Dr Michael L. Ginger, School of Health and Medicine, Lancaster University, Lancaster, LA1 4YQ, UK
Examiners Report: Ph.D. thesis Zdeněk Verner '*Mitochondrial energy metabolism in* Trypanosoma brucei'

Questions addressed in the report

1. Is an original intellectual input of the student apparent from the thesis?
Yes

2. In the referee's opinion, did the student work independently in his/her experiments as well as in their interpretation and writing?
Yes

3. Is the project original and what is its main outcome?
Yes, the project is original, with the major outcomes being ***(a)*** *insight into the entry route for electrons into the mitochondrial respiratory chains of the trypanosomatid parasites* Trypanosoma brucei *and* Phytomonas serpens *and* ***(b)*** *characterisation of three putative sub-units/assembly factors for one of the enzymes catalyzing the terminal electron transfer in the* T. brucei *respiratory chain, cytochrome* c *oxidase*

4. Would this thesis earn PhD at the referee,s own Institution – please answer Yes or No.
Yes

5. How would the referee evaluate this student relative to all PhD candidates he/she has previously known: among top 10%, among top 25%, within the top 50%, among the worst 20%.
It is not in the top 10%, but I certainly rate this as a good thesis. The science describes a good blend of classical biochemical analyses and trypanosome molecular genetics to predominantly address the long-standing conundrum of how electrons are transferred from NADH into the trypanosome respiratory chain. Experiments are controlled and the blend of appropriate biochemistry and molecular genetics techniques provide a good contemporary training. Zdeněk's experiments have contributed to three published manuscripts, and three manuscripts appropriate for publication (likely with minor revisions) are in the appendices.

6. Please do not grade the thesis, only state whether or not it meets criteria for PhD.
In my opinion, this thesis meets the criteria for PhD.

Examiner's more detailed overview of the thesis

In his thesis, Zdeněk Verner largely focuses on the biochemical and genetic characterisation of the enzymes that transfer electrons from NADH into the mitochondrial respiratory chain of trypanosomatid parasites. Given the long-standing debate over whether a 'classic' mitochondrial complex I operates in trypanosomatids and is capable of pumping protons across the inner-mitochondrial membrane, this is a very worthy topic for a thesis dissertation (*see Q3 above*). The experimental data provided by Zdeněk clearly show

(i) The essentiality of alternative NADH dehydrogenase in procyclic *Trypanosoma brucei*
(ii) The apparent dispensability, at least in culture, of the classic mitochondrial NADH dehydrogenase activity provided by respiratory complex I in procyclic *T. brucei*
(iii) Biochemical characterisation of two isoforms of glycerol-3-phosphate dehydrogenase in *T. brucei*
(iv) Biochemical evidence for a proton-pumping mitochondrial complex I in *Phytomonas serpens*

Collectively, these data are sufficient to make the original intellectual input *asked in Q1 above. In response to Q2 above,* the student's independent intellectual contribution is evident in a light, but engaging prose. It is important to acknowledge the general quality of writing since as far as I am aware English is not the native language of the Ph.D. candidate. I do not necessarily agree with all of the candidate's interpretations for some of his work, but since these are provided within draft manuscripts it provides further evidence that this

is the original work of Zdeněk Verner. I have also seen Zdeněk describe some of this work as an oral presentation at a COST-supported conference of trypanosome metabolism and drug discovery.

In addition to studies of how electrons are transferred to ubiquinone, Zdeněk also describes a novel and clever RNAi screen to identify novel proteins contributing to mitochondrial respiratory chain assembly and function. His contribution to a well-executed characterisation of three putative sub-units/assembly factors of *T. brucei* cytochrome *c* oxidase (or mitochondrial complex IV) is also evident in Appendix 10.7.

My comments are restricted to the three draft manuscripts and are to be found within the three accompanying .pdf files although I am also willing to declare that I was one of the supportive referees for the peer review of Verner et al. (2011) Mol. Biochem. Parasitol. 175:196-200. Data presented in the three draft manuscripts is in my opinion are suitable for publication; points raised and suggested revisions are therefore reflective of the type of critical, yet still supportive, comments I would make as an anonymous peer-reviewer.

