Amsterdam, 01-05-2023

After reading the proposed thesis work of Vendula Rašková (PhD student in the Biology Centre, Institute of Parasitology, Czech Academy of Sciences, and Faculty of Science, University of south Bohemia, České Budějovice, Czech Republic), I am happy that I can say that, in my view, the work is clearly sufficient to grant a PhD.

I really enjoyed reading the manuscript, as almost all of the subjects are related to subjects I am passionate about (what is not to like about "aspects of mitochondrial evolution"?). So, thank you for giving me the opportunity to read the thesis.

I want to congratulate both the candidate and the direct supervisor of the work, Durante Ignacio Miguel, Ph.D, with the quality of the work and the number of (possible) articles that are coming out of these studies (2 already have been published; one in PLoS ONE and the other even in Molecular Biology and Evolution, an important and high-impact journal in our field). Modern day research is a collaborative effort, so I would like to extend these congratulations to the other people in this research network as well.

Upon stating this, however, I am obliged to point out that I also have further remarks (and questions!) regarding the content of this thesis. These will be scientific in nature (I could list remarks concerning English language errors in the manuscript at hand, but as these are relatively minor, *and do not interfere with efficient information transfer*, I will not do so). I will mostly focus on those projects presented here which are close to research questions I am most acquainted with (and are close to my heart, I might add).

Remarks and questions.

1. Only two remarks about grammatical errors (see also remark 7), as these are not really that extensive in the current thesis. Thus, it is a bit of a pity that one of the first passages one reads (the "Annotation") contains quite a few…

2. On page 10 it is stated that "OXPHOS is completely dependent on electron transport complexes (ETC) I, III and IV (Fig. 2) as they pump protons across the inner membrane into the intermembrane space against the proton gradient." Formulated thus, the statement is *wrong*, as both complex I and IV can be replaced by non-proton pumping substitutes (the alternative NADH-dehydrogenase and alternative oxidase -AOX; referred to on page 19 below-, respectively). Of course, LECA had the full complement, but it is interesting to contemplate with regard to the evolution of the ETC (far before eukaryogenesis), that only complex III cannot be substituted when using molecular oxygen as the final electron acceptor. Here differences in mechanisms of proton displacement (III uses vectorial (!) transport) might be pointed out as well. But we are straying from the real subject(s) of the thesis here.

3. Alas, on page 11 a highly confusing description is given. I quote in full: "In aerobic energy metabolism, electrons coming from succinate generated in the Krebs cycle are transferred to ubiquinone through complex II. Complex II catalysed the oxidation of succinate to fumarate and transfers its reducing equivalent to Q, an is also responsible for the reduction of fumarate. Fumarate serves as the terminal electron acceptor and electrons are transferred in the reverse direction (Tielens et al., 2002; Lancaster, 2002)." The last part of the description has nothing to do with aerobic energy metabolism, as fumarate is the terminal acceptor now, *instead* of molecular oxygen! Fumerate reduction can e.g. occur in helminths, by transfer of electrons from *menaquinone* to fumarate and thus converting fumarate into succinate, instead of the other way around.

4. On page 12, it is stated that: "The mammalian complex IV consists of 14 subunits and exists in two different states (assembled into supercomplexes or dispersed on mitochondrial inner membrane) (Zong et al, 2018)." *Only* mentioning the involvement of supercomplexes here, gives the impression that complex IV is special. It is not (complex III, itself always a homodimer, is involved in supercomplex formation, as is complex I).

Also, on page 12: "Mitochondrial ATP synthases occur in dimers (Dudkina et al., 2005), this structure induces curvature of the inner mitochondrial membrane and governs cristae formation (Arnold et al., 1998; Davies et al., 2012). This is rather unnuanced: the dimerization is only one of the influences governing cristae formation. For instance, certain Gregarines do not have Mitochondrial ATP synthases anymore, but retain cristae (Mathur et al., Parallel functional reduction in the mitochondria of apicomplexan parasites; https://doi.org/10.1016/j.cub.2021.04.028).

5. Page 15: "Overexpression of TbDLP1 is able to rescue endocytosis and growth defects in bloodstream form of the parasite, while in the insect-dwelling procyclic form, both TbDLP proteins are indispensable (Benz et al., 2017)." Rescue what precisely (probably absence of TbDLP2)?

6. Page 19: "As a substitution of the respiration,…" No, it is still respiration, but with a different oxidase.

7. Page 25: Just one more example that a final round of proofreading might have been beneficial. "Pam18 and Pam16 belong to the belong to the DnaJ and DnaJ-like families of proteins,…".

Overall, regarding the introduction, I have to admit that, because of the many, highly diverse, aspects of kinetoplastid mitochondrial function that are dealt with in this thesis (see "2. Objectives"), the introduction has to be somewhat superficial in nature. This cannot be helped.

The following chapters are either published, or on the way to be published.

"ZapE/Afg1 interacts with Oxa1 and its depletion causes a multifaceted phenotype." is the PloS ONE article coming out of objective 1. "Study of the functions of ZapE in T. brucei."

"Vestiges of the bacterial signal recognition particle-based protein targeting in mitochondria."is the Molecular Biology and Evolution publication coming out of objective 2. "Analysis of the presence of homologs of the bacterial Ffh and FtsY proteins in various unrelated plastid-lacking unicellular eukaryotes."

"Orthologues of presequence translocase-associated motor subunits are essential for kinetoplast DNA replication in procyclic form of Trypanosoma brucei." is to be published as a result of objective 3. "Investigation of the role of TbPam18 and TbPam16 in T. brucei."

"Novel protein complex involved in kinetoplast DNA replication and maintenance" is to be published as a result of the eponymous objective 4. "Research into a novel protein complex involved in kinetoplast DNA replication and maintenance."

These chapters represent an impressive amount of diverse research objectives, using a wide variety of techniques to highlight both unique kinetoplastid developments *and* generate some findings in trypanosomes that help explain (mitochondrial) processes in other eukaryotes. The PhD student seems to have to made a worthy contribution to all this work. I am looking forward to discussing the chapters.

Thus, it is with pleasure that I can recommend the thesis for defense.

With kind regards,

Dave Speijer

PhD Room K1 - 263

Amsterdam UMC; University of Amsterdam, Medical Biochemistry, Meibergdreef 15

1105 AZ Amsterdam, The Netherlands, e-mail: d.speijer@amsterdamumc.nl, tel: 31205665122.

Officially signed: