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- TO WHOM IT MAY CONCERN -

Michaela Czerneková performed her graduate studies under the supervision of Dr Petr Svoboda and Pr Ingemar Jönsson. Her graduate work focuses on the physiology of storage cells of the eutardigrade *Richtersius coronifer* and is presented in a doctoral thesis entitled "Storage cells and their role in tardigrade physiology". Four articles published in peer-reviewed and well-recognized international journals validate the quality and excellence of Michaela's scientific research.

PUBLICATIONS:

The *first* publication of Michaela addressed the question of whether the storage cells divide, and in case of mitosis, whether cell division can be related to a specific body phenotype. Her results reveal a strong correlation between storage cell division and moulting, suggesting that mitosis is more related to animal growth than cell renewal.

In her *second* publication, Michaela characterized the ability of *Richtersius coronifer* to survive multiple (up to six) cycles of desiccation/rehydration, without feeding the animal between cycles. Her results show that the survival rate declines smoothly during the first four cycles of desiccation/rehydration and drops abruptly at the fifth cycle. The abrupt decline seems to be related to the inability of the tardigrades to enter properly in a tun state.

The *third* and *fourth* publications compare the structure of *Richtersius coronifer* in three states: (i) hydrated, (ii) desiccated or (iii) after desiccation and heat stress, with a special emphasis on the structure of storage cells. The major breakthrough of these studies is the discovery of two different types of storage cells in the hydrated animal. One type (type I), observed in male and female, corresponds to cells with the expected function of storing reserve material. In hydrated animals, the ultrastructure of the nuclei and cytoplasm of these type I storage cells, characterized in terms of chromatin type, nucleolar vacuoles, mitochondria, ribosomes, RER, spheres of reserve material and autophagosomes, changes during oogenesis. The major (and expected) changes observed on these cells during desiccation and heating are in their size (shrinkage), and in the electron density of most organelles (higher electron density). The second type of storage cells (type II) possibly corresponds to stem cells due to their undifferentiated phenotype, and is observed only in females.

THESIS MANUSCRIPT:

The thesis manuscript itself starts with a broad introduction on tardigrades (first chapter). The second chapter is dedicated to the materials and the methods used during the research. The third chapter describes the major findings and results of the doctoral research. The thesis manuscript ends with a conclusion and the perspectives that are open by this research (fourth chapter).

Overall the thesis manuscript is well written and easy to read. There are a few **minor** points and questions that I would like to mention and that could help read the manuscript.

In the “Introduction” chapter, Michaela starts with the biology and morphology of tardigrades. Personally I would have discussed morphology after phylogeny. With that order, the reader already knows what are eutardigrades and heterotardigrades when these names are mentioned in the “Morphology of tardigrades” section. Alternatively, it would be possible to refer to the section on “Phylogeny of the tardigrades” when these two families of tardigrades are mentioned in the “Morphology of tardigrades” section. In the section “Morphology of tardigrades” I would have liked to have a scheme of a tardigrade with all organs shown. One chapter of the introduction is dedicated to cryptobiosis but the chapter covers more than cryptobiosis. I would, for example, have entitled this section “Cryptobiosis and other forms of inactive states” and have maybe included encystment. The last section of the introduction is on storage cells with a single paragraph, 1.6.1, but no 1.6.2.

The second chapter entitled “Materials and methods” provides experimental procedures with high level of details, and analysis methods. I have wondered why the statistical models that are used in manuscript I are neither reported nor explained. It might have been useful since no detail on the statistical models is provided in the manuscript itself.

The third chapter of the thesis manuscript describes the results obtained during the doctoral studies.

The *first* part focuses on the mitosis of the storage cells (manuscript I). The subject of such a study is without any doubt essential and, I believe, it would have been helpful to **start** this part by explaining why it is important to characterize whether storage cells are able to divide or not. Furthermore, the parameter of mitotic index is used to characterize to which extent storage cells divide, but I wondered how the total number of the cells in *Richtersius coronifer* that is required to calculate the mitotic index has been measured. I also would have liked to have a figure showing the different phenotypic parameters that have been measured (juveniles *versus* adults/ different stages of egg development (stage I, II, III, IV)/ moulting stages (simplex *versus* double cuticule)/ gut content (empty, medium, full)).

