Verification of *Hypsibius exemplaris* Gąsiorek et al., 2018 (Eutardigrada; Hypsibiidae) application in anhydrobiosis research

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Abstract: Anhydrobiosis is considered to be an adaptation of important applicative implications because it enables resistance to the lack of water. The phenomenon is still not well understood at molecular level. Thus, a good model invertebrate species for the research is required. The best known anhydrobiotic invertebrates are tardigrades (Tardigrada), considered to be toughest animals in the world. *Hypsibius. exemplaris* is one of the best studied tardigrade species, with its name "*exemplaris*" referring to the widespread use of the species as a laboratory model for various types of research. However, available data suggest that anhydrobiotic capability of the species may be overestimated. Therefore, we determined anhydrobiosis survival by *Hys. exemplaris* specimens using three different anhydrobiosis protocols. We also checked ultrastructure of storage cells within formed dormant structures (tuns) that has not been studied yet for *Hys. exemplaris*. These cells are known to support energetic requirements of anhydrobiosis. The obtained results indicate that *Hys. exemplaris* appears not to be a good model species for anhydrobiosis research.

Keywords: cryptobiosis, model species, Tardigrada

Introduction

One of the most prevalent adaptations to water deficiency is anhydrobiosis, often called simply 'life without water', tolerance to desiccation or waiting for water to return [1-5]. More precisely, anhydrobiosis is described as the ability to dry to the point of equilibrium while exposed to moderately to very dry air (i.e., to 10% water content or even less) and then recover to normal functioning after rehydration without sustaining damages [6]. This denotes a series of coordinated events during dehydration and rehydration that are associated with preventing oxidative damages and maintaining the native structure at different levels of organism's organization [7-8].

Anhydrobiosis is also described as an adaptation to unstable environmental conditions including drought or freezing, that allows the organism to survive when the environment becomes hostile to active life. Therefore anhydrobiosis is considered to be a phenomenon of important applicative implications, enabling biostabilization and biopreservation as well as human disease treatment (e.g. [9-15]). Anhydrobiosis occurs in prokaryotes (e.g. [16]) and eukaryotes, with the latter including many microorganisms (e.g. [17]) as well as plants (e.g. [8]) and some small invertebrates (e.g. [18]). Among animals the best known example are tardigrades (e.g. [19]), indicated lately as an emerging source of knowledge of importance for medical sciences [13].

Tardigrade anhydrobiosis includes entry, dormant and exit stages, that correspond to the dehydration (i.e., tun formation), tun and rehydration stages, respectively [18, 20]. On the organismal level, the tun formation and return to the active stage have been quite well described and are understood fairly well [3, 21-26]. The key morphological changes during tun formation are longitudinal contraction of the body, invagination of the legs and intersegmental cuticle that are then reverted during rehydration. However, responsible cellular and molecular mechanisms are not yet fully described.

At the present, the genomes of only two tardigrade species are available i.e. *Hypsibius exemplaris* Gąsiorek, Stec, Morek & Michalczyk, 2018 [27] (previously known as *Hys. dujardini* (Doyère, 1840) [28] and *Ramazzottius varieornatus* Bertolani & Kinchin, 1993 [29] [30-32], both representing the eutardigrade lineage [33]. The genomes enabled identification of proteins significant for tardigrade anhydrobiosis including some intrinsically disordered proteins regarded as unique for tardigrades (for review, see [26, 34-35]). Moreover, both genomes allowed for comparative transcriptomics that corroborates experimental data indicating that different evolutionary tardigrade lineages may exhibit unique physiological and molecular adaptations to survive anhydrobiosis [36]. Accordingly, *Ram. varieornatus* is

regarded as more tolerant to anhydrobiosis than *Hys. exemplaris* [13, 26, 37-39]. Nevertheless, *Hys. exemplaris* is one of the best studied tardigrade species, with its name "*exemplaris*" referring to the widespread use of the species as a laboratory model for various types of studies, ranging from developmental and evolutionary biology, through physiology and anatomy to astrobiology (e.g. [27, 40-44]).

It is frequently suggested that *Hys. exemplaris* requires a period of preconditioning to mobilize protectants needed to undergo a successful anhydrobiosis. However, the available protocols are based on different time windows and values of relative humidity (RH) for the preconditioning and dehydration. They also differ in the applied walking surface substratum as well as rehydration process and the reported levels of recovery following rehydration ranging between ca. 22 and 100% (e.g. [37, 39, 40, 45-47]). The second approach consists in slow dehydration under conditions of decreased RH but the recovery is not stated [48]. Therefore, we decided to verify the anhydrobiotic capabilities of *Hys. exemplaris,* which is crucial for the species applicability as a model in research of anhydrobiosis. For this purpose, we tested three different protocols, i.e. the protocol based on preconditioning, published by [49], our own protocol that we also use for other tardigrade species [50] and based on slow dehydration as well as a third one we termed "environmental drying" applied in two variants, using moist sand or a pond sediment as substrates. The obtained results indicate that in *Hys. exemplaris* anhydrobiosis, slow dehydration may be a better strategy than preconditioning. However, despite being a useful model in studies of other aspects of tardigrade biology, *Hys. exemplaris* appears not to be a good model for anhydrobiosis research.