If I am to find fault with the thesis, then whilst the Introduction covers all the appropriate points regarding trypanosome energy metabolism and molecular cell biology and cites all relevant recent publications, the depth of coverage is somewhat lighter than I was anticipating. The discussion of the data is also very trypanosome-centric; some wider comparison the data with what is known about mitochondrial respiratory chain composition and function in other aerobic parasites – *e.g.* apicomplexans – would have been of relevance.

Questions raised by this reviewer

1. The biochemical and molecular evidence for the apparent dispensability of mitochondrial complex I and the essentiality for the smaller alternative NADH dehydrogenase in procyclic *T. brucei* are convincing. Yet, these data would appear to be at odds with the retention in a 'streamlined' parasite of the many sub-units encoding mitochondrial complex I. Genes encoding homologues of known accessory factors of mitochondrial complex I are also evident in the trypanosomatid genome databases. Can the candidate therefore speculate on the function of mitochondrial complex I in *Trypanosoma brucei*?
2. The putative FAD-dependent G3PDH of the *T. brucei* endoplasmic reticulum: how well conserved is gene in other protists, notably parasites? For instance, is it found in many or a few protists? Is it found only in aerobic parasites or parasites capable of extensive lipid biosynthesis?
3. The putative FAD-dependent G3PDH of the *T. brucei* endoplasmic reticulum: was it not considered a target for inducible RNAi? Based on the likely function of this enzyme, would a phenotype be predicted in either cultured procyclic or bloodstream *T. brucei*?
4. The putative FAD-dependent G3PDH of the *T. brucei* endoplasmic reticulum: the results from biochemical fractionation are described, although as acknowledged in the draft manuscript the data can only points towards specific sub-cellular localisations. Is the V5-tag not suited to immunofluoresence analyses? If not, why not?
5. Characterisation of the essential alternative NADH dehydrogenase: on p83 of the thesis an increase in G3PDH activity in the *NDH2* RNAi mutant is described. What do you think is the significance of this observation and what experiments (if any) would you consider appropriate to follow up this preliminary evidence of metabolic flux changes.
6. Insight into the functional organisation of trypanosome cytochrome *c* oxidase: how likely is it that Tbcox6080 has catalytic glycerolphosphoryl diester phosphodiesterase activity? Does bioinformatic analysis of the amino acid sequence allow no prediction to be made?
7. Insight into the functional organisation of trypanosome cytochrome *c* oxidase: is the evidence that any or all of the three newly characterised proteins are *bona fide* sub-units of complex IV indirect? Could these proteins not be involved directly in complex IV activity, but be involved more generally

in mitochondrial biogenesis/function? What other experiments might be carried out to address these possible concerns?

M.L. Cinque.

24th April 2017 - report submitted.

Evaluation of Ph.D thesis by Zdeněk Verner

When I received the file with Zdeněk's thesis worth over 140 pages I felt overwhelmed and not entirely happy. Am I really supposed to read and evaluate seven papers and over 30 pages of introductory review, when, as I learned, one quality paper with defendant as a first author is sufficient to qualify for Ph.D? The pile of paper became more understandable when I realized that two published reports with Zdeněk being the first author are short communications, one is a book chapter and one is a four years old paper principally by Petra Čermáková and Anton Horváth. Three manuscripts are as yet unpublished, with Zdeněk being first author on one of them.

