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EVALUATION REPORT OF ALENA ZIKOVA'S HABILITATION THESIS

Dr Alena Zikova dedicated her research career to the atypical mitochondrion of the parasitic protist *Trypanosoma brucei*. After a successful PhD thesis in the laboratory of Professor Julius Lukes, she moved to the prestigious Seattle Biomedical Research Institute in the USA to work with Professor Ken Stuart. There, she had major contributions reporting the molecular composition of mitochondria, with surprising results such as the presence of numerous atypical proteins including in "classic" protein complexes. The most striking example is the case of the F0F1 ATP synthase, an enzyme that is conserved in animals, plants and bacteria. These seminal findings led her to set up her own research group in the Czech Republic, with the main goal of understanding the function of the bizarre subunits and to decipher their biological significance.

These investigations led to fascinating results, revealing that the trypanosome ATP synthase functions on a significantly different manner compared to other organisms. Furthermore, she worked out that this enzyme itself functions very differently between two life cycle stages of the parasite, with unique regulators. In one case, it indeed produces ATP, in the other one it consumes ATP to maintain membrane potential. A major finding was the discovery that the enzyme is essential for the bloodstream stage of the parasite, a rather unexpected finding knowing the then-thought minor function of the mitochondrion. All these works are of remarkable quality, carefully executed and very well presented.

In the recent past, she pushed on towards precise molecular and structural studies, collaborating efficiently with experts in X-ray crystallography (Walker group, Cambridge) or cryo-electron microscopy (Amunt lab, Stockholm). These projects have been extensively worked out and Dr Zikova did much more than providing samples: she herself worked in these labs or sent her staff members to work side-by-side with the collaborators. This led to new discoveries and demonstrated that some commonly accepted hypotheses (for example the proposed role of the p18 protein) were not correct. Her last work went even further, determining by cryoEM the molecular organisation of the ATP synthase isolated from trypanosome mitochondria, revealing once again novel exciting features.

Most people work with the two trypanosomes stages that can be grown easily in the laboratory. However, the trypanosome life cycle is much more complex than that, with multiple intermediate stages during development in the tsetse fly. These have resisted exhaustive biochemical characterisation due to the difficulty in obtaining sufficient material. This

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limitation can be overcome thanks to an *in vitro* differentiation approach triggered by the overexpression of an RNA-binding protein. This system remains quite delicate to manipulate, but Dr Zikova and her team achieved that successfully with an exquisite study where they followed the progression through differentiation towards the mammalian-infective form, encapsulating every aspect, from transcriptomics to proteomics. Moreover, they discovered an exciting pathway by which the production of ROS contributes to reinforce the differentiation process, in a so-called retrograde signalling process. This is supported by a clever approach, using inducible expression of a catalase enzyme from a distant parasite to control ROS production, leading to an arrest of differentiation. This study is definitely a landmark for the field.

I would also like to mention that Dr Zikova dares exploring less commonly studied aspects of mitochondria, such as this nice collaborative project with Dr Butikofer in Bern where they investigated the role of a lipid (cardiolipin) in mitochondrion structure, function and composition. Once again, this was a tight bidirectional collaboration that led to unexpected results.

In summary, the research achievements of Dr Zikova are truly exceptional. Throughout her career, she displayed the right combination of creativity and rigor, driving multidisciplinary projects and setting up appropriate and tremendously successful collaborations. She can think outside the box and is not afraid to challenge dogmas. All the papers presented in her manuscript are well-constructed, figures are crystal clear, making them easy and enjoyable to read. Finally, her work has strong potential for drug development. She has established herself as a leader in her field, with international recognition.

Please do not hesitate to contact me if you need more information.

