

Ceske Budejovice, Monday February 20th 2012

To the Chairman of the Thesis committee.

Subject: Reviewer comments about the thesis defense of RNDr. Zdenek Franta entitled "Blood meal digestion in the hard tick *Ixodes ricinus*".

The PhD thesis dissertation of RNDr. Zdenek Franta uncovers mainly the molecular and biochemical events that mediate blood meal digestion in the midgut of the hard tick *Ixodes ricinus*. Working with guts of semi-engorged *I. ricinus* females the author has also mapped the morphological changes of female midgut during feeding and he disclosed the specific roles of individual proteolytic enzymes during this process. This piece of information was clearly missing upon the initiation of his PhD studies and therefore it is an original and creative intellectual input of the author.

I have read the PhD dissertation and find it a high quality scientific contribution that combines a variety of disciplines. The work would be considered superior dissertation material in any university that I have been affiliated with and it clearly meets the requirements of the PhD degree. I strongly support the approval of thesis defense at the University of South Bohemia, Faculty of Science because RNDr. Zdenek Franta excelled during his doctoral studies as judged by:

- i) his two publications as a first author in top-ranked journals in the field of studies.
- ii) his co-authorship in another five publications in top-ranked journals in the field of studies.
- iii) his co-authorship in another four publications in the field of tick physiology.

The thesis has a nice blend between descriptive and mechanistic work and its published results practically demonstrate the ability of the author to pioneer individual projects in the field, while his participation as a co-author in numerous other projects undoubtedly verifies his ability to function as a valuable unit within a research infrastructure. The concluding remarks of the presented thesis demonstrate that the author has a clear envision of the field and he is ready -as an individual research scientist- to test many of his ideas experimentally. The dissertation is comprehensively discussed and the aims of the study are described in a logical and straightforward manner. The introduction and the figure material could easily be considered a review to be submitted for publication elsewhere. The experimental approach chosen by the candidate appropriately answers the most relevant questions that were raised, and the presented results provide a platform to gain a valuable insight into the evolution of hematophagy within the arthropods or different blood feeding ectoparasites. It is clear that the achievements of the candidate were the result of hard work, ability to collaborate within the group and with other groups worldwide, creativity and knowledge.

Accordingly, I have some questions regarding the dissertation and the related papers:

- 1) Based on the presented data and the related literature I would like to have a mechanistic model about the regulation of specific proteolytic activities in the midgut of semi-engorged *Ixodes ricinus* females. What could be the mechanisms that control the spatial and temporal distribution of the herein presented proteolytic activities?
- 2) Transcriptome projects has demonstrated that tick salivary glands are rich in proteases. How would you explain these findings in terms of tick biology/host interaction and then with respect to the results presented in this thesis?
- 3) Why ticks digest blood similar to parasitic platyhelminthes or nematodes? What could be the advantage for this proteolytic mechanism to be similar to nematodes rather than to mosquitoes and flies? How this mechanism may relate to *I. ricinus* vectorial capacity?

Based on the presented studies, I am confident that RNDr. Zdenek Franta has gathered the necessary expertise and knowledge to sufficiently address my questions, to receive the PhD degree and to become a leader in the presented field of research. To conclude, I most certainly support this scientific work by approving his thesis defense with most of my enthusiasm.

Yours Sincerely,



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Review of PhD thesis submitted by RNDr. Zdeněk Franta

Blood meal digestion in the hard tick *Ixodes ricinus*

Supervisor: RNDr. Petr Kopáček, CSc.

PhD Defence at, Institute of Parasitology, Biology Centre, Academy of Sciences of the Czech Republic, České Budějovice.

Reviewer: Mohammed Sajid, PhD, Leiden Malaria Research Group, Department of Parasitology, Leiden University Medical Centre, Leiden, Netherlands.

14th March 2012

The PhD thesis by RNDr. Zdeněk Franta is built on a solid foundation of seven published papers in relevant and respectable scientific journals. The papers represent a substantial international collaborative effort involving several groups that are experts in their respective fields. RNDr. Zdeněk Franta is first author on two papers and also second author on two papers, which are indicative of the substantial advances that has been made to previous research. This work makes it possible to fully delineate the cascade of interactions of a cadre of endo and exo-peptidases that comprise a complex enzymatic network essential in blood meal digestion in the hard tick, *Ixodes ricinus*. The introduction provides a thorough and concise synopsis of tick classification (including a detailed *Ixodes ricinus* biology) and a comprehensive description of hematophagy in ticks, which was very useful to a non-tick expert such as myself. The reader is further given a detailed run down of each of the key enzymatic players in tick gut peptidolytic cascade, and heme detoxification; ending with the potential of a functional hemoglobinase vaccine. The introduction and background text adequately meshes together the published papers by RNDr. Zdeněk Franta.

The précis of the findings in the thesis 'Annotation' statement are well supported by the publications cited.

First named author papers

Paper IV. This elegant work uses biochemistry, immunolocalisation, and temporal transcriptional mapping to define the dynamic respective roles of members of the cascade of peptidases that are necessary in post-blood meal hemoglobinolysis in *Ixodes*. The work concludes that the hemoglobinolytic read out positively correlates with the *in vivo* synthesis of the peptidases known to be involved in hemoglobinolysis. Further, the relative rate of hemoglobinolysis is measured and is shown to increase with time reaching a maximum activity with fully fed ticks at around days 6-8 days post blood meal ingestion. This work also confirms that indeed digestion occurs intracellularly in digestive cells that line the gut lumen. I especially enjoyed the design of the protease active site titration

assays using the fluorometric substrates in conjunction with selective inhibitors to back off undesired activities.

Question 1. Could you comment on the practicalities of monitoring real time *in vivo* hemoglobinolysis in the presence of either one or more cocktail of inhibitor or by using RNAi in the tick.

Question 2. Is there evidence of dipeptides being generated by the peptidyl dipeptidase activity of the gut resident tick cathepsin B? how would these be identified?

Question 3. Are all the proteases involved in blood meal digestion activated by autolysis? Is *trans*-processing required for activation (by legumain or IrCL1 for example)?

Paper V. This is a complete and very robust manuscript looking at a key cathepsin L-like endopeptidase from *Ixodes*, termed IrCL1. The work is indeed thorough, and covers many facets of molecular biochemistry and tick biology; namely, cloning, functional heterologous over-expression (in *E.coli* and yeast), transcriptional knock-down, temporal qRT-PCR in the tick, intracellular immunolocalisation to the gut wall, pH optima (small molecule peptides, hemoglobin and BSA), positional scanning substrate assays and stability profiles. Importantly the RNAi work revealed that this peptidase is central to degradation of host macromolecules in the gut; the pivotal role of which makes IrCL1 an excellent target for chemo and/or immunotherapeutic intervention. The only aspect missing, and the icing on the cake, would have been mapping the cleavage site sequences and order of peptides generated within hemoglobin and BSA. An excellent all-round piece of work.

Question 4. What are the pros and cons of using information gleaned from a positional scanning combinatorial peptide library to predict cleavage sites in known or predicted macromolecular substrates?

Overview of papers I-III, VI and VII. RNDr. Zdeněk Franta has been involved in a number of additional relevant papers all on tick gut peptidase and iron metabolism in the tick gut; further adding to RNDr. Zdeněk Franta's expertise in this field. The work undertaken comes across as a substantial contribution to the published papers (especially in papers II and VII).

Finally, the reader was left somewhat perplexed why the title promised 'blood meal digestion' however, the lion's share of the thesis is on hemoglobin digestion (hemoglobin, around 10-12g/100ml and serum proteins around 7-8g/100ml) . The author does briefly mention serum protein uptake and digestion in the

uptake of a blood meal constituents section in the thesis and BSA digestion in paper V. I do understand that historically hemoglobin catabolism has been studied in more detail because of malaria and other hematophagus parasites, perhaps more information (if available) would be warranted.