In case of presenting the Ph.D. thesis as a collection of publications where the defendant is not the corresponding author I am usually curious how the introduction looks like, as this should be the part that is really written by the student. Since there is an abundant literature on the topic of trypanosoma biochemistry and molecular biology available, according to the general attitude of the student (likely heavily influenced by his supervisor) the introduction could be either a detailed and knowledgeable review or a pragmatic chapter written in the "way of least resistance" to meet the formal requirements. Fortunately, introduction by Zdeněk does not fully qualify to be the latter, but stays somewhat short of being the former either. The chapter that opens up with necessary overview of trypanosoma biology reads well, is written in good English (as far as I can tell) and is peppered only with few minor typos, grammatical errors or cumbersome formulations. To comply with my reputation of a nitpicker, though, I must mention that procyclic stage is not defined by the position of flagellum (bloodstreams are the same trypomastigotes as most vector stages, page 4). It would have been useful to mention the number of *T. brucei* genes when describing the RNAi library (page 11) and it was not clear to me which two subunits were essential, when only one line above the text mentions three affected subunits (page 13). Is it for sure that procyclics survive thanks to anabolism of amino acids (page 15)? Sincere question concerns the same page, few lines below. Is it really that bloodstreams lack cytochromes entirely? Now I do not mean the flagellum-associated cytochrome b, but the cytochrome in complex II, that may be present in bloodstreams? What hurts a bit more in a review dealing with biochemistry of trypanosomes is the description of glycosome metabolism. This part omits completely the unique feature of non-regulated kinase reactions within the glycosome, possibly the raison d'etre of the localization of glycolysis within the organelle, and also overall glycosome metabolism including its redox aspects, metabolic products and enzymatic activities that change in different *T. brucei* stages could have been described more carefully. Electrons from glycerol-3-phosphate are not passed to ubiquinole (page 15, 19) and the expression changes do not concern phosphoglycerol kinase (page 16). The associated metabolic map lacks names of most enzymes as well as of depicted compartments and the color codes of some metabolic products do not fit the legend. The statement that proline requires pyruvate (page 18) may be relativized by the possibility that oxaloacetate may take part in the reaction instead or that glutamate dehydrogenase could replace aminotransferase reaction. In the part dealing with FAD-dependent glycerol-3-phosphate dehydrogenase, it is suggested that some dihydroxyacetone phosphate could be diverted into endoplasmic reticulum. What would be the effect of this on overall glycosome metabolism? What possible experiment could be used to test whether putG3PDH participates in lipid biosynthesis?

The description of complex 1 and its function, the major subject of this thesis, should have been much more detailed than presented. The reader learns nothing about proton extrusion, electron flow, rotenone binding etc. What inhibitor is meant by the abbreviation DPI (page 23)? The name Q2 of the coenzyme is unexplained (page 25). What surprised me in the introductory chapter was that after starting as a standard review it suddenly transformed into a blend of results, review and discussion. Not that I object that results and discussion were presented in the introduction, not at all

and to the contrary, but I feel these parts should have been placed at the end of the chapter and the review itself could have been more thorough.

The following published papers were literally pain to read. Due to low-quality copy process the printouts with diminutive fonts are almost unreadable and on-screen magnification does not help. The likely reply of the supervisor "it was published, so download it for yourself" is not something the reviewer supposed to provide favorable assessment wants to hear. Apart from that, the three published research papers leave little room for criticism. All went through a tough review process in quality parasitological journals which speaks for itself, and all present top science dealing with the most challenging aspects of trypanosoma biochemistry. I sincerely envy the trypanosoma researchers their methodology, which enables functional studies that the poor guys without the possibility to use RNAi can only dream of. The work demonstrates the experimental skills of Zdeněk Verner and also uncovers the original thinking of the authors. On the page 67 the authors mention that by using ferricyanide and DCIP as acceptors possibly able to uncover other enzymatic activities, they observed much higher activities than those measured with Q2 as acceptor, and ascribe this to the presence of other redox enzymes. Is it possible that ferricyanide and DCIP act as higher efficiency acceptors for the same enzymes as those using Q2? Also, what is known about the effect of DPI on the activity of complex 1, which contains flavine? And is dihydrolipoate dehydrogenase able to use Q2 as an acceptor?