Best regards,

Dr Philippe Bastin, Director of Research, P.I., Trypanosome Cell Biology Unit

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The reviewer's evaluation of the habilitation thesis

Candidate	**RNDr. Alena Panicucci Zíková, Ph.D.**
Habilitation thesis	Mitochondrial adaptations throughout the *Trypanosoma brucei* life cycle
Reviewer	Professor emeritus Fred R. Opperdoes
The reviewer's department, institution	de Duve Institute, Université catholique de Louvain, Brussels, Belgium

The scientific contributions by Alena Zikova are remarkable. in view of her age she has an impressive CV with an excellent track record of well over 50 publications in international, peer-reviewed journals (PUBMED). She received training in a number of top laboratories in the field of trypanosome biology, such as those of Julius Lukes, Ceske; Rob benne, Amsterdam; Ken Stuart, Seattle; John Walker, MRC, Cambridge. She made many important contributions to science especially related to the structure and function of the trypanosome mitochondrial ATPase. She has presented many invited lectures and has been, and still is, involved in many international collaborations. In spite of her relatively young age (45) she can be considered already as one of the most successful younger scientists in her field with well over 2000 citations, an interest score of 937, and an h-index of 23 (Research Gate and Google Scholar). She has supervised many graduate and PhD students and gained a wide experience in teaching of both undergraduate and graduate students. Also she has succeeded to collect large amounts of funding from both national and international organisations.

It is important to mention that this thesis has already gone through peer review since it was published last March as an invited review in the Journal of Eukaryotic Microbiology (doi 10.1111/jen12911), an additional indication that the contributions to science by Alena Zikova are highly appreciated by her peers.

Questions of the reviewer on the defence of the habilitation thesis

From the work by Pjotr Slonimski we know that yeast cells may loose parts (petite mutants) or their entire (rho(-) mutants) mitochondrial DNA. Such cells are still capable of anaerobic fermentation converting glucose into alcohol without involvement of the mitochondrion. The cells remain viable for a considerable time, but need access from time to time to traces of molecular oxygen for the synthesis of essential sterols. In your work you describe a similar situation with vertebrate trypanosomes of the genus Trypanosoma. In T. brucei parts, or all, of its mitochondrial DNA may be deleted by mutation, while T. evansi and T. equiperdum are believed to be natural dyskinetic trypanosomes. Is it correct to infer that the dyskinetoplastic trypanosomes on the one hand and the petite or rho (-))

yeast mutants on the other hand represent comparable biological systems, or do there exist significant differences between these two research models?

In your thesis (page 23 "The Conundrum of the TCA cycle") you address the functioning of the TCA cycle as a real cycle, or not. One of the problems with the cycle is that under physiological conditions Louis Tielens and coworkers have clearly shown that the enzymes of this cycle do not operate as a full cycle. Several enzymes such as citrate synthase, aconitase and isocitrate dehydrogenase are found to be absent or very low in activity, in agreement with the absence of such a full cycle. On page 23 (middle) you discuss an experiment in favour of such a cycle where the TCA cycle seems to be fully active in the presence of 10 mM malate. I would like to make two remarks. First, 10 mM is not very physiological and second in T. brucei there are two isocitrate dehydrogenase (a mitochondrial and a cytosolic/glycosomal isoenzyme), both of which are NADP-dependent enzymes and work preferably in the reversed direction, contrary to the NAD-dependent isoenzyme, the true TCA cycle enzyme. This is in support of the absence, rather than in the presence of a functional TCA cycle under physiological conditions. Such situation is also encountered for most other trypanosomatids as well, except in the case of some Leishmaniae. Please comment.

In the early studies of the respiratory chain of trypanosomatids there was frequently mention of the presence of an o-type cytochrome. In recent publication no cyt o is mentioned anymore, and moreover there is no cyt o gene reported in any of the published trypanosomatid genomes. Do you, as an expert of the mitochondrial respiratory chain, have an explanation for this absence?

