specimens were enclosed in small plastic bags and kept in the laboratory (room temperature) for 6 months. Immediately after the 6 month period, specimens in the heating group (animals determined for heating in incubator) were heated in an incubator at 50°C for 24 h. Half of them (n = 14) were then fixed in the desiccated state and prepared for microscopy, and the other half (n = 14) were rehydrated individually in circa 4 ml of distilled water in Petri dishes (60 x 15 mm). The same procedure, except of heating, was used for the non-heated specimens. Specimens used for post-rehydration analyses were rehydrated individually in Petri dishes (60 x 15 cm) with distilled water for 3 or 5 h before fixation for ultrastructure analysis. Specimens used for survival analysis were checked after 3 and 5 h post-rehydration. Animals were recorded as alive if they were active (slowly moving and fully moving or fully active) and were still moving after 2 more hours (5h and 7h).

Light and electron microscopy. Ten desiccated (6 from the experimental and 4 from the control group) and ten rehydrated (6 from the experimental and 4 from the control group) specimens were prepared for analysis with a transmission electron microscope (Hitachi H500 at 75 kV) as described earlier (see I. Non-experimental analyses of storage cells in desiccated and hydrated specimens, light and transmission electron microscopy).

Histochemical analysis. Detection of polysaccharides (PAS method). Semi-thin sections (from 4 active specimens and 3 tuns) were used for detection of polysaccharides. The same method as in the non-experimental study was used; see the description above.

Detection of proteins (Bonhag's method). Semi-thin sections (from 4 active specimens and 3 tuns) were used for detection of proteins. The method was described earlier (see the non-experimental study, the description above).

Detection of lipids. Semi-thin sections (from 4 active specimens and 3 tuns) were used for detection of lipids. The same method as in the non-experimental study was used; see the description above.

Ethics statement: The study did not involve endangered or protected species, and moss samples were not collected within an area where permission was required.

Results

Non-experimental analyses of storage cells

Storage cells of hydrated specimens. The body cavity of *R. coronifer* was filled with fluid and storage cells (Fig 1B). The cells of examined specimens had ameboidal or spherical shapes (Fig 1B and 1C). The average diameter of cells in the five specimens examined for cell size was 15.36 μm (S1 Table, S1 File). Among all analysed specimens (eighty active), we found only one male. All desiccated animals (twenty-five tuns) were females.

Storage cells of the male. Only one type of storage cells (Type I) was observed in the male. These cells had an ameboidal shape. The large nucleus (Fig 1D and 1E) with a non-homogenous nucleolus was located in the centre of each cell (Fig 1E). The nucleolus was composed of two types of material with different electron density. A small nucleolus vacuole with

low electron density was observed in the nucleolus (Fig 1E). The cytoplasm was filled with organelles, such as ribosomes, mitochondria and short cisterns of rough endoplasmic reticulum (Fig 1D and 1E). Moreover, non-homogenous spheres of different size and electron density were observed in the cytoplasm (Fig 1D and 1E). Most of the electron-dense spheres were filled with granules of lower electron density (Fig 1D and 1E). Medium electron-dense spheres and spheres of high electron density were also distinguished in the cytoplasm of the storage cells (Fig 1D and 1E).

Storage cells of females. Two types of storage cells were found in females. The cells of the first type (Fig 1F) were similar to those observed in the male, thus of Type I. Their ultrastructure differed in relation to the stages of oogenesis (see below). The cells of the second type (Type II) had an ameboidal shape (Fig 1G), and their ultrastructure was similar during all stages of oogenesis. The centre of each cell of Type II was occupied by a large lobular nucleus with a large non-homogenous nucleolus. The external part of the nucleolus had a higher electron density than its internal part (Fig 1G). The cytoplasm of these cells was poor in organelles. It contained ribosomes, mitochondria, a few short cisterns of rough endoplasmic reticulum and several small electron-dense granules. Among the observed storage cells, we found on average 7.2% cells of the Type II.

Ultrastructural differences in storage cells of Type I in relation to stage of oogenesis. The process of tardigrade oogenesis can be divided into three major stages: previtellogenesis (organelle accumulation and mRNA synthesis), vitellogenesis (early, middle and late vitellogenesis—yolk synthesis and accumulation) and choriogenesis (egg shells formation) [34, 35, 36, 37]. To see if storage cell structure differed between oogenesis stages, we analysed 10 specimens in previtellogenesis, 24 specimens in vitellogenesis, and 10 specimens in choriogenesis. During previtellogenesis, the central part of each storage cell was occupied by a large nucleus with a large non-homogenous nucleolus (Fig 2A). The internal part of the nucleolus had a lower electron density than its external part. Moreover, a small nucleolus vacuole with a low electron density was present (Fig 2A). At this stage the cytoplasm was filled with ribosomes, short cisterns of rough endoplasmic reticulum, few mitochondria and a small amount of reserve material (Fig 2A). The reserve material had the form of smaller and larger spheres of different electron density. Smaller spheres were electron-dense, while the larger spheres had lower electron density (Fig 2A).

Subsequently, during vitellogenesis, an increase in the number of mitochondria and spheres of the reserve material were observed in the cytoplasm of the storage cells (Fig 1F). The central part of each cell was still occupied by the large nucleus with a large non-homogenous nucleolus. However, the nucleolus vacuole was not observed at this stage (Fig 1F). The stored spheres of the reserve material had different sizes and electron density. Most of the spheres had medium electron density. They possessed a high electron-dense external ring and granules of lower electron density. Moreover, smaller homogenous electron-dense and medium electron-dense spheres were observed (Fig 1F).

