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Stillwater, April 19 2009

To whom it may concern,

I had the pleasure to review the Ph.D. thesis entitled "Functional analysis of metabolic and immune proteins in the tick *Ixodes ricinus* by RNA interference" authored by Mr. Ondrej Hajdusek and supervised by Drs. Libor Grubhoffer and Petr Kopacek from the Biology Center of the Academy of Sciences of the Czech Republic, Institute of Parasitology, Ceske Budejovice.

The thesis presented by Mr. Hajdusek is an excellent work on the study of tick biology at the molecular level. Until recently, functional studies of tick molecules were limited by the lack of tools to genetically manipulate ticks. The discovery and application of RNA interference (RNAi) have opened a new venue for functional studies in ticks. Mr. Hajdusek used this technology to address basic questions in tick biology. The discovery of the putative genes involved in RNAi in *I. scapularis* allowed Mr. Hajdusek to propose a mechanism for RNAi in ticks. Then, Mr. Hajdusek used RNAi to characterize the function of tick molecules that play important roles in tick iron metabolism, immunity and digestion. The results of these studies constitute an important advance towards understanding the molecular events that control tick physiological responses and processes. Finally, I would like to stress the applied aspect of the thesis. The results presented in the thesis have implications for the development of new vaccines for the control of tick infestations, one of the most important areas in tick research.

The work presented in the thesis was published in five papers in top ranked journals such as The Proceedings of the National Academy of Sciences, U.S.A., Developmental and Comparative Immunology, Insect Biochemistry and Molecular Biology and the International Journal for Parasitology. The thesis is well organized into chapters with an introduction to the topic addressed by the papers and concluding remarks at the end of the work.

I have the following comments/questions/suggestions:

1. The section on research objectives would benefit from defining a hypothesis to unify the work presented in the thesis.
2. Although the results on defining the tick RNAi pathway in ticks were partially published recently (Kurscheid et al., BMC Mol Biol. 2009;10(1):26), this group did not address functional studies of putative RNAi pathway genes. I would encourage Mr. Hajdusek to complete his studies with functional analyses and publish the results.

3. How the results presented in the thesis on the characterization of RNAi pathway in ticks explain the fact that when dsRNA is injected into engorged females, RNAi is detected in the eggs and larvae but not in the nymphs?
4. Mr. Hajdusek discussed the properties of an ideal tick vaccine antigen on p. 38 of the thesis. However, he did not discuss the possibility of using intracellular proteins as tick protective antigens. Recent results have suggested that this is indeed a possibility that would likely overcome the limitations imposed by antigen cross-reactivity with host proteins.
5. Typographical errors:

p. 32, text line 11: "phylogenetical" should be "phylogenetic"
p. 35, text line 19: "lack" should be "absence"
p. 42, text line 6: The sentence should start with "In"

In addition to the review of the work presented in the thesis, I had the opportunity to collaborate with Mr. Hajdusek in designing experiments to study tick RNAi and vaccine antigens. I can speak with full confidence when I state that Mr. Hajdusek is a very creative, strongly motivated scientist with excellent technical skills.

In summary, it is my opinion that the thesis presented by Mr. Hajdusek meets the criteria for a Ph.D. degree.

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Evaluation of Thesis – Referee's Report (José de la Fuente)

We ask the referee to kindly answer the following points:

1. In this thesis, have you found an original and creative intellectual imput of the student?

Yes, in these experiments the student has shown creativity and an important intellectual contribution.

2. In your opinion, has the student been autonomous in performing the experiments as well as in interpretating and presenting them?

Yes, I believe that the student have had an important input into planning, performing and interpreting the experiments included in the thesis.

3. Are results of the project original, and what is their main outcome?

As indicated above, the thesis presented by Mr. Hajdusek is an excellent work on the study of tick biology at the molecular level. The discovery of the putative genes involved in RNAi in *I. scapularis* allowed Mr. Hajdusek to propose a mechanism for RNAi in ticks. Then, Mr. Hajdusek used RNAi to characterize the function of tick molecules that play important roles in tick iron metabolism, immunity and digestion. The results of these studies constitute an important advance towards understanding the molecular events that control tick physiological responses and processes. Finally, I would like to stress the applied aspect of the thesis. The results presented in the thesis have implications for the development of new vaccines for the control of tick infestations, one of the most important areas in tick research.

4. Would this thesis be adequate for PhD degree in your home institution/country – please answer Yes or No.

Yes.

5. How would you evaluate the work of this student considering other PhD candidates whom you have known: among best 10%, among best 25%, average, among worst 25%.

Among the best 10%.

6. Please do not grade the thesis, but state clearly whether or not it meets criteria for PhD degree.

In summary, it is my opinion that the thesis by Mr. Hajdusek meets the criteria for a Ph.D. degree.

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April 24, 2009

Report on **Mgr. Ondřej Hajdušek** for PhD thesis defense.

"Functional analysis of metabolic and immune proteins in the tick *Ixodes ricinus* by RNA interference"

It is a great pleasure to write an assessment on Ph.D. thesis of Ondřej Hajdušek. As a reviewer who is expected to come with some criticism, I read the work very carefully and I tried hard to find any flaw I can point on, however, I did not find any. I have to admit that this is an outstanding work of very high level, which represents fundamental contribution not only to the filed of tick research, but with important impact on more general area such as iron transport in eukaryotes.

The thesis consists of three chapters containing 5 original research articles published in impacted journals including PNAS, which is one of the most prestige journals in life sciences. The first chapter is focused on general introduction concerning tick evolution and taxonomy, tick life cycles, physiology as well as tick-borne diseases. The second chapter summarized current knowledge on RNA interference as a cellular pathway as well as as a method for gene silencing. This chapter is not only a review but it also contains unpublished results concerning the establishment of RNAi in ticks, the method which opens the field of functional genomics in these organisms, and bioinformatic search for components of RNAi pathway in *Ixodes scapularis* for which a genome sequence is now available for exploration. Although these two chapters did not pass peer-review process as published articles, they are both of very high quality, clearly logically written with worthwhile illustrations and they could be parts of any modern parasitological textbook or they could be published as independent reviews.

To this part I have the only question:

Page 25 "..to get an efficient knock-down in the progeny, females could be injected with a vector DNA…" Does it incorporated to the genome or does it replicate as episome?

The third chapter contains research articles with prefaces to iron metabolism, tick immunity and tick digestion. After the last article the author summarized achieved results in part named "Concluding Remarks" which is followed by the list of references, the supporting information and the CV of the author.

I have following questions:

Tick iron metabolism

Page 35 – "…ticks are exposed to an enormous amount of free (non-heme) iron." What author means by free iron? In which form "free" iron is present?

Page 35 – the author speculates that lack of mitochondrial ferritin could be connected with the reduction of the heme synthesis. If so, how to explain lack of mitochondrial ferritin in majority of unicellular eukaryotes even when they retain heme synthesis?

Page 35 – the author claims that transferrin is the only iron source for ticks. Which data ruled out contribution of heme iron?

Page 36 – The author believes that the tick transferrin is not involved in iron transport as most of the conserved iron binding residues are altered. If so, how TF2 may play a role in the innate immunity by iron sequestration?

Article I: In very elegant study on iron metabolism, the author described and studied by RNAi a new secreted ferritin FER2. My only formal criticism is that the author did not attach supplementary data to the thesis. I have a number of questions concerning to this beautiful piece of work, but I selected following:

As ferric-transferrin is likely the major source of iron in tick gut, is it known how ferric iron is reduced to ferrous iron which is likely transported across a cell membrane?

Does native FER2 form multimeric complexes as FER1?

Is FER2 intracellularly loaded with iron before it is excreted to hemolymph?

Do you expect that peripheral tissue cell possess specific receptors for FER2 and what is expected mechanism for iron acquisition from FER2?

As FER2 is considered for vaccination, what is its similarity/identity when compared with vertebrate orthologs?

Article II: How specific is 1,10 phenantroline to Zn?

In conclusion, I fully recommend thesis of Ondřej Hajduk to be accepted for PhD award. This work is certainly among top five I have an honor to review and I would like to congratulate to the author to presented results.

Prof. Jan Tachezy, PhD.

Fanis MISSIRLIS (UK)

23 May 2009

Review of the Ph.D. thesis of ***Ondřej Hajdušek*** entitled "Functional Analysis of Metabolic and Immune Proteins in the Tick *Ixodes ricinus* by RNA interference" presented to the Department of Parasitology, Faculty of Science, University of South Bohemia.

