Copenhagen, 17. January 2024

Review of PhD thesis submitted by
Amit Kumar Nagwani, Adam Mickiewicz University in Poznań, Poland

The PhD thesis entitled "Searching on aging markers of the tardigrade *Paramacrobiotus experimentalis*" submitted for evaluation by Amit Kumar Nagwani explores hallmarks of aging in tardigrades, using the dioecious tardigrade species *Paramacrobiotus experimentalis* as a model.

The thesis is based on three first authored peer-reviewed articles, including two original research articles (one published and one under review) dealing with recovery in *Paramacrobiotus experimentalis* following anhydrobiosis and exposure to hypomagnetic fields, respectively, as well as a published review investigating potential markers related to aging in tardigrades. The thesis includes co-author statements from the five co-authors involved in the three publications (all from the Adam Mickiewicz University in Poznań), confirming that Nagwani performed a major part of the work presented in these articles. The thesis, moreover, includes a summary in Polish, an abstract, a list of abbreviations, an overview of academic achievements reached by the candidate during his PhD study, as well as an introductory chapter. The abstract provides a nice introduction, framing the theme of the thesis and putting it into perspective. Among the academic achievements listed are two personal grants (amount not provided), four oral and seven poster presentations at international and national meetings, as well as a one-month internship at the University of Wyoming (USA) hosted by Prof. Thomas C. Boothby. The candidate presents preliminary data obtained during his training at University of Wyoming, and further lists two co-authored publications, which were published during his PhD period, but not formally included in the thesis.

The introductory chapter, which has the title "*Description of results of doctoral thesis*", provides an overview of the candidate's scientific profile, aim of the thesis and a more thorough introduction to the field of research, as well as an overview of the results obtained during the PhD study. The chapter provides a nice framework for the three articles constituting the basis of the thesis, although I note that statements often appear somewhat superficial. Generally, the chapter would have benefited from a more accurate presentation

and detailed discussion of selected themes, including a more thorough discussion on opposing theories underlying tardigrade aging, e.g., exemplified by "the Sleeping Beauty" versus "the picture of Dorian Gray" hypotheses. Based on the results obtained in the current thesis, which hypothesis does the candidate think best explains aging in cryptobiotic tardigrades? Also, I lack a presentation of aging hallmarks normally used within animals. Hence, I would expect that the candidate can explain the choice of aging markers and briefly outline what other markers have been used in vertebrates and invertebrates, respectively. Moreover, as the mitochondrial membrane potential plays a central role in the investigations underlying the current thesis, I would have expected are brief explanation to the magnitude and nature of this potential, i.e., what generates this potential and how large is it? I also note that referencing is not always adequate or correct and I would have expected that more literature had been consulted. Generally, the reference list is too short, and it does not appear fully curated—it would have benefited from the author using more time on securing its accuracy. Below follow specific examples of statements that are in need of better explanation.

On page 17 the candidate states: "The phylum is generally divided into two classes, Eutardigrada and Heterotardigrada, distinguished mainly on the basis of claws, dorsal cuticle, body appendages, and reproductive organs". What exactly does the candidate mean with "body appendages" and what traits within the reproductive system distinguish these two major clades? Are there other anatomical structures that distinguish the two groups? Also, I am a bit surprised that the candidate only mentions morphological and anatomical data. Does the candidate not recognize the importance of molecules in revealing sister-group relationships within the tardigrades? I would expect that the candidate is aware of and can mention marker genes frequently used in tardigrade phylogenetics. The candidate further mentions that tardigrade genomes are small (also page 17), but I lack information on the exact size of these genomes and what exactly the author defines as small, *i.e.*, small compared to what?

On page 18, the candidate states that factors known to influence anhydrobiotic survival are "overall body size, dehydration conditions, duration of desiccation, air humidity, temperature and strength of surrounding magnetic field". Again, I find this statement needs elaboration. What does the candidate mean by "dehydration conditions" and who exactly has shown that the mentioned factors are important for anhydrobiotic survival in tardigrades? Do these factors apply to all investigated cryptobiotic tardigrade species? The candidate continuous with stating that "the importance of sex and group influence were rather not investigated till the date". However, Ivarsson and Jönsson, already in 2004 investigated the

influence of aggregation on anhydrobiotic survival in *Richtersius* cf. *coronifer*, which should have been acknowledged here.

On page 19, the candidate argues that aging "can be considered at different levels of organism organization", but what levels of biological organization is the candidate referring to, and can he give examples of studies investigating these different levels? The candidate further states that the species in focus (*i.e.*, *Paramacrobiotus experimentalis*) has a long average lifespan and high anhydrobiosis capability, without explaining how long the lifespan is and what he means by "a high anhydrobiosis capability". I note that the candidate provides information on lifespan on page 20, but this information should have been given together with the above statement to ease readability. Also, there are no references to the statement "The mean (19-360 days) and maximum lifespan (1-24 months) vary between known tardigrade species" on page 20. I further find the following statement (page 20) "In tardigrades, the parthenogenesis (a self-fertilization strategy) is the most common reproduction strategy" needs elaboration. Is this correct? Please elaborate and explain in which habitats you normally will find parthenogenetic tardigrades. And along this line please explain whether the species used as model in the current thesis (*i.e.*, *Paramacrobiotus experimentalis*) can reproduce by parthenogenesis.