During late vitellogenesis and the beginning of choriogenesis the number of mitochondria, cisterns of rough endoplasmic reticulum, and the amount and type of reserve material accumulated in the cytoplasm of the storage cells did not change with respect to the stage of vitellogenesis (Fig 2B). The amount of reserve material decreased significantly at the end of choriogenesis (Fig 2C). Moreover, the number of mitochondria increased at this time. Additionally, some autophagosomes with fibrous medium electron dense material inside them were observed in the cytoplasm (Fig 2C). The amount of reserve material decreased until the end of oviposition. A very small amount of proteins (Fig 2D) and large amounts of polysaccharides (Fig 2E) and lipids (Fig 2F and 2G) were accumulated in the cytoplasm of the storage cells of the analysed species.

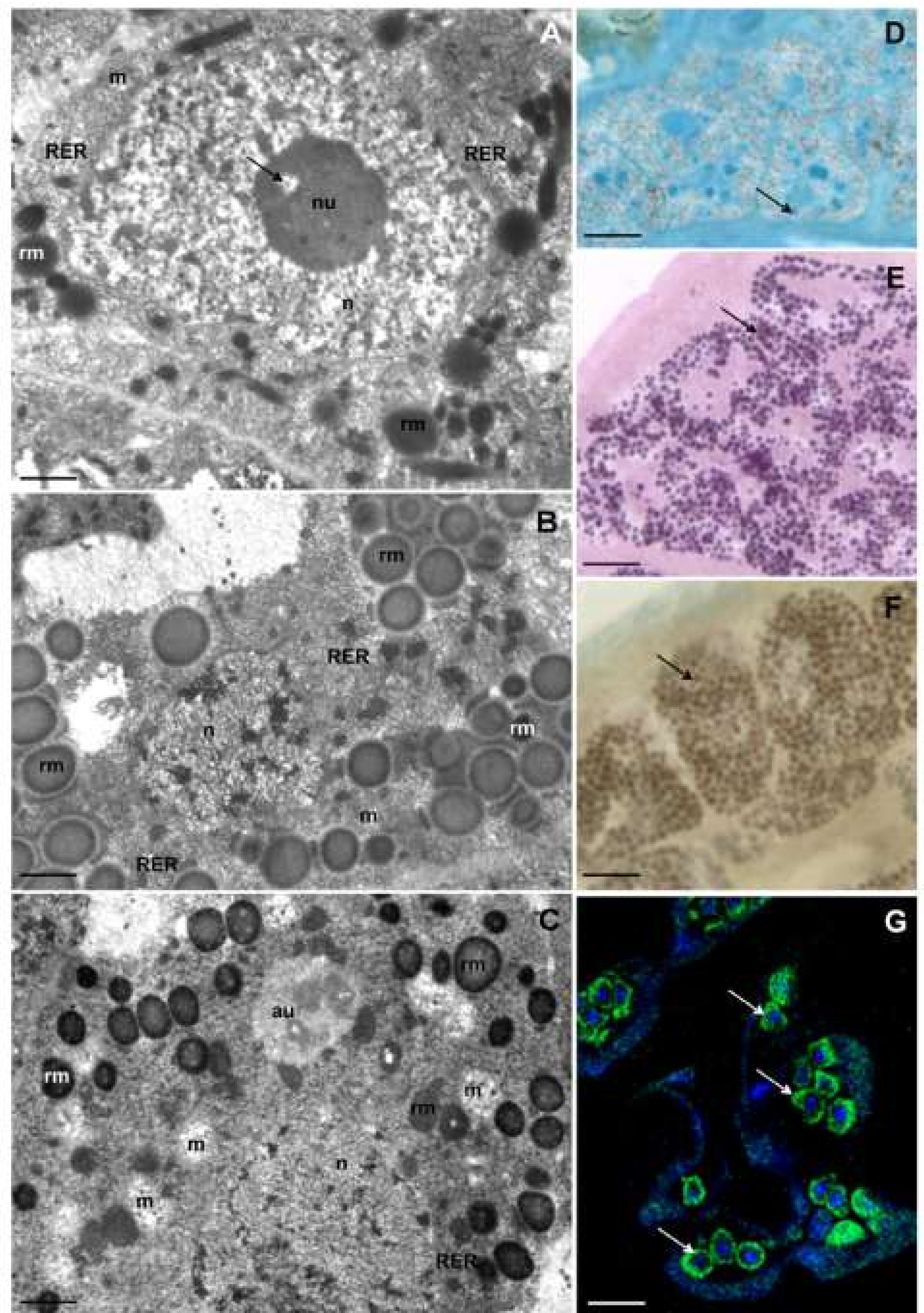
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Fig 2. Ultrastructure and histochemistry of the SC of the first type during different stages of oogenesis. (A-C) Ultrastructure of SC, TEM: nucleus (n), nucleolus (nu), mitochondria (m), rough endoplasmic reticulum (RER), spheres of reserve material (rm). (A) Previtellogenesis, nucleolus vacuole (arrow). Bar = 0.47 μm. (B) Late vitellogenesis. Bar = 0.57 μm. (C) Late choriogenesis, autophagosome (au). Bar = 0.65 μm. (D-G). Histochemical staining of SC, arrow indicates positive reaction: (D) BPB staining, LM. Bar = 4 μm. (E) PAS method, LM. Bar = 3.5 μm. (F) Sudan Black B staining, LM. Bar = 3 μm. (G) BODIPY 493/503 and DAPI staining, confocal microscopy. Bar = 10 μm.