In saying the aforementioned, I believe RNDr. Zdeněk Franta has achieved a high level of scientific competence as evidenced by his seven publication (two first author papers). RNDr. Zdeněk Franta has also significantly added to the substantial body of high quality research that was carried out prior to the thesis being undertaken. With this in mind I have no hesitation in highly recommending to accept the thesis for RNDr. Zdeněk Franta's PhD defense. I would finally add that this work will not only be of importance to tick peptidase aficionados, but also be an invaluable resource for biochemists studying interacting peptidase catabolic cascades in other organisms, allowing extrapolation and interpolation of key enzymatic activities in the respective pathways.

Question 5. how does the tick proteolytic blood meal digestion cascade cope with the relatively large amounts of ingested blood resident macromolecular protease inhibitors such as macroglobulins and serpins.

Question 6. Would using drugs, such as quinines, that interfere with heme aggregates (in the hemosomes) be a practical option to control ticks - used in topical cream for example?

Question 7. What are the pros and cons of using a low pH to digest protein and peptide substrates.

Question 8. Why are no blood clots seen in the tick gut?

A handwritten signature in black ink, appearing to be 'Z. Franta', located at the bottom center of the page.

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The review of the dissertation thesis of Zdeněk Franta „Blood meal digestion in the hard tick *Ixodes ricinus*“

The thesis of Zdeněk Franta deals with the role of individual proteolytic enzymes of different tick species during various feeding phases. The thesis consists of several parts. The first part of 36 pages provides an overview on the current knowledge of the biology and biochemistry of blood digestion in hard ticks and summarizes author's contribution to the field, specifically the characterisation of digestive aspartic and cysteine proteases of tick gut. The second part of the thesis consists of the reprints of seven experimental papers directly related to the topic of this thesis, to which the dissertant substantially contributed. The papers have been published in respectable peer-reviewed international journals and the dissertant contributions are specifically outlined. Finally, several other papers co-authored by the candidate dealing with other aspects of the biochemistry of tick are listed.

The dissertant convincingly proves that the tick digestion of blood is not executed by a single protease responsible for hemoglobin hydrolysis but by a cascade of well regulated proteolytic events. I regard the genetic and biochemical screens leading to the identification of individual enzymes of this cascade a very important part of the thesis (Paper II of the thesis). The second result that I regards very relevant and interesting is the characterization of legumain, an asparaginyl endopeptidase, as an important part of this cascade in the gut of hard tick (Paper I). Also the findings concerning iron metabolism in in tick that might lead to the development of an effective vaccination strategy against tick-borne diseases are very relevant and potentially useful; however, they are slightly off the main topic of the thesis and the candidate's contribution to these results seems to be less significant.

The candidate proved that he masters a wide variety of experimental techniques of molecular biology and protein biochemistry, namely PCR and RT PCR, primer design,

expression, isolation and refolding of recombinant proteins, antibody preparation, enzyme characterisation and gene silencing by siRNA.

The overview of the thesis is very clearly written and provides interesting and entertaining primer to the field for uninitiated. I did not find many typos or other formal problems and the English of the text seems to be very good to me (as far as I can judge). From the formal point of view, I only have one question: are all the figures used in the first part of the thesis (pages 1-37) in fact the candidate's own work? If not, the authors of the original photographs or artwork should have been acknowledged.

Specific questions for the discussion:

- manuscript VII describes the cloning, expression and characterization of cathepsin D-like protease from *Ixodes ricinus*. What was the overall yield of recombinant expression in *E. coli*? Is it enough for structural study by X-ray crystallography?
- IrCD1 was expressed in *E. coli* in inclusion bodies, solubilized and refolded in 6M GmCl and then purified using metal chelation chromatography in 8M urea. Is this really the procedure that was used? How was the protein transferred from 6M GmCl to 8M urea and why?
- is there any structural information on the IrCL1 (paper V)?
- which of the proteases described in the thesis (if any) might be a useful therapeutic target for the tick borne diseases, and why?

Finally, I am glad to conclude that I regard the thesis of Zdeněk Franta a very valuable contribution to our knowledge of the digestive apparatus of blood-feeding parasitic vectors. This work meets all the criteria for the dissertation thesis in biological sciences and I recommend to accept it as a basis for further proceedings without any hesitation.

Prague, March 11., 2012

Jan Konvalinka