On page 12 (lines 8-10) you mention the enigmatic situation of a functional complex I in trypanosomatids. Most seem to have such a complex but only in a few of them the complex seems capable of OXPHOS. There exists also an alternative to complex I: alternative NADH dehydrogenase or NAD2, which may replace this complex. Now that we have at our disposal so many complete genomes (nuclear and mitochondrial) have you ever tried to compare the subunit composition of the various complexes I (Trypanosoma and certain Leishmaniae, against Phytomonas and Vickermania) to correlate the absence or presence of one or more Complex I subunits with the presence or absence of OXPHOS capability?

You propose the cardiolipin biosynthetic pathway as a potential target for drug development. I would like to stress the cardiolipin is also essential for mammalian mitochondria. Is there anything known about this pathway that is specific to trypanosomatids? Have you identified all the individual enzymes involved and how much do the Trypanosome enzymes differ from the respective enzymes from mammals?

Fig. 1 of your thesis (page 9, long slender form). According to this figure the LS form of T. brucei converts glutamine into 2-oxoglutarate. This is exactly what I expected to find in the seventies when I started to study the sub-cellular distribution of trypanosome enzymes by differential and gradient centrifugation. To do so, one needs unambiguous marker enzymes for each of the different organelles. In those days the marker for mitochondria was NAD-dependent glutamate dehydrogenase (GDH), but I could not measure any activity ever in extracts of T. brucei (this was the monomorphic strain 427). Interestingly a GDH sequence was later described in Leishmania by Bringaud and Simpson as a guide RNA binding protein. Later it turned out that there is a NADP-dependent isoenzyme, but this does not operate in the mitochondrion. Similarly I was also unable to measure activity for citrate synthase, aconitase and threonine dehydrogenase, while there respective genes are all present in the T. brucei monomorphic 427 Lister genome. So I have some difficulties

with this fifth panel of Fig. 1, which is maybe based on the presence of gene sequences, or transcription data, rather than on measured enzyme activities.

In your paper (Panicuci et al., 2017) on the F1-ATPase inhibitor protein you show that this protein inhibits the hydrolysis of ATP by the F1-ATPase and that therefore it is expressed only in the procyclic insect stage capable of OXPHOS and not in the bloodstream form where a proton gradient is maintained by hydrolysis of cytosolically produced ATP by reversal of the ATP synthase reaction. I carried out a quick BLAST search in the monomorphic T. brucei 427 Lister proteome and the other trypanosomatids and this revealed that a TbIF1 orthologue is absent in 427 but is present in Phytomonas. This nicely correlates with the the absence of OXPHOS by complexes I, III and IV in T. brucei 427 Lister and the presence of a demonstrated OXPHOS-capable complex I in Phytomonas. Interestingly the same Blast search revealed also that the only other trypanosomatid without an F1-ATPase inhibitor gene is Vickermania. However, Vickermania which lacks the complexes III and IV has a predicted complex I. Do you agree with me that one may conclude from this observation that the Vickermania Complex I will not be capable of any OXPHOS and creates its proton gradient by ATP hydrolysis?

Oligomycin sensitivity of the mitochondrial ATPase is a property dependent on the presence of an intact mitochondria DNA both in yeast, mammals and tryps. In the latter several mitochondrial DNA sequences have been published but it is not clear to me which gene, if any, is responsible for the OSCP (oligomycin-sensitivity conferring protein) in trypanosomatids. Could you fill me in?

Signalling (page 28). In my humble opinion signalling could turn out into a very promising line of research.The fact that the inactivation of ROS involves a number of enzymes that are related to or are themselves trypanothione-dependent enzymes suggests the presence of a host of novel enzymes, pathways and mechanisms. I look forward to hear about this in the near future.

Conclusion

The habilitation thesis of RNDr. Alena Panicucci Zíková, Ph.D. "Mitochondrial adaptations throughout the *Trypanosoma brucei* life cycle" **meets** the standard requirements for habilitation theses in the field of Molecular and Cell Biology and Genetics.