https://doi.org/10.1371/journal.pone.0201430.g002

We observed indications (not quantified) of degeneration of some individual storage cells. The cytoplasm of these cells was electron-dense; many clusters of heterochromatin occurred in the neighbourhood of their nuclear envelope, and their nuclei underwent fragmentation (Fig 3A). The fragmentation of DNA in nuclei (Fig 3B) indicates an apoptotic cell death of these cells.

Sporadically, divisions of the storage cells were observed (Fig 3C). Since we did not obtain images of the dividing cells by transmission electron microscopy, it was not possible to determine if dividing storage cells belonged to the first or second type.

Storage cells in desiccated specimens. We analysed storage cell ultrastructure in fifteen tuns during different stages of oogenesis. The storage cells of tuns were shrunken and had an ameboidal shape (Fig 3D). The average diameter of the cells in the five tuns examined for cell size was 11.8 μm (S1 Table), which is significantly smaller than cells of hydrated specimens (Mann-Whitney U-test, $U = 0.0$, $P = 0.005$, $N = 10$). The general characteristics of desiccated storage cells of *R. coronifer* were reported in our previous article [38].

Immunolabelling. We detected cell divisions in 2 specimens in late stage of oogenesis with the use of immunolabelling with anti-phosphohistone H3, a mitotic-specific antibody (for detection of cell proliferation). In the first and second specimens, 7 nuclei and 5 nuclei, respectively, were found in a mitotic stage. In one specimen in a late oogenesis stage, 4 nuclei were detected with TUNEL labelling for detection of cell death.

Experimental study on long-term desiccation and heating

Survival of specimens. The survival of specimens desiccated (but not heated) for six months was 100% ($n = 14$). All of the non-heated specimens ($n = 14$) were fully active (coordinated body movements, directional movements forwards as well as to the side angles, using all legs, moulting of cuticle) within 3 h after rehydration. The survival of heated specimens was 40% (6 survivals, $n = 14$). Among the heated survivors, 50% were fully active after 3 h of rehydration, whereas the other specimens showed only some slow moves in some legs, and required 5 h of rehydration to resume full activity.

Storage cell ultrastructure of heated and non-heated specimens. The storage cell ultrastructure of heated and non-heated desiccated specimens appeared similar (Fig 4A and 4B). The cells were shrunken with an amoeboid shape, and the cytoplasm was electron dense and entirely filled with membrane coated spheres (Fig 4A and 4B). The centre of all observed cells was occupied by an irregular nucleus with a distinct nucleolus and dense heterochromatin masses (Fig 4A). Large autophagosomes were present in the cytoplasm of the storage cells of both heated and non-heated specimens (Fig 4A and 4B). Differences between cells were only found in the density of spheres. In the heated specimens, the non-homogenous larger spheres were filled with granules of lower electron density, while the spheres of non-heated specimens were homogenous (Fig 4A and 4B).

All rehydrated cells had a circular or amoeboid shape, and there was no apparent difference in ultrastructure between non-heated and heated specimens. After 3 h of rehydration, the cytoplasm was electron lucent and containing a circular nucleus with a distinct nucleolus (Fig 4C and 4D). The cytoplasm of both heated and non-heated specimens contained non-