Materials and Methods

Hypsibius exemplaris rearing

Hypsibius exemplaris Z151 strain (Fig. 1) was purchased from Sciento (Manchester, United Kingdom) in 2015. To maintain the culture, specimens were kept in POL EKO KK 115 TOP+ climate chamber (photoperiod 12h light/12h dark, 20°C, relative humidity (RH) of 50% on Petri-dishes (55 mm in diameter) with their bottoms scratched using sandpaper to allow movement of tardigrades. They were covered with a thin layer of the culture medium obtained by mixing double-distilled water and spring water (Żywiec Zdrój S.A., Poland) in ratio of 3 to 1. *Chlorella vulgaris* Beijerinck 1890 [51] (SAG211-11b strain) was served as a food once per week after the dish cleaning. Animals were transferred to a new culture dishes every few months (for details see [52]). The algae strain was kindly provided by Marcin Dziuba (Department of Hydrobiology, Faculty of Biology, Adam Mickiewicz University, Poznań, Poland) and was

obtained from the culture collection of algae (Sammlung von Algenkulturen (SAG)) at the University of Göttingen, Germany.

Anhydrobiosis protocols

For tun formation, fully active (displaying coordinated movements of the body and legs) adult *Hys. exemplaris* specimens of medium body length (approximately 200–250 μm) were extracted from the culture. After removal of debris, tun formation was performed using three different protocols, designated as A, B and C. In protocol A, provided by Boothby [49], specimens were transferred onto 2% agar-coated lids of Petri dishes of 3.5 cm in diameter, in the minimal amount of the culture medium. The lids, termed "agar plates", were transferred for 16 h to a humidified chamber with RH 92%, obtained by application of 10% glycerol solution in a small plastic box with a lid (Fig. 1). After the preconditioning, the agar plates were transferred to POL EKO KK 115 TOP+ chamber and kept in 40% RH for 24 h. Then, obtained tuns were kept in a desiccator for 7 days at 22% RH. All stages of tun formation were performed at controlled temperature of 20℃. Protocol B consisted in application of slow dehydration of specimens by transferring them into 3.5 cm (in diameter) covered and vented Petri dishes with qualitative filter paper CHEMLAND 150 (06-00A102.150) placed on their bottom (Fig. 1). Specimens were transferred in 400 μl of the culture medium and were left to dry slowly in the Q-Cell incubator (40–50% RH, 20℃) for 72 h. The obtained tuns were kept in the incubator for 7 days. Protocol C, termed "environmental drying" was applied in two variants, i.e. C1 and C2. In both variants specimens were placed, together with 400 μl of the culture medium, into 3.5 cm (in diameter), covered Petri dishes containing ca. 5 ml of previously autoclaved (121℃, 20 minutes, 100 kPa) substrate and were left to dry in Q-Cell incubator (40–50% RH, 20℃) for 72 h. The dishes were kept in incubator for 7 days following drying of the substrate. In protocol C1 the substrate consisted of terrarium sand (Vitapol), with ca 3 ml of the culture medium added to moisturize it, while in protocol C2 sediment collected from a pond near the Faculty of Biology, Adam Mickiewicz University in Poznań, Poland (52° 28' 7.3956"N; 16° 56' 1.356"E), containing soil and decomposing plant matter was used as the substrate. The numbers of specimens and repeats used for estimation of survival rate as well as substratum for each of the applied protocols are summarized in Table 1. In the case of the A and B protocols, each plate contained five additional specimens selected randomly for tun microscopic analysis (see below). In the case of the C1 and C2 protocols, microscopic analysis of tuns was impossible due to the applied walking surface substratum which made observation and extraction of tuns impossible.

The animals' survival rate after 24 h following rehydration was observed in small glass cubes under the stereomicroscope (Olympus SZX7 and SZ51). In case of A and B protocols, the rehydration was performed by addition of 2 ml of the culture medium to each dish. Tuns were then transferred from their dishes to separate glass cubes and kept at 20 ℃, and 40–50% RH. In the case of C1 and C2 protocols, contents of each dish were placed in larger Petri dish filled with culture medium to allow extraction of animals to the separate glass cube kept at 20 ℃, and 40–50% RH. Successful survival was defined as the presence of coordinated movements of animal body and legs (crawling). Statistical significance of results was tested using unpaired t-test.

Tun microscopic analysis

Tun formation by application of protocols A and B was observed under an Olympus SZ61 stereomicroscope connected to Olympus UC30 microscope digital camera. Randomly selected representative tuns were photographed. Tuns were fixed in solution of 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4, room temperature, 2 min). Then tuns obtained by protocol A were photographed on agar plates using an Olympus SZ61 stereomicroscope. Tuns obtained using protocol B were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, mounted in a drop of water on a slide, covered by coverslip, and photographed with the use of Olympus BX60 stereomicroscope and OLYMPUS DP50 camera.

Ten tuns obtained using protocols A and B were randomly selected for ultrastructure analysis under transmission electron microscopy (TEM). Tuns were selected just before rehydration and then fixed in 2.5% glutaraldehyde prepared in 0.1 M sodium phosphate buffer (pH 7.4, 4℃, 24 h), postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4, 4℃, 1.5 h), dehydrated and embedded according to the protocol by [41]. The material was cut into ultrathin (50 nm) sections on a Leica Ultracut UCT25 ultramicrotome. These sections were mounted on formvar covered copper grids, stained with uranyl acetate and lead citrate, and analyzed with use of a Hitachi H500 transmission electron microscope at 75 kV.