The *second* part focuses on the characterization of the anhydrobiosis of *Richtersius coronifer* and covers the second, third and fourth publications. One aspect of this study was to measure the survival rate after multiple (up to six) cycles of desiccation/rehydration (manuscript II). The two things that might be lacking in this section are:

(i) a picture of tun, semi-tun and extended states of tardigrades in order to see how they differ.

(ii) the relationship between states of the tardigrades (tun, semi-tun and extended) and survival.

I have also wondered why 95% of relative humidity (RH) has been chosen. Why not go to a lower level of RH?

Another aspect of the study of anhydrobiosis of *Richtersius coronifer* has been to reconstitute in three dimensions *Richtersius coronifer* in a tun state by aligning semi-thin sections of tuns into correct order and position (publication III). The results are impressive.

The *third* part focuses on the ultrastructure of the storage cells in hydrated and desiccated animals with the description of two types of storage cells in hydrated animals. This part includes data from manuscript III and IV. It must be emphasized that this is the first time that different kinds (two) of storage cells have been described (among which undifferentiated cells, possibly stem cells?). Whereas the structure of type I storage cells in hydrated animals, desiccated animals, with or without heat stress, is well described, that of type II cells seems to be lacking. Why is that? Could storage cells between type I and II (under differentiation?) be detected? Images obtained by transmission electron microscopy show autophagosomes, both in hydrated and desiccated animals. Is there some evidence that their activity has increased upon a cycle of desiccation/hydration? Furthermore, I am quite not sure I understood why the vitrification hypothesis of the storage cells upon desiccation is favored.

One last question concerns the state of the DNA either in desiccated animal or during mitosis. In both cases, the state of the DNA is called “heterochromatin”. I am not sure whether the use of heterochromatin to describe mitotic DNA is proper. I would have called it “condensed DNA” instead. But may be there is a rational behind the use of heterochromatin for mitotic chromosomes that I am not aware of.

The fourth section “Conclusions and perspectives” lists the major discoveries of this work and the perspectives of research that they open, including the role of lipids in desiccation survival and their contribution in controlling and preserving cell integrity.

At the end of the thesis manuscript, a side study in the form a poster entitled “Primary Culture of Tardigrade Storage Cells from *Richtersius coronifer*” is presented. My feeling is that, although still unpublished, these results deserve more attention and should be included in the “Results and discussion” section. From my point of view, the possibility to culture tardigrade cells will represent a major and significant step in the study of the tardigrades, since editing of the genome of the tardigrade itself still represents a big challenge.

Finally, there are a few typographic errors that I will be happy to mention.

In conclusion, the amount of work that Michaela has been accomplished during her doctoral study is impressive. With her four manuscripts signed as the first and corresponding author, she has acquired, under the supervision and guidance of Pr. Ingemar Jönsson, a variety of experimental skills (*e.g.* tardigrade sampling, anhydrobiosis induction, light microscopy, scanning and transmission electron microscopy, histo- and immuno-chemical staining, 3D reconstruction, statistics), and a scientific rigor and has, without any doubt, developed an independent open minded and critical mind that is essential to any young scientist. Her work and contribution during her doctoral study are very important and break new grounds in the field of tardigrades and the physiology of their storage cells. The few minor points and questions that I have raised above will be easily fixed and answered during the defense. There is no doubt that Michaela will be able to take advantage of all these acquired expertise, strength and knowledge to perform a “state of the art” science.

I therefore certify that Michaela Czerneková exhibits expectation for independent scientific work and for awarded of the academic degree doctor.

I remain at your disposal for any additional information.

Sincerely,

Emmanuelle DELAGOUTTE