The three so far unpublished manuscripts present valuable data that seem in some aspects still preliminary or difficult to interpret. What would be the explanation for the fact that overall NADH:Q2 activity was lower than the sum of the activities recovered from glycerol gradient fractions of RNAi-induced cells (page 83)? It seems that in case of NDH 2 a careful functional study using recombinant protein and a variety of possible acceptors is needed. On the page 88, the legend to table 1 states that the activities are expressed in U (international units). This cannot be the case as the Unit means the change of micromoles per minute, but the legend mentions only the change of absorbance without calculating the amount of consumed NADH. The manuscript principally by Ingrid Škodová and Anton Horváth is a neatly and carefully written contribution dealing with an enigmatic homologue of FAD-dependent glycerol-3-phosphate dehydrogenase. What I do not understand is why the antibody against the V-5 tag fused with G3PDH was not used to determine the protein localization in the subcellular fractions obtained by differential/isopycnic centrifugation of the cell lysate and by using immunofluorescence microscopy on slides. The last manuscript of the thesis by Anna Gnipová and supervised by Alena Zíková is a very interesting piece of work on novel subunits of trypanosoma cytochrome c oxidase. I only miss here the description of cytochrome c reductase assay and feel that other mitochondrial activities besides cytochrome c oxidase and cytochrome c reductase should have been measured as controls, e.g. threonine dehydrogenase or others.

In conclusion, the thesis by Zdeněk Verner represents an impressive amount of work that translated into high quality publications with others on the way. What needs to be appreciated is that Zdeněk and his coauthors adventured into the very challenging fields of trypanosoma biochemistry and scored well. My overall impression is somewhat clouded by less-than-perfect introductory review (in my view, Jula may say whatever he wants) and by poor quality of reproductions of published papers, but the result is nevertheless clear and unambiguous: great work that should easily win Zdeněk a Ph.D.

Ivan Hrdý

Prague, 23.4.2011

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In Budweis, Czech Republic, April 27, 2011

Review of the PhD Thesis by RNDr. Zdeněk Verner "Mitochondrial energy metabolism in *Trypanosoma brucei*"

Thesis supervisors:
Prof. J. Lukeš (University of South Bohemia, Budweis)
Dr. A. Horváth (Comenius University, Bratislava)

The structure and volume of the thesis. The submitted 139 page thesis in English is composed of two main parts: 1) a general section including abstract (one page), preface (one page), followed by the 22 page text that mainly represents the literature review (but see also below), and the references (nine pages); and 2) the appendices representing four published papers and three manuscripts in preparation. Thus, excluding the manuscripts, which clearly represent a separate endeavor with a collective input of several coauthors, the part of the thesis proper that can be regarded as the original creative writing is limited to review section. The text is written in a mostly correct and clear language that the candidate is commended for.

Review of the individual sections of the thesis. The abstract (section 1) represents a brief description of the research projects (papers or manuscripts) included in the thesis. Although all are clearly related by virtue of addressing various aspects of energy metabolism in *T. brucei* (RNAi screen for proteins involved in mitochondrial transmembrane potential, functional evaluation of three subunits of Complex I, similar analyses of alternative NADH dehydrogenase, glycerol-3-phosphate dehydrogenase and cytochrome c oxidase), neither the abstract, nor the following text clearly spell out what the main question(s) of the entire work was (were). The last sentence of the abstract indicates that "all the data gathered" would then be "discussed in a wider context of mitochondrial metabolism".

However, as follows from considering the subsequent parts of the thesis, this promise was not supported by a summarizing section that would integrate all the results obtained and draw the conclusions pertinent to the entire work. Without such a concluding discussion, the impression being made is that of the candidate's work mostly representing forays into various aspects of the energy metabolism. If there was a unifying hypothesis, it remains obscure to the reader. There is an obvious logical link between the analyses of Complex I and the alternative dehydrogenase, but the other aspects of the work are not so clearly related. Although interesting and important results were obtained during each individual project, yet, for a PhD thesis such a lack of cohesiveness (at least in the presentation of results) is not considered advantageous. The candidate is advised to present

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the unifying hypothesis (or hypotheses) and include the concluding section in the final version of the thesis.

The text following the abstract and the preface (the 'literature review') is divided on the several chapters. Section 3 represents a description of *T. brucei* biogeography, life cycle and virulence. It is well written overall except for a factual error (p.4) – the procyclic stage is not defined by the relative position of kinetoplast, nucleus and flagellum (that would define whether the cell is an epimastigote or a trypomastigote) but just denotes a certain position in the cell cycle ('procyclic' means moving along with the cycle).