The first of the three publications, forming the basis of the thesis, is a review article with the title "*Applicable life-history and molecular traits for studying the effects of anhydrobiosis on aging in tardigrades*" published in the MDPI journal *Diversity* in 2022. The review provides an evaluation of markers that potentially can be used to study the influence of anhydrobiosis on aging in tardigrades. Specifically, it involves a discussion of tardigrade life-history traits, with focus on lifespan and fecundity, as well as an overview of markers of cellular aging that potential could be used in tardigrades. The latter includes reactive oxygen species (ROS) and various markers of antioxidative defense, markers of mitochondrial function, as well as epigenetic modulations. The review is novel in the sense that it highlights a perspective of tardigrade research that has not yet received much attention, *i.e.*, the possibility of using tardigrades as a model for research into mechanisms underlying aging. The review rests on a comprehensive number of references and it forms the basis for the two original research articles included in the thesis.

The second article entitled "*Recovery from anhydrobiosis in the tardigrade* Paramacrobiotus experimentalis*: better to be young than old and in a group than alone*" is not, yet, published.

Specifically, this original research article is currently submitted to the Cell Press journal, *Heliyon*. The article builds on the review mentioned above and has the overarching aim of providing sufficient data on *Paramacrobiotus experimentalis* to allow this tardigrade to be implemented as a model for investigating molecular and cellular mechanisms underlying anhydrobiosis. While most investigations into extreme stress tolerance in limno-terrestrial tardigrades have been performed on parthenogenetic females, the current investigation distinguishes itself by focusing on a dioecious species that allows investigation of both males and females. Specifically, the authors investigate the influence of selected life-history traits, *i.e.*, sex and age, on post-anhydrobiotic survival, as well as the influence on survival of desiccation alone or with other individuals during repeated desiccation cycles. The methods are generally well described and seem straight forward. Nevertheless, the term "vitality rate" would benefit from a more comprehensive explanation. Hence, I expect that the candidate can explain the rationale behind this variable. The authors find that survival is highest among younger individuals and in tardigrades desiccated in groups. Also, the duration of the desiccation period matters, with higher survival following repeated short desiccation periods as compared to repeated longer periods.

The third publication entitled "*The effect of hypomagnetic field on survival and mitochondrial functionality of active* Paramacrobiotus experimentalis *females and males of different age*" was published in the Frontiers journal *Frontiers in Physiology* in 2023. This is an interesting publication addressing a novel subject, *i.e.*, hypomagnetic fields as an extreme environment. Specifically, in this article the authors investigate the effect of exposure to a hypomagnetic field (HMF) on survival as well as on the magnitude of the mitochondrial membrane potential in tardigrades of different age and sex. The authors find that the tardigrades have a high tolerance (≥ 87% survival) towards exposure to hypomagnetic fields, but that survival, nevertheless, depends on the duration of the exposure, and in males also on age, with older males being more sensitive to the exposure. In order to investigate the influence of hypomagnetic fields on mitochondrial function, the authors subsequently evaluate the intensity of stainings with the cell-permeant, cationic fluorescent dye TMRM and find that intensity declines upon exposure to HMF, indicating a depolarization of the mitochondrial membrane potential. I would expect that the candidate can explain the applied method for quantification of the mitochondrial membrane potential. The results indicate that both age and sex influence the magnitude of changes in mitochondrial membrane potential. The data support the assumption that mitochondrial function in tardigrades, and more specific the

mitochondrial membrane potential can be used as marker of age, but also sensitivity towards HMF and possibly other extreme stress conditions.

In addition to the three articles mentioned above, the author presents preliminary data on accumulation of ROS using a cell-permeable fluorescent probe in *Paramacrobiotus experimentalis* females and males of different age and in their isolated storage cells. This data was generated during the candidate's internship at the University of Wyoming with the purpose of investigating whether ROS can be used as an aging marker in tardigrades. The candidate reports that the intensity of the fluorescence signal increases with age in both females and males and in their isolated storage cells, with males generally showing higher signal intensity. Confocal laser scanning microscope images of tardigrades and isolated storage cells stained with the probe (DCFH2-DA) are presented in the Appendix (page 133-135), but the author does not provide any quantitative data that can support his conclusion. Hence, these data are indeed preliminary, but they, nevertheless, demonstrate an important aspect of the current thesis, *i.e.*, that it does not simply build on repeating methods and investigations performed by others—it has a more experimental focus, directed at developing methods with potential importance for future directions within tardigrade research. Accordingly, I hereby confirm that the candidate, Amit Kumar Nagwani, can proceed to the final stages towards the award of a doctoral degree.

Kind Regards,

Nadja Møbjerg