Results and Discussion

Applied anhydrobiosis protocols result in formation of correct tuns, but differ in survival rate

The applied anhydrobiosis protocols differed from each other at tun formation (dehydration procedure) while the rehydration procedure was similar (Table 1). As shown in Figure 2, reasonable survival rate, defined as coordinated movements of the body and legs (crawling) after 24 h following rehydration, was observed for protocol B (slow dehydration on

filter paper). In the case of protocol C1 (environmental drying in sterile moist sand) survival was variable whereas in the case of protocol A (preconditioning on agar layer) and C2 (environmental drying in pond sediment) survival was very low. To explain these differences in survival rate we decided to check the appearance of formed tuns. It should be mentioned that for C1 and C2 protocols, microscopic analysis of tuns was impossible due to the applied walking surface substratum which made observation and extraction of tuns impossible. As shown in Figure 1, protocols A and B led to contraction of the body and withdrawal of legs into the body cavity accompanied by loss of water from the body, resulting in a distinctly shrunken body shape. Thus, the protocols allowed for formation of tuns with typical appearance. However, the typical appearance did not guarantee successful return to full activity after rehydration following 7 days spent in a tun stage. Thus, typical appearance of tuns cannot be regarded as indicative of successful anhydrobiosis for *Hys. exemplaris* specimens. Therefore, we decided to analyse ultrastructure of ten randomly selected typical tuns obtained by A and B protocols.

Typical tun appearance does not rule out degeneration at ultrastructural level

Ultrastructural analysis of randomly selected correctly formed tuns performed using transmission electron microscopy (TEM) was based on storage cells regarded as representative cells for this kind of analysis [25]. The obtained TEM images of storage cells allowed to assign three stages of degeneration of typical tuns: 0 – tuns with no signs of degeneration; 1 – tuns with the first signs of degeneration; 2 – tuns with highly advanced degeneration (Fig. 1 and Fig. 3, Tab. 2). In stage 0, the storage cells had oval or ameboid shape and their electron-dense cytoplasm was filled with spheres of reserve material. Between the spheres, ribosomes, cisterns of rough endoplasmic reticulum, and shrunken mitochondria with electron dense matrix were visible (Fig. 3A). In stage 1, the storage cells had the same shape and ultrastructure as described for stage 0, but in their cytoplasm, single vacuoles and autophagosomes appeared (Fig. 3B). Accordingly, in the distinguished stage 2, the storage cells underwent severe vacuolization, and in their cytoplasm numerous autophagosomes were observed, and some of the autophagosomes were also disintegrated (Fig. 3C-D). Moreover, the cell membrane of some cells was degraded (Fig. 3D) and mitochondria had electron lucent matrix (Fig. 3C). The latter is observed for damaged mitochondria with impaired functionality (e.g. [53]), which and may result in cell death. All the stages were observed for tuns obtained by protocol B, i.e. out of 10 analysed tuns, five displayed features of stage 0, two of stage 1, and three of stage 2, whereas for tuns obtained by protocol A only stage 2 was observed (Fig. 1. Tab. 2). Interestingly, these observations

corroborated with the survival rate determined for tuns obtained by A and B protocols (Fig. 2). Thus, it could be assumed that only tuns without visible degeneration of storage cells appear to be able to successfully return to active life.

According to our knowledge, it is the first report indicating possibility of degeneration in *Hys. expemplaris* tuns of typical appearance, resulting in their decreased survival. Available data on *Hys. exemplaris* tuns of comparable duration [47] concern only tuns of classical cellular structure. Moreover, the functional state of anhydrobiotic *Hys. exemplaris* storage cells has not been studied yet although the cells are known to accumulate polysaccharides and lipids (e.g. [42]), and to be related to anhydrobiosis success because of their role of energy supplier (e.g. [19, 54]). We ca assume that the observed damage to mitochondria may distinctly impair the role. However, it should be mentioned that in different tardigrade species the effect of anhydrobiosis on storage cells may be different as reflected by differences in changes of storage cells' size observed after dehydration [55]. Moreover, our data indicate that preconditioning is not a necessary element of *Hys. exemplaris* anhydrobiosis protocol as slow dehydration appears to provide even a better outcome. It should be remembered that application of different definitions of *Hys. exemplaris* recovery from the tun stage may hinder the comparison of the applied protocol effectiveness. For example there is a difference between "We defined recovered animals as those exhibiting spontaneous movements or at least responding to touch stimuli" [37] and the approach applied in this report, i.e. "coordinated movements of the body and legs (crawling)". Additionally, some of the available papers do not contain clear definition of the recovery (e.g. [46]), estimation of survival rate [48] or the indication of time window for survival estimation following rehydration [39] as well as duration of anhydrobiosis [37, 39, 45, 48].

Summing up, *Hys. exemplaris* is able to form tuns of typical appearance, but the process of internal degeneration decreases tun survival distinctly. Thus, the species does not appear to be a good model in anhydrobiosis research.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Data generated and analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

These studies were supported by the research grant of National Science Centre, Poland, NCN 2016/21/B/NZ4/00131.