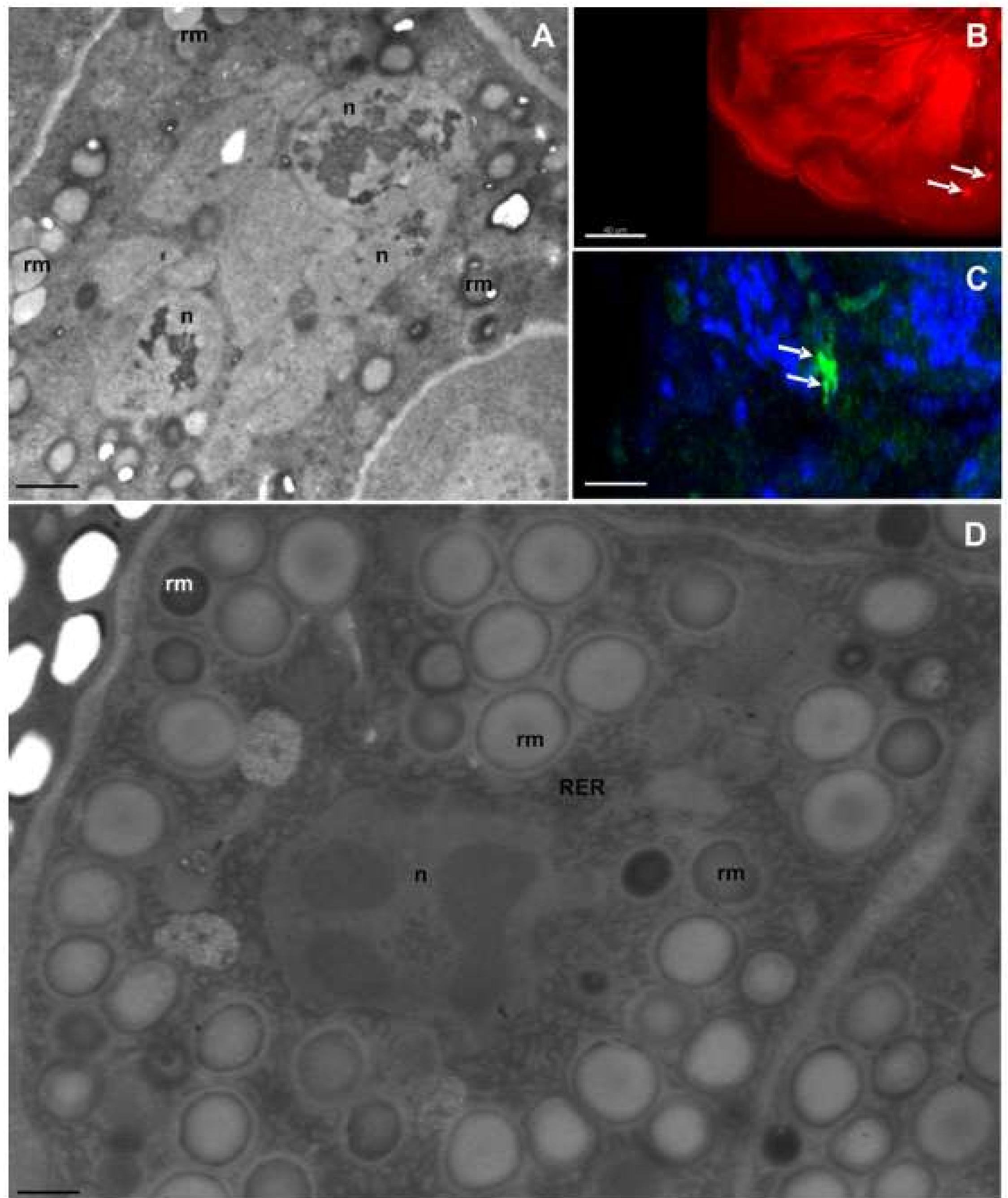
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Fig 3. Ultrastructure of storage cells (SC) of desiccated specimens. (A) Degeneration of SC, nucleus (n), spheres of reserve material (rm), TEM. Bar = 0.6 μm. (B) Detection of the cell death, arrow indicates nucleus of the apoptotic cell, TUNEL, confocal microscopy. Bar = 40 μm. (C) Detection of the cell proliferation, arrow indicates nucleus of the proliferating cell, anti-phosphohistone H3 staining, confocal microscopy. Bar = 25 μm. (D) Ultrastructure of SC of desiccated specimen: nucleus (n), mitochondria (m), rough endoplasmic reticulum (RER), spheres of reserve material (rm), TEM. Bar = 0.36 μm.

https://doi.org/10.1371/journal.pone.0201430.g003

homogenous and homogenous membrane coated spheres of various electron densities (Fig 4C and 4D). Autophagosomes were observed in the cell cytoplasm (Fig 4C). After 5 h of rehydration, the storage cells contained nuclei with a distinct nucleolus in both non-heated and heated specimens (Fig 4E and 4F). The nuclei of some cells were fragmented and degraded (not shown). The spheres with reserve material, filling the cytoplasm of 5 h rehydrated cells, were non-homogenous and contained electron lucent and medium electron dense bodies (Fig 4E and 4F). Large autophagosomes were also observed in the cytoplasm after 5 h rehydration (Fig 4F).

Large amounts of lipid and polysaccharides and a low amount of protein were detected in the storage cells of all examined specimens (not shown).

Discussion

Storage cells in active and anhydrobiotic animals

We compared storage cells of active and anhydrobiotic specimens of *R. coronifer* in all oogenetic stages. We observed dividing as well as apoptotic storage cells in active animals. Some differences between storage cells of active and desiccated specimens of *R. coronifer* were observed. During desiccation, the storage cells slightly changed their shape as the water evaporated, and a low water content and condensed cytoplasm resulted in a higher electron density of condensed cytoplasm and nucleoplasmic matrix [38], which confirmed the observations of Walz [39]. The storage cells of the desiccated specimen also had significantly smaller cells than the active animals. In cells of active specimens, we observed a higher number of autophagosomes at the end of choriogenesis and after 3–5 h of rehydration.

Autophagic pathways allow cells to eliminate large portions of the cytoplasm, aberrant protein aggregates, damaged organelles or invading bacteria. Structures targeted for degradation are gradually surrounded with the phagophore, and double membrane vesicles called autophagosomes are formed [40]. Since autophagy is known to be a major factor in the turnover of long lived proteins, the presence of autophagosomes indicates degradative pathways during dehydration and rehydration processes in cells as a response to damage and/or starvation. Autophagy might therefore be more common in cells that have undergone dehydration than in the cells of healthy, well fed animals [40]. In tardigrades, autophagy was also observed in the digestive cells of the midgut epithelium, and in trophocytes, at the end of oogenesis [36, 40, 41]. In case of the midgut epithelium, initially, when the stressor (infection by pathogens, starvation) was weak, autophagy was activated. However, when the stressor was too strong, autophagy initiated necrosis [36, 41]. In trophocytes, autophagy is the first step of cell degeneration, which is followed by apoptosis [40].