Leuven, Belgium on October 1st, 2022

Signature of the reviewer

The reviewer's evaluation of the habilitation thesis

Candidate	**RNDr. Alena Panicucci Zíková, Ph.D.**
Habilitation thesis	Mitochondrial adaptations throughout the *Tryponosoma brucei* life cycle
Reviewer	Boris Striepen, PhD Mark Whittier and Lila Griswold Allam Professor of Microbiology and Immunology Professor of Pathobiology School of Veterinary Medicine Professor of Microbiology Perelman School of Medicine University of Pennsylvania
The reviewer's department, institution	.....

Please refer to my enclosed letter.

Questions of the reviewer on the defence of the habilitation thesis

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Conclusion

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In Phialadelphia, PA on 10/10/2022

Signature of the reviewer

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Philadelphia, October 11, 2022

Dear Colleagues,

You recently asked me to evaluate the habilitation and overall professional standing of **Dr. Alena Zikova**, and it is with great pleasure that I respond today. Let me start by apologizing that I cannot take part in the formal meeting of the committee in November in person. I agreed to give a seminar at Hebrew University in Jerusalem that week, and I will travel back to the United States on the very day of the meeting. I spoke to the chair of the committee, Professor Jan Tachezy, and he offered to represent me, for which I am grateful.

Dr. Zikova received outstanding scientific training as undergraduate and graduate student in Ceske Budejovice conducting research in the laboratory of Professor Lukes, and as a postdoctoral fellow in Seattle with Professor Ken Stuart. She was remarkably productive during her training, and published eight articles, from her thesis and before, and seven from her postdoctoral fellowship. That is a very strong record, and it thus not surprising that she was appointed as a group leader in Ceske Budejovice upon her return from the US in 2009.

I am happy to report that her independent research group was just as successful as her time as a trainee. Dr. Zikova is an internationally recognized expert in the cell biology and biochemistry of protozoan parasites, and the consistent focus of her research program has been the mitochondrion of *Trypanosoma brucei*, the causative agent of African sleeping sickness in humans and Nagana in cattle. To complete their lifecycle, trypanosomes must colonize both mammalian and insect hosts, which provide vastly different metabolic environments. In her research Dr. Zikova has revealed dramatic changes in the mitochondrion, as the parasite progresses through its lifecycle, and adapts to changing nutrient availability. In turn, the parasite uses metabolic cues to time and elaborate

developmental programs, and Zikova's work has highlighted multiple such interactions. Dr. Zikova's work is grounded in her deep understanding of biochemistry, solid molecular biology and genetic engineering, and the overall rigor of her experiments. Over the twelve years of her laboratory's work, she drew in new approaches as they became available, ranging from biochemical assays and mutant analysis to sophisticated measurements of the abundance of transcripts, proteins, and metabolites, and most recently, high resolution structural approaches. While open to the power of new technologies, her work never lost sight of answering fundamental questions of cellular evolution and physiology. Studying the respiratory chain of trypanosomes and in particular the ATPase is of great interest, as this machinery is highly divergent from our primary knowledgebase in mammalian cells and baker's yeast. Perusing her CV and pubmed record, I noted at least 36 articles from her independent career. These articles were consistently published in excellent peer-reviewed journals, speaking for the high quality of the work. Out of the many articles that form the basis of her habilitation, I only have time and space here to discuss a selection in detail.

The 2015 Subtrova *et al. PLoS Pathogens* paper is focused on the mitochondrial F_0F_1-ATPase of African trypanosomes, which is required in both hosts (even in the absence of active oxidative phosphorylation), as it switches from ATP synthesis to hydrolysis to maintain the organellar membrane potential in the mammalian host. This study provides a detailed genetic and biochemical analysis of the unique Tb2 subunit in stages that do not rely on oxidative phosphorylation. Zikova's team shows with rigorous biochemistry that in these parasites, the protein is indeed a mitochondrial protein, and a *bona fide* functional part of the fully assembled ATPase. Ablation of the protein potently suppressed parasite growth by decreasing the mitochondrial membrane potential. Making clever use of laboratory derived and natural petite mutants they show that the profound growth defect does not depend on a fully functional mitochondrion, and that the Tb2 subunit is critical for complex assembly and, importantly, membrane association. The paper not only offers insight into the function of the ATPase, but also a thoughtful model of how this function shifts in the context of different lifecycle stages and mutants.