Authors' contributions

IP, MR, ŁK and HK came up with research ideas. HK, IP and ŁK supervised the performed analyses. IP, ŁK, TB, MR, AK and HK wrote the final version of the manuscript. ŁK, MR, TB, AK and WE carried out the tardigrade cultures and collected animals for experiments. ŁK, TB, WR, AK and MR performed experiments with anhydrobiosis. ŁC, IP, AK and MR prepared microphotographs and figures. All authors read and approved the final manuscript.

Acknowledgements

These studies were supported by the research grant of National Science Centre, Poland, NCN 2016/21/B/NZ4/00131. The technical contribution of Kamil Janelt is highly appreciated.

Studies have been conducted in the framework of activities of BARg (Biodiversity and Astrobiology Research group).

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Figures

Figure 1. Scheme of the experimental setup of A and B protocols used for ultrastructural analyzes. Protocol A is represented by the sketch of preconditioning procedure and protocol B by a plate used for slow dehydration. **A.** small plastic box with lid; **B.** 2% agar-coated lids of Petri dishes ("agar plates"); **C.** scaffold for agar plates; **D.** a glass watch dish containing 10% glycerol solution; **E-F.** digital hygrometer; TEM, transmission electron microscopy; 0, 1 and 2, the distinguished three stages of degeneration of typical tuns.

Figure 2. Survival rate of *Hys. exemplaris* specimens after 7 days in tun stage. The survival rate corresponds to percentage of specimens able to return to full activity after 24 h following rehydration. A, B, C1 and C2 - symbols assigned to applied anhydrobiosis protocols. A, preconditioning on agar; B, slow dehydration on filter paper; C1, environmental drying in moist sand; C2, environmental drying in pond sediment. Data represent mean values ± SEM (see also Table 1).

Figure 3. Ultrastructure of storage cells in tuns of *Hys. exemplaris*, TEM. A. Storage cells in stage 0 – tuns with no signs of degeneration: m- mitochondrion, rm- reserve material, sc – storage cell, arrow – cisternae of rough endoplasmic reticulum, scale = 0.38 µm; **B.** Storage cells in stage 1– tuns with the first signs of degeneration: au– autophagosome, m- mitochondrion, rm- reserve material, sc – storage cell, v- vacuole, scale = 0.53 µm; **C-D.** Storage cells in stage 2– tuns with highly advanced degeneration: au– autophagosome, m- mitochondrion, rm- reserve material, sc – storage cell, v– vacuole, arrow– degraded cell membrane; **C.** scale = 0.36 µm; **D.** scale = 0.42 µm (see also Table 2).

452 # Tables

453 **Table 1.** Summary of the applied anhydrobiosis protocols.

Protocol	Quantitative details	Mode of dehydration	Substratum
A.	10 repeats, each for 20 individuals (20 specimens per plate)	preconditioning	agar layer
B.	5 repeats, each for 50 individuals (10 specimens per plate)	slow dehydration	filter paper
C1.	5 repeats, each for 50 individuals (50 specimens per plate)	environmental drying	moist sand
C2.	5 repeats, each for 50 individuals (50 specimens per plate)	environmental drying	sediment from the pond

454

455 **Table 2.** Summary of ultrastructural analysis of typical tuns based on storage cells. The analysis was performed
456 for 10 randomly selected typical tuns obtained using protocols A and B.

Protocol	degeneration stage	Description	Approx. percentage [%]
A	2	highly advanced degeneration	100
B	0	no signs of degeneration	50
	1	first signs of degradation	20
	2	highly advanced degeneration	30