We verified ultrastructurally two types of storage cells, which differed in cellular organelle complexity, amount and content of reserve material and connection with oogenetic stages. The Type I occurred in both the male and females, while Type II was found only in females. One of the features of Type I storage cells was the presence of nucleolar vacuoles. Nucleolar vacuoles, also called nucleolar cavities or interstices, are rather characteristic of plant cells, are rarely visible in animal nucleoli, and represent high nucleoli activity (RNA synthesis) [42, 43, 44]. In plant cells, they are possibly connected with mitosis, particularly in condensation and decondensation of chromosomes [45]. In females of *R. coronifer*, the nucleolus vacuoles were

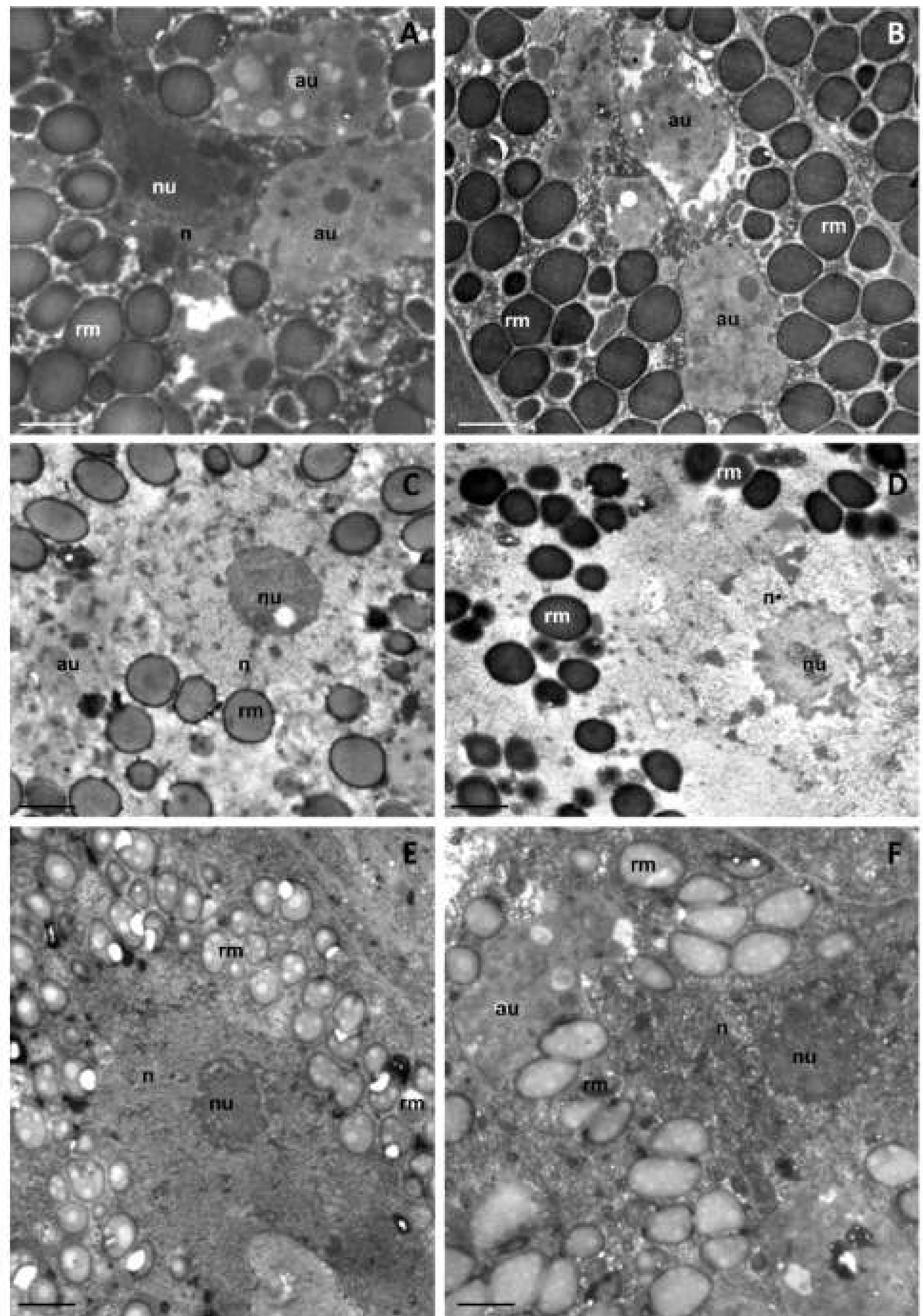
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Fig 4. Ultrastructure of storage cells (SC) of experimental specimens. Autophagosome (au), nucleus (n), nucleolus (nu), spheres of reserve material (rm), TEM. (A) SC of non-heated 6 month old desiccated specimens. Bar = 0.65 μm. (B) SC of heated 6 month old desiccated specimens. Bar = 0.65 μm. (C) SC of non-heated 3h rehydrated specimens. Bar = 0.8 μm. (D) SC of 3h rehydrated specimens, which were heated prior rehydration. Bar = 0.8 μm. (E) SC of non-heated 5h rehydrated specimens. Bar = 0.95 μm. (F) SC of 5h rehydrated specimens, which were heated prior rehydration. Bar = 0.8 μm.