Section 4 represents a synopsis of trypanosome peculiarities on the cellular and molecular levels. Apparently, it was a challenge to squeeze this immense topic into a 2.5 page write-up, yet errors should have been avoided. Thus, using the authors terms (p. 7), RNA editing can be better described in terms of discrepancies between what is encoded and what is translated (instead of 'transcribed' – because the original, pre-edited, transcript still is a faithful copy of its gene). It took long time to identify ND2 (= MURF1) as such not due to pan-editing (it is a non-edited type) but due to sequence divergence. Moreover, apologies for citing a 1968 paper as the earliest on the web to mention the term "kinetoplast" are not accepted. This is not because a PubMed search could yield at least a 1960 paper, but, more importantly, because a PhD candidate is expected to know (or at least be aware of the existence of) the classical literature in the field of study (in our case that would be the reviews of Vickerman) that can be used to help in cases like this. Further on, the description of polycistronic transcription and mRNA processing (p. 8) contains inaccuracies: 1) the statement that it "rather resembles prokaryotic transcription" is misleading and should be avoided; 2) polycistrons are not "subsequently" but co-transcriptionally processed; 3) an mRNA with a trans-spliced leader and no introns can hardly be viewed as "classical"; 4) the term 5'-cap does not refer to an entire spliced leader RNA but to a part of it (co-transcriptionally added m^7G and four methylated nucleotides downstream). Overall, this section is rather superficial.

The next chapter (section 5) is devoted to characterization of the RNAi system and its applications (inducible knock-down, conditional knock-out, overexpression, and RNAi library) in Trypanosomatidae and that is of an immediate relevance to the experiments. Following a brief survey of the subject, there is a presentation of the unpublished results of the candidate which represented an unsuccessful attempt to use the RNAi library for a screening of novel components of the respiratory chain by selection of cyanide insensitive cells. Regardless the actual causes of this failure, the inclusion of the negative result in the thesis is commendable. The drawback, however, is that for a better clarity of presentation, a clear separation had to be made between the literature review and the results. The section concludes with a description of a successful screening of the by staining for membrane potential and FACS (published in Parasitology Research, 2010). Given the

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apparent success of this approach, it is surprising that the work has not been followed up by search and analysis of additional clones.

Chapter 6 (Energy Metabolism, four text pages and a large figure) is the central part of the literature review and represents the most thoroughly written part of thesis. Yet, it is not without minor glitches: the abbreviation G3P in the text refers to glycerol-3-phiosphate, while in the figure it refers to glyceraldehydes-3-phosphate; the references to Chaudhuri et al. (1995 and 2006) at first appeared to be missing until found listed out of order elsewhere.

In Chapter 7 the biochemical role of mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase is reviewed and a potential functions and localization of the second enzyme (putG3PDH) are discussed. This section is cursory albeit satisfactory. The chapter ends with a list of experimental questions that could help to delineate the functions of each enzyme. The reader is then referred to a manuscript in preparation (Appendix 10.6) for the answers to "at least some of these questions". This is not appropriate for a thesis. Besides providing the appendix, the candidate should have presented a summary of the work in the concluding section and showed what exactly had been achieved. Evaluation of the aforementioned manuscript does show that the putG3PDH is most likely not a component of the mitochondrial membranes. Given a preliminary nature of the experiment (solubilization of whole cells with increasing amounts of digitonin and evaluation of the residual amount of the protein in the insoluble material) and the potential technical issues (the lack of appropriate markers for ER, mitochondrial matrix, outer mitochondrial membrane; omission of the solubilized material from the analysis), this analysis can only represent the beginning of the investigation, not a full story. Moreover, considering the claimed contribution of the candidate (suggestion of experiments, supervision of another student) it is not clear to what extent this work can be regarded as part of the thesis in question, instead of the future thesis of the other student.

