
Evaluation of thesis:

I would divide the Jan Mach’s doctoral thesis into two major parts: an introduction directly written by the candidate and a set of three publications that he co-authored. As a whole, the publications are of excellent quality, each representing an important advance in our understanding of *Trypanosoma brucei* mitochondrial (mt) biogenesis, or extra-organelar pathways that influence this process. The candidate is first author on two and second author on the third articles, reflected in the significant contribution he made as documented on page 53 of the thesis.

The first paper published in Genome Biology and Evolution is a very nice piece of work not only characterizing the subunits of mt processing peptidase (MMP) itself in *T. brucei*, but also the related respiratory complex III core proteins cp1 and 2. The paper has an impressive array of techniques spanning cell biology, reverse genetics, protein biochemistry and bioinformatics. The last set of methods was used effectively to reveal the underlying properties of mt signaling peptides that are recognized by MMP as well as the evolutionary history of the subunits and cp proteins as well. I learned a lot from this paper and have no doubt it will be well cited due to its wide scope and subject. The main conclusion that *T. brucei* MMP and its substrates do not represent ancestral forms but are highly derived is well supported.

The second paper published in the Journal of Parasitology is the shortest of the three. It has also been cited eight times since publication, which is a testament to its importance. For its length, it is quite rich in data, establishing an iron-uptake assay in PCF *T. brucei* to show that this process occurs as in yeast by a reductive mechanism. I see the potential for this assay allowing future researchers to further investigate this process. This paper importantly shows that Jan is capable of optimizing a new assay in *T. brucei*, which is a very difficult process requiring skill and patience.

The final paper to be published this year in Molecular Microbiology, in which Jan made significant contributions as second author, characterizes the recently discovered mitochondrial pyruvate carrier (MCP) in the two *T. brucei* lifecycle stages PCF and BSF. It is a timely article showing that the function of MCP is well-conserved *T. brucei*. The paper goes along with the main theme of Jan’s thesis that *T. brucei* is not a living fossil but a diverged eukaryote that evolved in its own milieu. In support of the latter idea, they also present two surprising findings: evidence that pyruvate may be imported into the *T. brucei* organelle by another pathway; pyruvate uptake is essential even in BSF, in which the Krebs cycle further metabolizing the keto acid is absent. As with the first paper, an impressing spectrum of methods was employed. I believe that it will be a key paper in field of *T. brucei* metabolism.

In summary, all of these publications demonstrate that Jan has become a capable researcher during his doctoral studies, possessing knowledge about a wide range of methods and topics in mitochondrial biology. Because the papers have been through rigorous peer review in high quality journals, I will focus mainly on the introduction that Jan has written himself.
I commend Jan for choosing to write this part in English. Overall, I am happy with the use of English in the introduction. While it is not perfect, the grammatical errors are those I commonly encounter, e.g. the use of definite and indefinite articles. I have marked these errors in my copy of his thesis, which I am happy to trade if Jan is interesting in seeing these classical errors corrected.

However, I have to admit that the quality of the introduction was less than the excellent level displayed in the publications. In my opinion, the glaring errors, such as confusing statements and passages, poorly chosen citations, and other examples of what I can only call sloppy mistakes, needlessly detract from a potentially nice introduction. I have the feeling that this part of the thesis succumbed to being written under time pressure. However, advising Jan to take more time for this endeavor is preposterous to me, as I recently come to the realization that almost all the writing we do is under some sort of time pressure. I hope that Jan takes these criticisms as advice how to only better write in general, but also what to be careful of when it has to be done inevitably during a short time span.

- If you chose to write in English, then the references should be ordered in the English way as well. I noticed that names starting with “ch” were ordered after “h”, which would be confusing to many people who are not familiar with this type of ordering. Furthermore, names with prefixes such as “van der”, “von”, etc., should be placed with names starting with “v” in such a list. Also, I could not find one citation “Braun and Schmitz, 1995” in the references.

- While parts of the introduction about protein import and iron metabolism are well cited, other parts are not at all. For example, the paragraph discussing VSG in trypanosomes on page 8 does not contain a single citation!

- I also noticed some inappropriate citations in the introduction, such as referring to Immo Scheffler’s Mitochondria book in page 11 when speaking about mt carrier proteins. Since MPC is a topic of one of the publications, I think better references could have been chosen here. Another example is the van der Laan review about the MINOS complex as a reference for ERMES (page 23) or the Baltz et al., 1985 reference for BSF culture media when even the publications contained in the thesis reference the researchers that actually formulated the now used HMI, the Hirumi papers.
  - As a rule, I try to avoid referencing books, try to keep reviews citations to a minimum, restricting them to when I refer to a general phenomenon or a tangential topic in the text.

- Words are often repeated in the same sentence, which should be avoided (e.g. …small part of the lipids, while most of lipids…(page 14)).

- There were many passages that I found a bit confusing. For example, write on page 5 “Euglenozoa belongs to [a] group with unique cristae Discicristata…” Why is the last word in italics? You mean the superphylum or the actual disc-like cristae?

- You write on page 6 that nagana is a “livestock and wild animal disease”. If I understand correctly, wild animals serve as reservoirs for T. brucei, and thus are not symptomatic for nagana. This would be consistent with their co-evolution with the parasite, unlike introduced cattle.

- The section about mt energy metabolism was the most poorly written part of the introduction. The TCA cycle subsection discusses in depth about glycolysis. I understand glycosome and mt function are tightly integrated, but it would have been less confusing to devote its own subsection to the topic and then discuss how it relates to TCA. Furthermore, the glycolytic step catalyzed by phosphoglycerate kinase moves from the glycosome in BSF to the cytosol in PCF,
which is not mentioned in the introduction. Yet, when differences in the metabolism are discussed in this section, it is done in a way that is difficult for this reader to orient himself. Again, since MPC is a topic of one of the publications, this part should have been much better written as it pertains to pyruvate generation/catabolism.