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ultrastructurally related to oogenetic stages with respect to the presence/absence of this structure and the amount and type of reserve material. These cells were in general filled with plenty of mitochondria, cisterns of rough endoplasmic reticulum and specific spheres of different electron densities, particularly lipid reserve material. We therefore assume that storage cells of Type I have intense metabolic activity and that their main function is storage and distribution of energy [13, 16]. This is in line with previous studies on storage cells in other tardigrade species, indicating intense metabolic activity [8, 11, 12, 14, 46]. In relation to overall organelle complexity differences and oogenesis, it seems that the function of nucleolar vacuoles in tardigrades is related to changes in nucleolic activity of storage cells during different stages of oogenesis, which was previously suggested in other organisms [12, 42, 43, 44]. Moreover, the nucleolar vacuole serves as a diagnostic feature in some species, e.g., Caryophilliidea (Cestoda). Nucleolar vacuoles were also observed in storage cells of *Hypsibius exemplaris* Gąsiorek, Stec, Morek and Michalczyk, 2018, *Macrobiotus polonicus* Pilato, Kaczmarek, Michalczyk and Lisi, 2003 and *Xerobiotus pseudohufelandi* (Iharos, 1966) [14]. Nevertheless, their specific function in tardigrades cells (similar to other animal cells) is still unknown.

In tardigrades, yolk material accumulated in the cytoplasm of the oocytes is synthesized by the oocyte and their sister cells (trophocytes); however, sometimes the yolk precursors are synthesized by storage cells or the cells of the midgut epithelium [11, 12, 36]. The synthesis of yolk precursors by storage cells was reported in some Macrobiotidae species, e.g., *Dactylobiotus dispar* (Murray, 1907) [12], *M. polonicus* and *Paramacrobiotus richtersi* (Murray, 1907) [11], as well as in some other species, e.g., *Hypsibius exemplaris* and *Isohypsibius granulifer granulifer* (Thulin, 1928) [14]. In some tardigrades, the amounts of reserve material accumulated in the storage cells increases gradually during previtellogenesis and start to decrease during vitellogenesis and choriogenesis [11, 12, 14]. These observations indicate participation of the storage cells in yolk precursor synthesis. We observed that the fine ultrastructure of the first storage cell type is in general similar to other Parachela species [14] but differs in stored reserve material. During yolk synthesis (vitellogenesis), the amount of reserve material in storage cells of *R. coronifer* increases, but no changes were observed during late vitellogenesis and choriogenesis. Late vitellogenesis occurs at the simplex stage, a start of moulting stage, when the bucco-pharyngeal apparatus is absent or incomplete, while the late choriogenesis is connected with the moulting process [14, 47, 48]. At these stages, the animals do not eat, and the ovaries are large and oppress the midgut lumen. Since these storage cells during late vitellogenesis/choriogenesis are similar to cells at other stages, we conclude that they are probably not involved in production of vitellogenins. The observed decrease in reserve material after oviposition was caused by starvation due to lack of feeding during oogenesis and the moulting process. Evidence of energy reserve functions of storage cells during starvation periods (assumed also by Reuner et al. [15]) were observed in *Macrobiotus sapiens* Binda and Pilato, 1986 and other tardigrades, where storage cell size was found to be smaller after starvation [8, 9, 11, 14] and were also related to the stage of oogenesis [8, 12, 14, 46]. The size and content of reserve material in storage cells is also species dependent, e.g., three types of reserve material spheres were found in *H. exemplaris*, *M. polonicus* and *I. g. granulifer*, whereas only one type was found in *Xerobiotus pseudohufelandi* (Iharos, 1966) [14]. In active specimens of *R. coronifer*, we found large amounts of polysaccharides and lipids but low amount of proteins, similar to *X. pseudohufelandi* [14]. In

I. g. granulifer large amounts of polysaccharides but fewer lipids and proteins were observed, and in *H. exemplaris* and *M. polonicus*, primarily lipids were observed [14]. In contrast, with these species that inhabit limnic habitats, both *R. coronifer* and *X. pseudohufelandi* inhabit dry terrestrial environments and are able to survive long periods of drought in the anhydrobiotic state [49, 50]. This supports the suggestion by Hyra et al. [14] that interspecies variability in storage cells is related to habitats and anhydrobiotic properties.

The storage cells of Type II were found in much smaller numbers (7.2% in all analyzed specimens) and only in females but with a similar ultrastructure during all oogenetic stages. These cells had few organelles and did not contain nucleolar vacuoles. In general, the youngest nucleoli are homogenous and do not possess nucleolar vacuoles [43], and it is possible that these storage cells represent young undifferentiated cells, perhaps stem cells. In general, stem cells are characterized as undifferentiated, unspecialized cells with simpler morphology compared to specialized cells from the same lineage [51]. Polymorphism of coelomocytes has also been verified in earthworms [52, 53], nematodes [54], echinoderms [55] and sea urchins [56]. The classification of coelomocytes is mostly based on differential staining, ultrastructure, and granule composition, as well as on behavioural traits (such as a tendency to form aggregations or filopodia in some cell types) but is still uniformly unsatisfactory, mostly due to various functional states and stages of maturation [57]. The classification of coelomocytes in earthworms is not well standardized, and the number and size of different coelomocytes can vary from species to species [52, 58]. However, it is assumed that coelomocyte types are derived from a common stock of stem cells, and different types of coelomocytes may be produced by direct transformation from stem cells [59]. Our study might be the first to ultrastructurally indicate the possible stem cells of tardigrade storage cells.