457

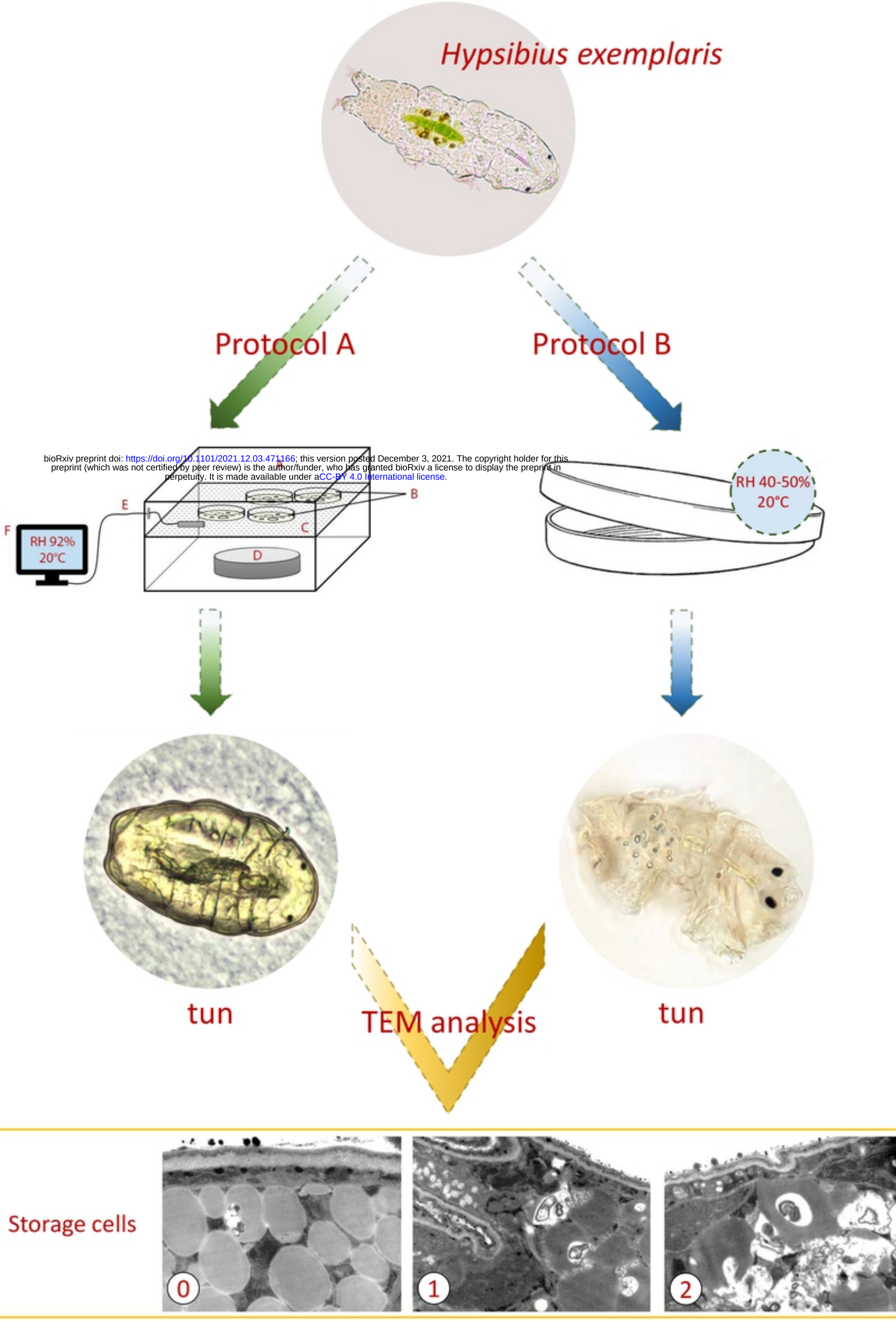


Figure 1