The 2020 *PLoS Biology* paper by Dolezelova *et al.* studies the transition from procyclic trypomastigote to epimastigote, and then to metacyclic trypomastigote forms. Using the previously

published RBP6 overexpression model the authors follow mitochondrial rewiring in unprecedented detail, observing both the mRNA and protein levels of enzymes, transporters, and respiratory chain components. A key finding was a profound shift to the use of proline oxidation funnelling into the TCA cycle during progress toward metacyclogenesis, leading to an emphasis on alternative oxidase respiration. Interestingly, this is reflected not only in complex composition but also in the relative level of the membrane potential and the overall profile of pharmacological sensitivity of the mitochondrion. Highlighting the rigor typical for Dr. Zikova's work, the team validated their RNA and protein-based findings through comprehensive studies that directly measured the abundance of the metabolites involved. The article ends on a most interesting hypothesis, proposing that the biochemical rewiring of mitochondrion may result in molecular cues, that ultimately lead to metacyclogenesis letting us appreciate the trypanosome mitochondrion not only as a biochemical hub, but also as a critical component of cellular and developmental signalling.

The 2021 *Journal of Biological Chemistry* article by Hierro-Yap *et al.* is focused on Tb1 one of the subunits unique to trypanosomes. The article silences Tb1 and then studies the structural, biochemical and physiological consequences of loss at different stages of the lifecyle. These changes are profound and rigorously documented in elegant experimentation. The team explores the relative requirements for Tb1, and I was impressed by how tightly they could titer the abundance of the protein, and how they use this to derive a very detailed understanding of the proteins role (in particular in blood stage trypomastigotes).

Just this morning, I noted in my twitter feed the very well received announcement of Dr. Zikova's brand-new paper in *Nature Communications* (which was included in the document as preprint). This article reports the cryo EM structure of the complete T. brucei ATPase dimer – a major accomplishment, and a terrific conclusion to a long-standing effort in her laboratory.

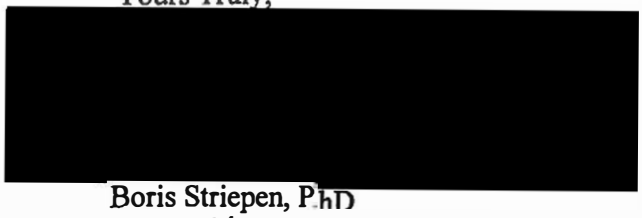
Given the productivity of her laboratory, it is not surprising to note that Dr. Zikova has been highly successful in seeking financial support for her research. She has been consistently funded for the time of her independent career by numerous Czech Research Agency grants in addition to additional ERC and EMBO awards.

Beyond her published record, Dr. Zikova is frequently invited to speak about her research by universities and research centers from around the world. She is also in high demand as a reviewer and editor for a wide range of scientific journals. Clearly, she has attained the international prominence and impact, that at US universities is seen as critical for appointment as, or promotion to, the position of professor.

Overall, I was deeply impressed by the document that I was asked to review. This is top-notch science on an topic equally important to parasitology as it is to evolutionary cell biology. The data shown are of excellent quality throughout, documenting not only Dr. Zikova's acumen as a scientist, but also her ability to train students and postdocs to perform experiments to the same level of excellence (her CV lists 3 PhD, 6 masters, and 14 bachelors students as trainees).

I found Dr. Zikova's to meet the requirements for habilitation. In case Dr. Zikova were to be considered for faculty appointment at my institution, the University of Pennsylvania, I would vote in the affirmative, and so I am sure would my colleagues. I congratulate Dr. Zikova to her accomplishments, and I very much look forward to continuing to follow the exciting work of her research team in the future.

Yours Truly,



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