General comments

In his thesis, Ondřej Hajdušek provides a concise and comprehensive introduction to the importance of ticks as vectors of human and cattle disease and their basic biology. His work focuses on *Ixodes ricinus,* which can transmit Tick borne encephalitis viruses and Borrelia spirochetes to humans. Ondřej Hajdušek has successfully developed RNA interference to silence genes in this species and monitor the functional consequences at the molecular, histological and whole-organism levels. He has performed a comparative genomic study of all the previously characterized genes implicated in double-stranded RNA degradation mechanisms and used the genomic information available for *Ixodes scapularis,* a sister-species of *Ixodes ricinus* that grows in America to suggest how conserved these mechanisms are in the tick. Curiously, RNA-dependent RNA polymerases were discovered for the first time in arthropods and the author discusses the implications of this finding and the possibility to target these proteins for vaccine development.

Ondřej Hajdušek is the first author in a very prominent research study published at the Proceedings of the National Academy of Sciences of the U.S.A. (2009; Volume 106, pages 1033-1038) that presented important breakthroughs in our understanding of tick iron metabolism. These include the demonstration that iron regulatory proteins control the levels of intracellular (cytosolic) ferritin in ticks and most notably that iron traffics in the hemolymph bounded to a secreted ferritin. His study is the first experimental evidence in the literature supporting an iron-trafficking role for ferritin and shortly after it was followed by the identification of a putative ferritin receptor present in mammals that may mediate ferritin uptake [Li *et al.* Scara5 is a ferritin receptor mediating non-transferrin iron delivery in Developmental Cell, 2009,16:35-46]. Ondřej Hajdušek's article ends with the first comprehensive attempt to describe iron metabolism in ticks and I consider the corresponding schematic representation (Figure 6A in the PNAS paper) as the most significant summary of the contribution of his thesis to science.

However, there is also an applied aspect of the work: a proposal based on the phenotype resulting from silencing of the Fer2 gene in ticks and its unique primary sequence that Fer2 could be an ideal target for generating antibodies that could block tick engorgement and therefore transmission of tick-borne disease. This idea has been made attractive thanks to the basic research presented in the thesis and perhaps in the future human lives can be saved following the ideas presented in Ondřej Hajdušek's thesis.

Finally, Ondřej Hajdušek has co-authored 4 publications on proteins involved in immune response and the digestive functions of the tick. The author's participation in such a wide range of studies is a remarkable achievement. Although it is hard to know

which parts of the work described in these papers was actually performed by Ondřej Hajdušek, I am confident that he has developed a very broad understanding of three important physiological functions of the tick (iron metabolism, immune responses and digestion) with novel contributions to all three areas.

Minor recommendations

Stemming from my last comment above, I believe that a brief disclosure on the author's specific contributions to each paper, printed in the thesis would be nice.

In addition, I suggest that the supplementary material of the PNAS paper should be included in the final version of the thesis.

Perhaps the section under "Tick transferrin" needs to be slightly less dogmatic in view of the preliminary nature of the experiments presented (see my question below)

Questions to the author

Chapter 1:
A) Is it known whether the same individual tick can transmit more than one disease at the same time?
B) Since the author has experience with iron metabolism and Borrelia spirochetes are introduced could he also give a critical comment on the paper published in Science 9 years ago by Posey and Gherardini claiming that there is a lack of a role for iron in the Lyme disease pathogen?
C) How common is intracellular digestion in chelicerates? Are there examples of insects that employ intracellular digestion? In other words, is this an adaptation that should be linked more to the mode of feeding or to the phylogenetic grouping of the organism?

Chapter 2:
D) Does the author have any suggestions on why RNA interference works less efficiently in the intestine?
E) Can the author elaborate on his predictions of what the function of RNA-dependent RNA polymerases may be in ticks?

Chapter 3:
F) The author's model of iron metabolism in ticks is based on solid experimental results. It is helpful and comprehensive. Could we highlight on that model what key players and/or steps in this model are not well understood and require further research?
G) Can the author substantiate his claims that insect/arthropod transferrins may not bind iron? What is the evidence that they may not play a role in iron trafficking? How does the author envisage a role for transferrin in the immune response if he does not believe they bind iron? Is tick transferrin predicted to be a secreted protein?
H) Could the author attempt to illustrate in a graphic form what is known of the tick's immune response as he has done with iron metabolism? If this is too much to ask, then a few words on how the specific roles of α_2-macroglobulins, lectins, thioester proteins etc. cooperate in securing protection against pathogens?

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