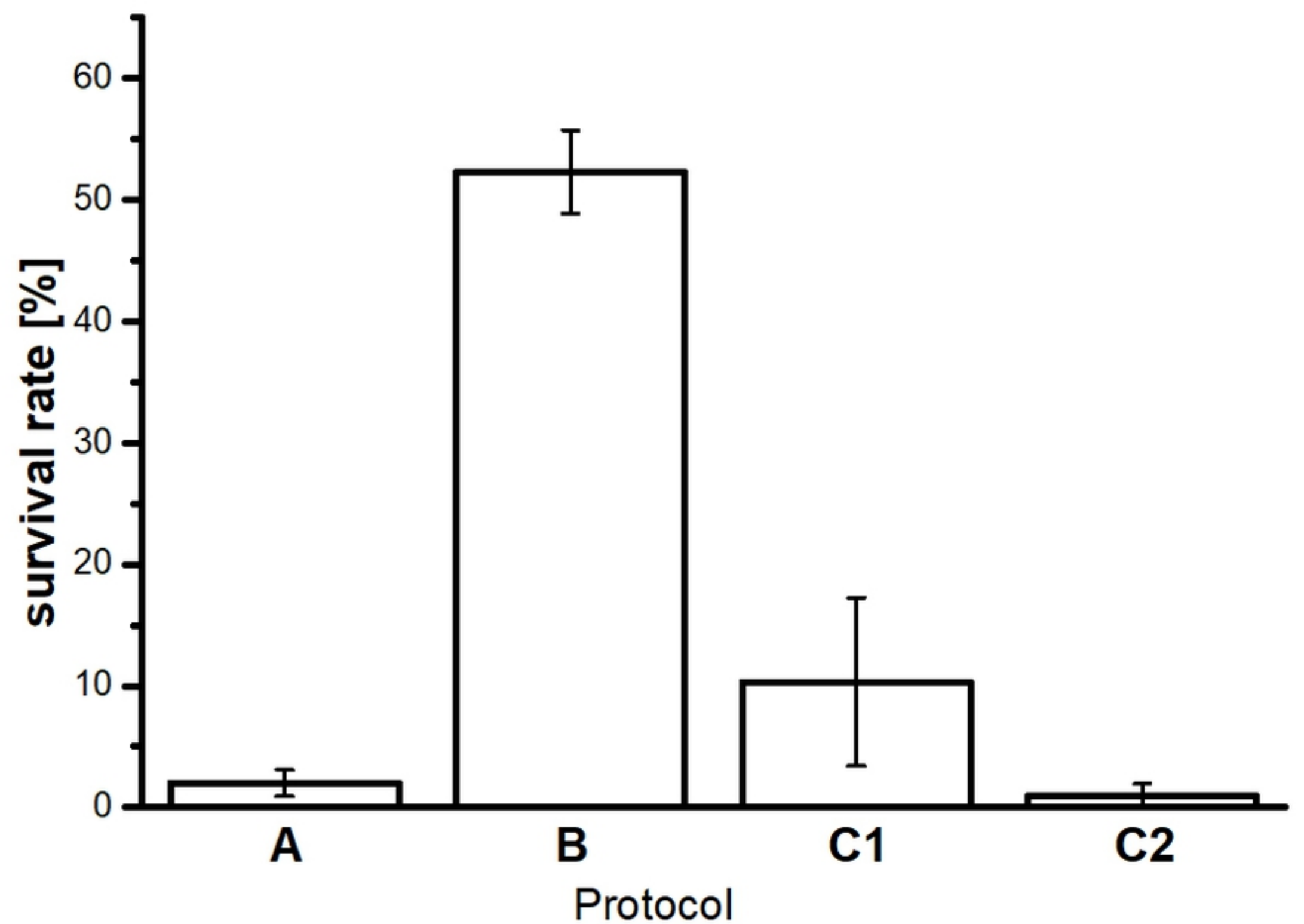
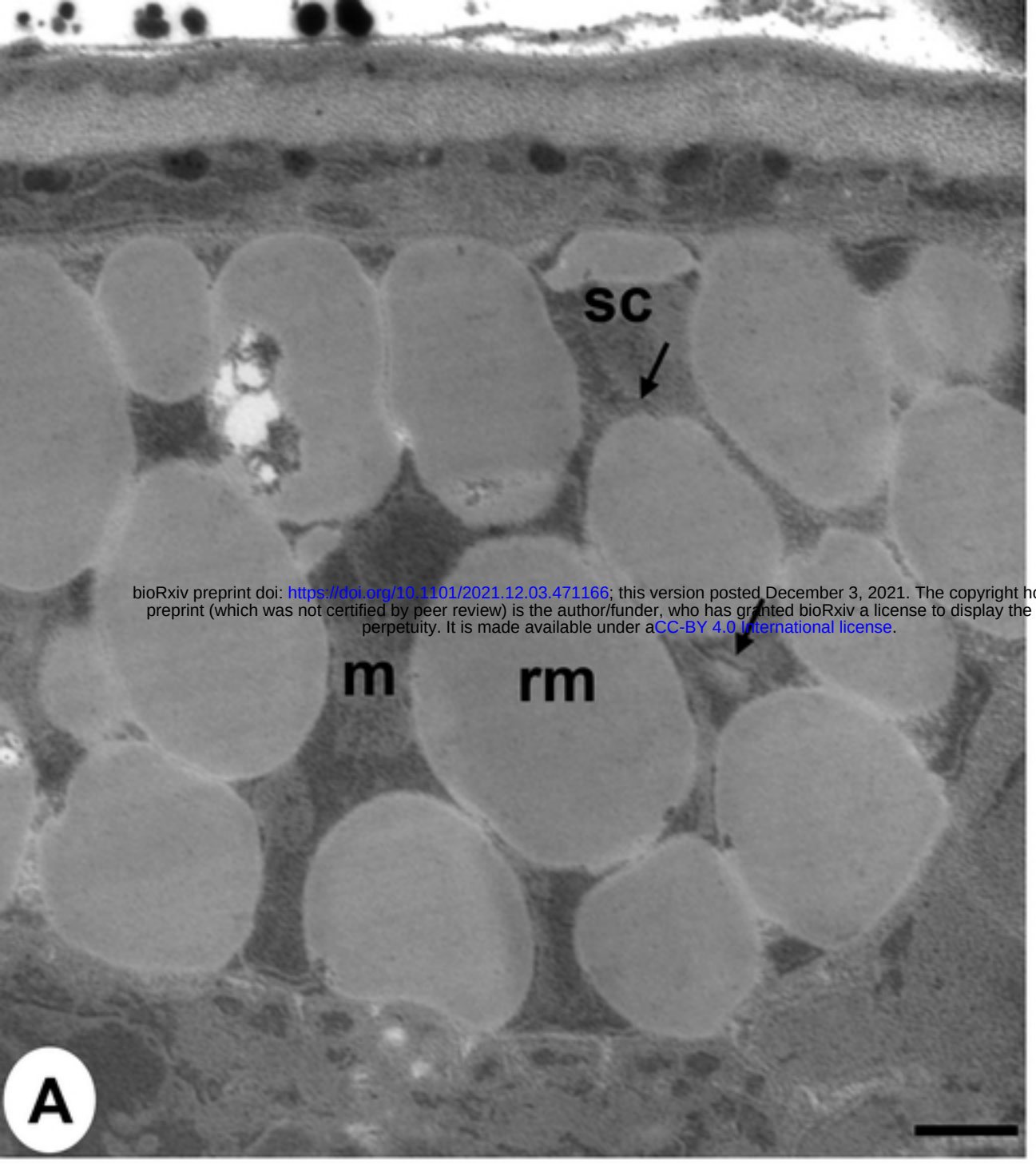
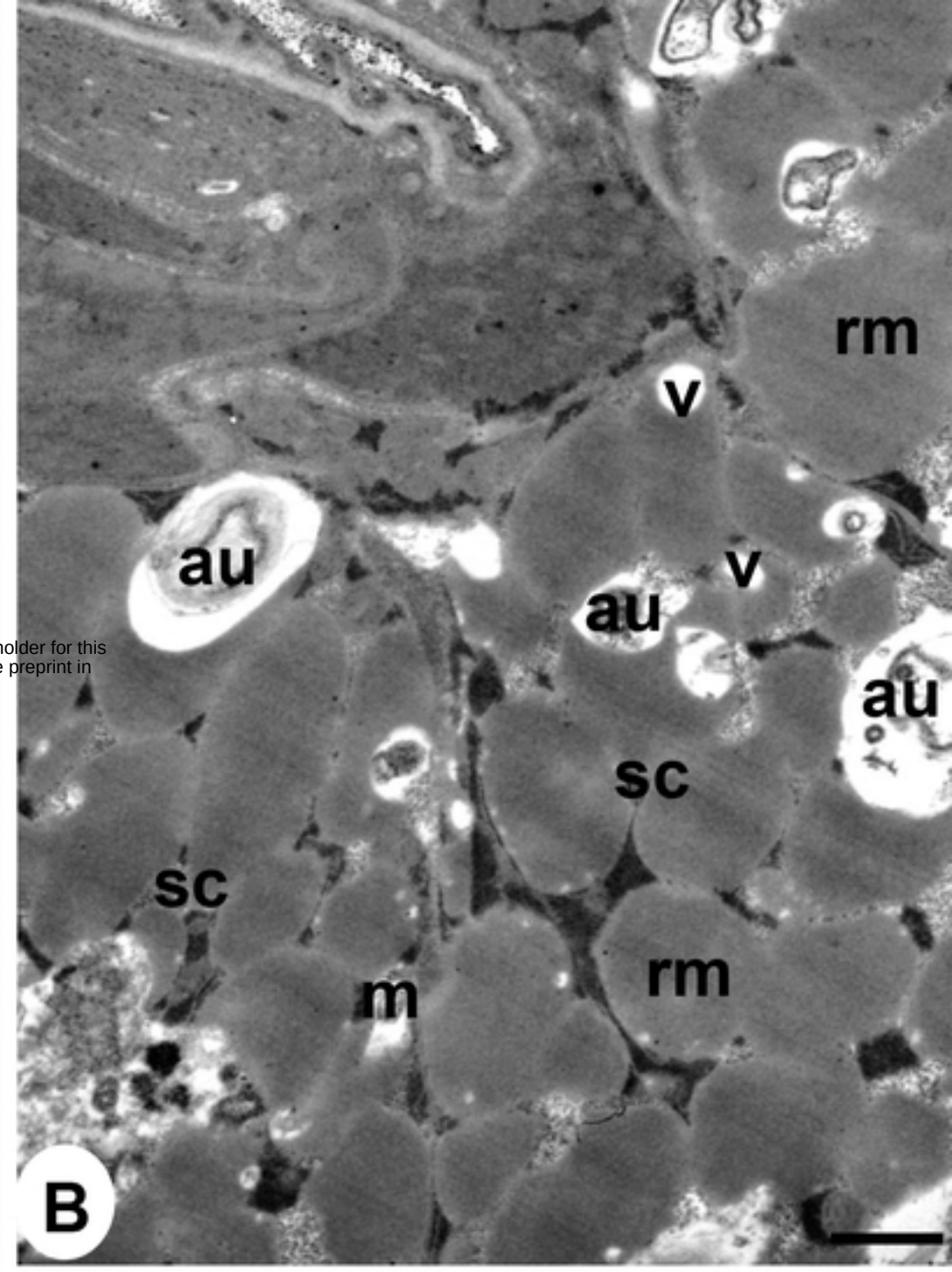
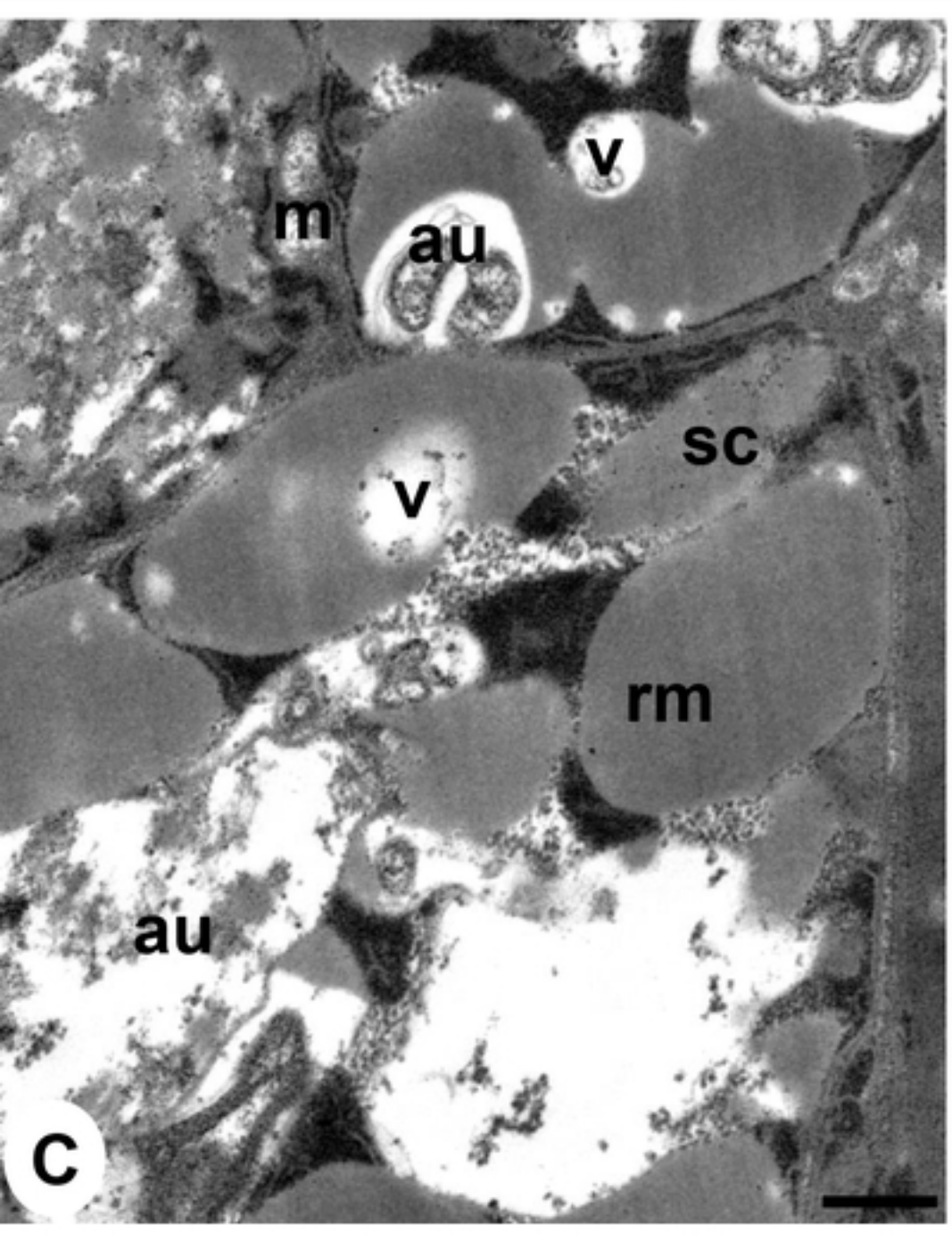