Exposures to long-term desiccation and heating

The results of this study suggest that storage cells of the eutardigrade *R. coronifer* are not affected ultrastructurally by six months of desiccation or by heating at 50˚C for 24 h. Still, heating of the tuns tended to considerably decrease survival of the animals. Additionally, the time of rehydration required to revive the animals tended to be longer for tuns exposed to heating. Thus, there were no indications that effects on viability of induced stress were connected with changes in the general structures of storage cells. Ramløv and Westh [25] did not find any effects on survival after heating *R. coronifer* for one hour at 50–70˚C, while survival declined to approximately 20% at 80˚C and to zero at 100˚C. Since in our study the specimens were desiccated for six months before heating at 50˚C, it is possible that this made them more vulnerable to heat stress. Since repair mechanisms are not working during anhydrobiosis, damage due to oxidative reactions with surrounding air accumulates over time [26]. Even if the non-heated animals did not express reduced survival after the six-month period, it may have made the body more vulnerable to damage by heat or unable to repair the inclusive damage from long-term desiccation plus heating. These detrimental effects apparently did not arise from damage to general cell structures but rather to molecular components necessary for cell survival. Protein denaturation occurs in cellular organelles during heat shock at temperatures of 42-45˚C [60, 61, 62], and sub-lethal heat shock may also inactivate transcription, splicing and translation of mRNAs into proteins and alters cell morphology [60].

Relative humidity is another factor that may affect survival in desiccated tardigrades exposed to heat. Ramløv and Westh [25] suggested that the relative humidity at which animals were kept before heating (even at RH levels as low as 50%) may cause damage to cell components such as proteins (denaturation) when exposed to high temperatures through residual water present in the tun. In the eutardigrade *Paramacrobiotus richtersi* (Murray, 1911), very

low humidity (0–3% RH) resulted in significantly higher survival after continuous exposure to 37˚C for up to 21 days. Animals desiccated within their natural substrate (leaf litter) seemed to be less sensitive than animals desiccated and kept on blotted paper and did not show reduced survival when kept at 30–40% RH [26]. In our study, specimens were desiccated at 95% RH but were then kept in plastic bags under ambient laboratory conditions (room temperature, RH not monitored) until the heating exposure.

Vitrification has been proposed as a mechanism for survival in the anhydrobiotic state, whereby membranes and other cell components are stabilized in the absence of water in a non-crystalline amorphous solid ("glassy") state that prevents cellular damage [63]. Evidence for the vitrification hypothesis was reported for the Macrobiotidae family within tardigrades [22], to which *R. coronifer* belongs. Although our observations of few differences in cell structures between hydrated and desiccated animals are in line with the prediction of the vitrification hypothesis that cells are "frozen" in a glassy state, they do not provide direct support for it.

We detected large amounts of lipids and polysaccharides but low amounts of protein in the storage cell cytoplasm of all examined specimens. Lipids have been proposed to have a key role in heat stress management of cells [64], but in anhydrobiotic processes, their role remains unclear. For anhydrobiotic nematodes, some have suggested that lipid reserves are not directly involved in processes of anhydrobiosis [65], whereas others have suggested a direct relationship between lipids/carbohydrates and successful anhydrobiosis [66]. Kinchin [46] proposed that different animal groups may have different mechanisms and that in tardigrades, lipids might be utilized during anhydrobiosis by conversion to glycerol or trehalose, which may stabilize membrane and protein structures [27]. Lipids might also serve as an energy source for metabolic preparations during anhydrobiotic induction or be used for energy after rehydration [67]. More studies on the role of lipids in storage cell physiology and anhydrobiosis would be valuable.

In conclusion, in our study we found (1) two types of storage cells in females of *R. coronifer*, while only one type in one male studied; (2) the ultrastructure of the storage cells of the first type changes during the process of oogenesis, while the ultrastructure of the second type of cells does not change; (3) that cells of the second type possibly represent stem cells for storage cells; (4) that storage cells (heated and non-heated specimens) accumulated large amount of lipids and polysaccharides, whereas the amount of proteins is low; (5) that exposure to 24 h of heating at 50˚C following six months of desiccation reduced animal survival to 40%, while all non-heated animals recovered; and (6) no large differences in the ultrastructure of the storage cells between heated and non-heated desiccated specimens.

Supporting information

S1 Table. The average diameter of storage cells in active and dehydrated animals. Estimates represent individual averages based on measurements of 50 cells per animal.
(DOC)

S1 File. Storage cells diameter (μm). Measurements of storage cells diameter in 50 active and 50 dehydrated specimens.
(XLS)

Acknowledgments

We would like to express our gratitude to Dr. Danuta Urbańska-Jasik, Dr. Lukasz Chajec, MSc. Marta Golas (Department of Animal Histology and Embryology, University of Silesia,

Katowice, Poland) and Dr. Jagna Karcz (Scanning Electron Microscopy Laboratory, University of Silesia, Katowice, Poland) for their technical assistance.