- On page 12-13, it is stated that PCF proline and glutamine from the tse tse fly hemolymph. Since PCF represent the midgut dwelling stage, how can they be exposed to the hemolymph?
- Figure 3 uses different abbreviations than used in the main text, e.g. BS and PS instead of PCF and BSF, respectively.
- Figure 5 shows the composition of the *T. brucei* mt protein import machinery, but the text referring to the figure discusses differences with this machinery examined in *Saccharomyces cerevisiae*. The figure would befit by showing the yeast system as well.
- A list of abbreviations used in the thesis is absent.
- Publications lack supplementary figures.
- While the conclusion chapter nicely summarizes the publications, there is no attempt made by the author to try to tie these three articles together and explain how the examined processes work together to contribute to *T. brucei* mt biogenesis or evolution.

I should reiterate however I applaud Jan for writing in English and there are parts of the thesis that are well-written. It is unfortunate that many of the mistakes I list above detract from the quality of the thesis. However, I am convinced from reading the introduction that Jan has the potential to learn from them and will be able to write scientific text under time pressure. In the final analysis, I would give the introduction a grade 2, which certainly does not mean he failed in this endeavor.

Overall, I feel that by the merits of Jan’s significant contributions to excellent publications and adequate albeit imperfect performance on the introduction shows that he has during his doctoral studies acquired the skill set and intellectual capabilities needed by a researcher at this stage of his career. I also should add that I enjoyed reading the thesis and look forward to seeing how Jan answers the following questions for discussion. I unequivocally recommend him for the title Ph.D.

Sincerely,

Hassan Hashimi Ph.D.
České Budějovice, 4 April, 2016
Questions for discussion:

Introduction

1. On page 5 of the introduction, you write, “Euglenozoa belongs to [a] group with unique cristae Discicristata…” What are the unique cristae exactly? How do they differ from other cristae forms?
2. On page 6, you mention T. vivax and T. congo. How do these trypanosomes differ from T. brucei?
3. On page 25, you write about OXA machinery that “…it was suggested that Oxa2 recently derived from Oxa1 by gene duplication (Preuss et al, 2005) whereas kinetoplastids are believed to diverge very early in eukaryotic evolution…” Can you please elaborate on this? Why do you think this finding is so intriguing?
4. On page 27, you write that the reason for Icp55’s localization also in the nucleus is that its substrate IscS is also there. I am not so satisfied with this logic. I would imagine that Icp55 would have to be present in the nucleus prior to IscS being targeted there, so the peptidase may have another reason for its dual localization. Can you please comment on this? Also, you state that IscS is still “somehow” processed by MPP in mitochondria before being exported to the nucleus. Also, any idea how the processed IscS would be translocated out of the organelle?
5. On page 28 you mention the high iron requirement for parasites since they more rapidly proliferate compared to the host. Do you think this system can inform cancer cell biology, since these also more rapidly divide compared to the somatic cells? Also, and perhaps relatedly, is the iron requirement for parasites really that high? You say yourself on page 6 that BCF most likely needs less than PCF due to the lack of the electron transport chain. Other excavate parasites such as Trichomonas and Giardia have reduced mitochondria and some helminth mitochondria are anaerobic in the host.

Genome Biol. Evol. article

6. Why were the Euglena cp1 and cp2 proteins used as a query for BLAST search of T. brucei MPP subunits instead of e.g. yeast?
7. You claim that cp1 evolved independently 3 times in metazoa, fungi and kinetoplastids based on the phylogenetic tree in Fig. 6. I do not dispute that cp1 emerged independently in kinetoplastids, but it is surprising to me that it did so fungi and metazoa. Is there a reason why the authors did not consider that cp1 was acquired at the root of the opisthokont clade and was secondarily lost in yeast lineages giving rise to or because of the emergence of the Neurospora-type system?
8. In Fig. 3A, you determine by western blot of digitonin fractionation T. brucei that both MPP subunits are downregulated in BSF as compared to PSF. According to Šmíd et al., 2006 reference, which describes the how the assay is performed, total cell number is measured as imput. Do you think that it is better to compare stage levels by western analysis by loading equal cell amounts, as I suume you did here, or equal protein amounts from both stages?
9. Any hypothesis as to the identity of the $^{59}$Fe-labelled mitochondrial protein complexes could be in Fig. 1B?
10. Did you look at whole cell fractions to see if different complexes/proteins are labelled than those in Fig 1B. What extra-mitochondrial proteins/complexes could potentially be $^{59}$Fe-labelled after the 1 hour incubation?
11. Did you consider trying a longer chase period after the 1 hour $^{59}$Fe-incubation to see whether other proteins/complexes incorporate the isotope? What would you expect the result of such an experiment would be if you did not?
12. This is a short paper establishing a very cool assay. Can you suggest another project (or projects) where this assay could be applied?

Mol. Microbiol. article
13. Why did you not consider growing the ∆MCP PCF mutants in glucose-poor or -depleted (depending on FBS used) SDM80, analogous to what you had done by growing BSF ∆MCP1 in CMM? What would you expect to be the outcome of such an experiment in terms of parasite fitness and metabolic end-products?
14. You hypothesize the presence of an alternative mitochondrial pyruvate uptake pathway based on the observation that the ∆MCP mutants or the specific MCP inhibitor UK5099 does not fully abolish the metabolite’s import. In the latter case, is it possible that UK5099 only partially inhibits the T. brucei ortholog, given its divergence from the opisthokont MCPs (e.g. P→A substitution in MCP1)? Can you provide any evidence to support/refute this idea?