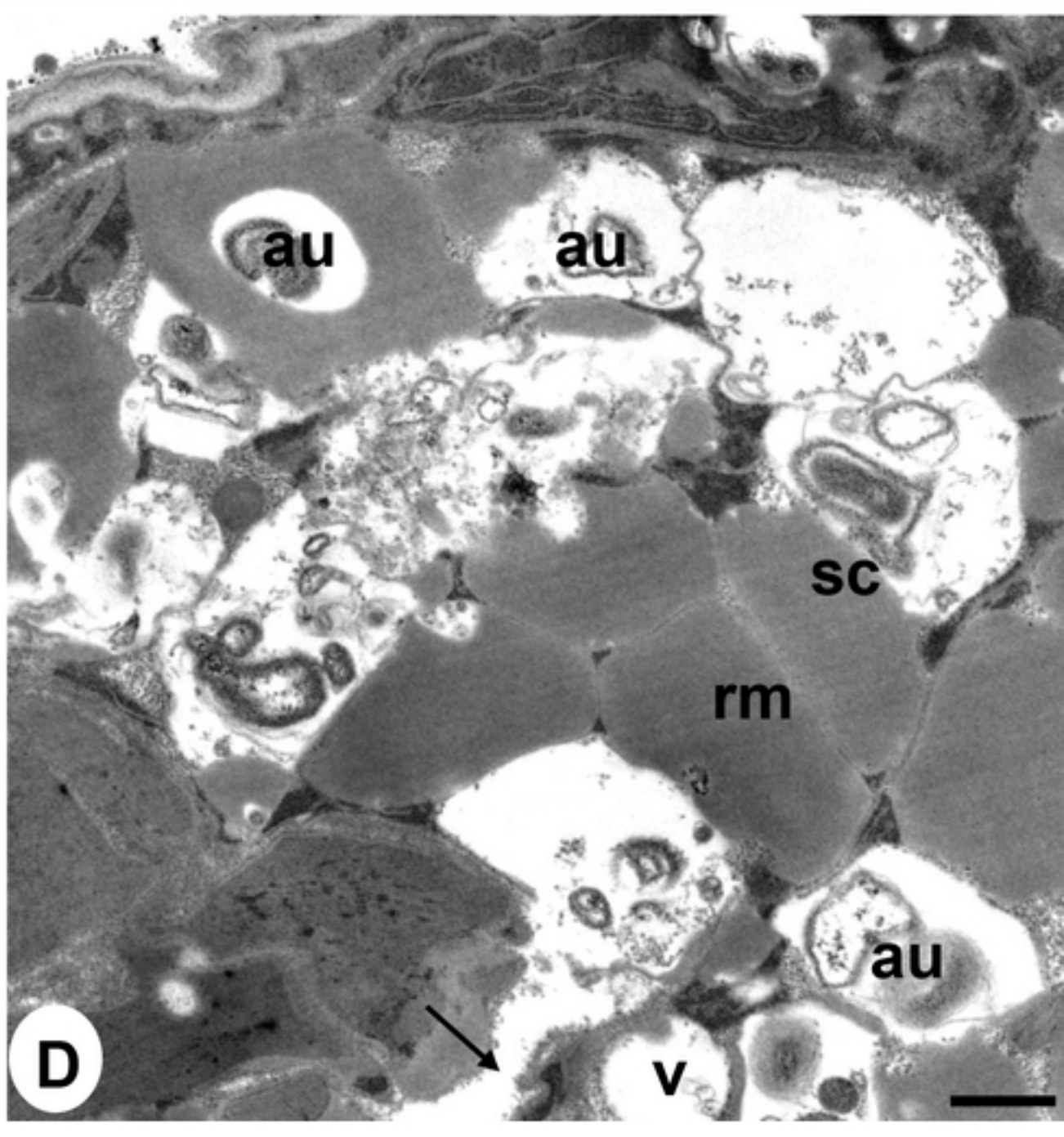


Figure 2









Figure