The final Chapter 8 of the thesis refers to the consideration of Complex I and 'alternative' rotenone-insensitive NADH dehydrogenase. Here, one page is devoted to a cursory introduction to Complex I, and the remaining two pages describe the experiments in a published paper (Verner at al 2011) and a manuscript in preparation (Appendix 10.5). Important observation was made during the analysis of inducible knock-down cells lines for three subunits of Complex I: there was no growth inhibition phenotype and there was no decrease in transmembrane potential or ROS production (Verner at al. 2011). Along with the small decrease of the specific NADH-ubiquinone oxidoreductase activity this suggested the presence but dispensability of Complex I and it negligible role (if any) in electron transport in culture procyclics of *T. brucei*. The published work also include analysis of the activity in a glycerol gradient, however, without an independent visualization of both enzymes the only reliable conclusion that can be made is that the RNAi led to a redistribution of the Complex I and (possibly) alternative enzyme which suggests some form of a physical alteration of the enzyme(s). Unfortunately, due the lack of efficient means to

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directly analyze intact or RNAi-affected Complex I this interesting beginning had no continuation.

As the previous work highlighted the importance of 'alternative' NADH dehydrogenase (NHD2), another major attempt was directed at this enzyme (the manuscript in Appendix 10.5). An important observation made was that knock-down of this enzyme resulted in growth retardation, decrease of the transmembrane potential and ROS production, strongly indicating the major role of NHD2 in NADH oxidation. The remainder of the manuscript presents the preliminary data. The authors need to solve the puzzle with the absence of the overall decline of the NADH-ubiquinone oxidoreductase activity in the NHD2 RNAi background. Besides testing other acceptors, a double RNAi knockdown (of both enzymes) might be fruitful. If the authors plan to produce a high-visibility publication, they need to develop a reliable antibody for the enzyme, as well as a Complex I subunit. These, as well as affinity tagging of NHD2 combined with the proteomics, would then allow for testing an interesting hypothesis of the interactions or uncover some other properties of these two enzymes including localization. For investigations of the overall impact on the respiratory chain, besides activity measurements, more direct analyses are in order including Blue Native gels and antibody probing.

With respect to additional experimental work, Appendices to the thesis also include the published work by Cermakova et al. (Appendix 10.4), and a manuscript in preparation by Gnipova et al. (Appendix 10.7) in which the candidate has not claimed to make the major contribution.

Conclusions and Recommendations. Judging by the standards that this reviewer is accustomed to (mainly by serving on the thesis committees in Cell, Molecular and Developmental Biology program in University of California – Riverside), the following conclusions are made. A significant part of the work has been published including two papers listing the applicant as a first author and there is one manuscript in preparation also with the applicant as the first author. A valuable contribution was also made to several other papers (manuscripts) granting to the candidate a co-authorship. Therefore, the presented thesis clearly represents a body of work expected from a candidate seeking a PhD degree in biochemistry or molecular/cellular biology. The quality of work that was published in the international peer-reviewed journal also receives a passing grade.

Unfortunately, by applying the same standards, the thesis itself would not be regarded as acceptable. A PhD thesis is the last but not the least part of the applicants work towards obtaining the degree. Learning how to write a thesis is the final training activity of a candidate. In doing so, a candidate demonstrates the comprehensive knowledge of the literature (Literature Review section), ability to present the obtained data in a logical and clear way (Results), and ability to analyze the data, draw conclusions and fit the obtained new knowledge into a larger picture (Discussion). The Results section does not need to

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reiterate the entire published papers but can briefly describe the major findings and make logical connection between the individual subprojects. Incorporation of entire manuscripts in preparation (not even submitted, leaving alone papers in press) is hardly a good practice. Those better be fully presented in Results with the clear indication of the contributions made by the candidate and others and logically linked to the other results.

From this perspective, the lack of the clearly defined sections in the presented thesis makes a negative impact on the cohesiveness of presentation. The literature review is rather cursory. The logical links between individual subprojects are not clearly shown. Importantly, the thesis does not include the discussion section that would integrate the results and draw the conclusions.

In conclusion, the quantity and quality of the candidate's work are sufficient for awarding him the sought PhD degree. However, it is highly recommended that this be done on a condition of significant improvement of the submitted thesis as outlined above.

D. Maslov
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