This work was supported by the Academy of Sciences of the Czech Republic (RVO: 67985823), the Mobility Fund, Charles University in Prague, and the Kristianstad University, Sweden. Microscopic analysis (confocal microscopy) was performed using the infrastructure that is supported by the POIG.02.01.00-00-166/08 and POIG.02.03.01-24-099/13 grant.

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Formal analysis: Michaela Czerneková.

Funding acquisition: K. Ingemar Jönsson.

Investigation: Michaela Czerneková, K. Ingemar Jönsson, Izabela Poprawa.

Methodology: Izabela Poprawa.

Project administration: K. Ingemar Jönsson, Izabela Poprawa.

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Writing – review & editing: Michaela Czerneková, K. Ingemar Jönsson, Izabela Poprawa.

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SIDE STUDY

Primary Culture of Tardigrade Storage Cells from *Richtersius coronifer*

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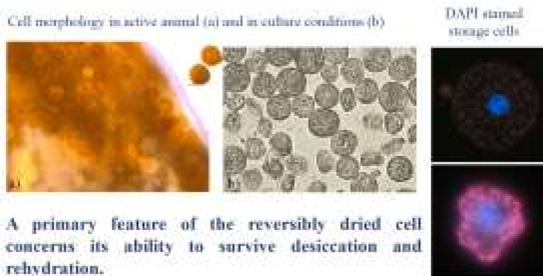
INTRODUCTION

Tardigrades are aquatic micrometazoans (adults size: 250-500µm) with extraordinary **stress tolerance**, such as **desiccation- or thermotolerance**. They are among the most **radio-resistant animals** and became the first animals in the history to survive the combined effect of space vacuum, cosmic radiation, and UV radiation in low Earth orbit (10 days exposure). **The cellular and molecular basis of these tolerances are not yet fully understood.**

STORAGE CELLS (COELOMOCYTES)

Storage cells (average size 10-20 µm) represent an important part of tardigrade physiology, storing and distributing energy and possibly also having immunological functions.

Only few studies of tardigrade cell biology have been reported and neither primary nor continuous cell cultures have been established. **Cell cultures of tardigrades would open up a new field of possibilities to study tardigrade stress responses at the cellular level.**



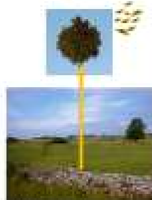
Cell morphology in active animal (a) and in culture conditions (b)

DAPI stained storage cells

A primary feature of the reversibly dried cell concerns its ability to survive desiccation and rehydration.

AIM of THIS STUDY:
to establish primary culture of storage cells

METHODS



Habitat of *R. coronifer*

ANIMAL COLLECTION

1. Extraction of animals from moss cushions
2. Wash animals in sterile water to get rid of debris particles

CELL COLLECTION

1. Sterilization of animals with 70% ETOH
2. Cutting animals under microscope directly into medium of use and collect cells
3. Seed cells into TC Plate 96 Well, Standard, F

Extraction of cells in laminar flow hood

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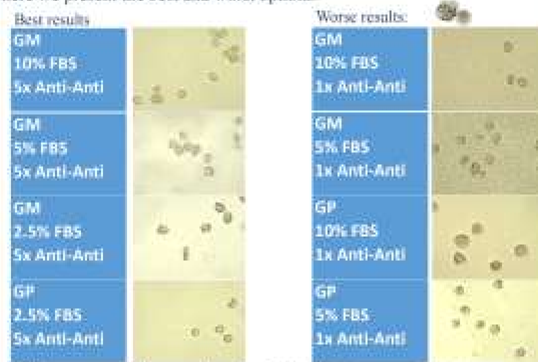
RESULTS: CHOICE OF MEDIA

In order to choose **convenient culturing medium**, we tested Grace´s insect medium (GM) 1x diluted in H_2O or 1x diluted in PBS (GP) and RPMI medium 1x diluted in H_2O (RPMI) in 18 combinations of different fetal bovine serum (FBS) and Antibiotics-Antimycotics (Anti-Anti) concentrations. Based on cell morphology and contamination occurrence, here we present the best and worst options.



All pictures were taken at culture-day 3 (40x objective magnification)

Maximal length of cell culture was 11 days, but fungi and bacterial contamination occurred.

CHOICE OF ANTIBIOTIC DOSES

In order to get rid of contamination, we tested different Antibiotics-Antimycotics doses with addition of Penicilin-Streptomycin. With use of high concentration (10x) of Antibiotics-Antimycotics combining with Penicilin-Streptomycin (5x) **we extended length of cultures up to 18 days.**

Some of the **cells changed their shape**, which was never observed in alive animals.

CONCLUSIONS AND PERSPECTIVES

- **Storage cells can be kept viable in vitro and some cell division was verified. Therefore these cells can be cultured.**
- **Grace´s insect medium diluted with H_2O is more suitable than diluted with PBS and than RPMI.**
- **Combination of Anti-Anti (10x) and Pen-Strep (5x) reduced bacterial contamination, but fungi were still present.**

This study provides the first evidence that primary cultures of storage cells from tardigrades are possible to establish, but the culturing method has to be refined to avoid contamination.

Fungi contamination was still present with the first signs after 4 days of culturing (40x objective magnification).

This work was supported by the Academy of Sciences of the Czech Republic (RVO: 67985823), the Mobility Fund, Charles University in Prague, and the Kristianstad University, Sweden.