FACULTY OF SCIENCE
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In Vestec, 7th of March 2017

Opponent review to the PhD thesis of Jan Perner

The PhD thesis of Jan Perner is a large piece of work. His experimental and intellectual achievements are wrapped into six publications of which the four are research papers (Jan is the first and also corresponding or co-corresponding author on two of them), one comment paper and one review book chapter. Five of the publications were published in 2016. There are also two manuscripts in preparation and the thesis also includes unpublished data and ongoing projects in the laboratory.

Together, the thesis is very compact work centered around the metabolism of haem in *Ixodes ricinus* - a haemotophagus ectoparasite. It involves wide range of neat experimental techniques ranging from the characterization of recombinant proteins, gene silencing by RNAi to actual biological consequences for the parasite. The presented scientific work as well as the interpretation of the obtained data are of very high quality. Key to the experimental achievements of Jan Perner´s PhD thesis is the *in vitro* feeding technique of tick , which to me is a great illustration that a seemingly simple methodological advancement opens up new possibilities when used to answer smart questions.

The thesis starts with brief introduction on haem biology in parasitic metazoans. It is basically an extract of information, which can be found in the following publications and as such does not bring new information or hypotheses to the reader. It is generally a well written text describing the key aspects of the haem biology of metazoan parasites without detailed molecular background. There is couple of typos, unclear expressions or missing explanation in figure legends, which slightly worsen the overall nice impression. While it is a well written text, I must disagree with the introductory sentence that "....all but one eukaryotic organisms (Kořený et al., 2012), require haem for living.... " There is a number of anaerobic protists, which live happily without haem and thus may feel offended by this expression.

I would like to ask one general question:

***Q**: In the thesis, the author often mentions the absence haem oxygenase in numerous parasitic and free living species? Does it mean that sequence orhologous to known haem oxygenases is absent or that the activity could not be determined biochemically? Is there only one haem oxygenase in nature so one can use the presence/absence of the gene as an indication of the actual activity? Is there a benefit in not having haem oxygenase?*

For the reader, it would be perhaps beneficial to put the book chapter (Paper III) in the beginning of the thesis so he/she get additional background information from the start.

PUBLICATIONS

Paper I Acquisition of exogenous haem is essential for tick reproduction. eLife 2016 (Jan Perner first and co-corresponding author).

This is a great paper describing tick´s need for the host haem solely for the embryo development. The parasite itself obtains iron from serum transferrin. It is a complex study involving wide range of the aspects of haem metabolism in *Ixodes ricinus* from the involved haem binding proteins, haem dependent gene expression to actual biological consequences for the parasite. The story is based on very solid experimental data.

Q: *How do actually vitellins bind heam? Do vitellins from different animals constitute a single protein family?*

Q: *What is the funtion of last three haem biosyntetic enzymes in tick?*

Paper II RNA-seq analyses of the midgut from blood- and serum-fed *Ixodes ricinus* ticks. Scientific reports 2016 (Jan Perner first and corresponding author).

It is a comprehensive transcriptomic study performed towards the characterization of the expression changes triggered by the full blood or serum only diet. Surprisingly, authors find only 15 genes, expression of which is linked to the presence of haem/haemoglobin in the diet. In addition, gene ontology analysis of the identified genes was performed.

Paper III Book chapter Molecular targets to impar blood meal processing in ticks. (Jan Perner second author).

The chapter summarizes current knowledge of the haem/haemoglobin and iron acquisition and their subsequent metabolism in tick.

Q: *Do antimalarials (e.g. chloroquine, arteminisin) work against tick?*

Paper IV Tick iron and heme metabolism - New target for an anti-tick intervention. (Jan Perner as third author).

The publication summarizes the bioinformatic search for iron and heme-binding proteins in *I. ricinus,* the validation of their expression *in vivo*, their silencing by RNAi and production of recombinant proteins as potential vaccine antigens. As a result authors identified

mitochondrial ferrochelatase as the most promising antigen when subjected to the experimental animals. It is a nice and straightforward paper with clear premises and unfortunately not fully satisfying results regarding the actual effect of the selected proteins. I have a one methodological question:

Q: *I am wondering how exhaustive the bioinformatic search was as this could be applicable to other organisms. How exactly were the genes identified? Did authors also search for the iron/heme-binding motives or just rely on blasting proteins known from other organisms?*

Paper V Multienzyme degradation of host serum albumin in ticks. (Jan Perner as third author)

This publication describes albumin degradation pathway in tick gut in relation to the presence and digestion of haemoglobin. The authors find that, while the acquisition of these two blood-derived molecules is different , the eventual proteolysis relies on the same multienzyme activities.

Paper VI Vector Biology: Tyrosine degradation protects blood feeders from death via *la grand bouffe*. The publication is a comment on the recently identified tyrosin-degradation pathway in hematophagus arthropods. Due to the essential character of the pathway, it represents a promising target specific to these blood feeding animals.

MANUSCRIPTS IN PREPARATION

1) Impact of serum-feeding on development of *Ixodes ricinus* nymphs.

A short study describing the optimization of the artificial feeding set up for *I. ricinus* nymph stage. Despite the lower efficiency when compared to adult animals, authors were able to follow the fitness of the parasite stages fed on full blood or serum only fraction. Analogous pattern of gene expression was observed for nymphs and adults fed on same source. The work extends the developed feeding technique to the immature stages of the parasite.

It is a almost finished manuscript with perhaps some stylistic work to be done.

2) GST as a putative endogenous haem-binder in tick gut.

It describes the characterization of glutathione S-transferase from *I. ricinus* using experiments performed in situ and on with the recombinant protein produced in *E. coli.* The authors find that while the protein expression corresponds to the RNA-seq data and also binds haemin in vitro, the silencing of the gene has no effect on tick viability. It is not a ready to published manuscript and it requires further discussion of the results and text editing.

To conclude, it was a pleasure to review Jan´s thesis. The overall work is of very high quality, proving that Jan Perner is talented and skilled young researcher, who clearly deserves, upon the successful defense, to be awarded by PhD degree.

Yours sincerely, Pavel Doležal

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Review of PhD Thesis: "Nutritional requirements of ticks" by Jan Perner

General Thoughts:

This Thesis represents a substantial body of work on nutritional dependence of the tick, *Ixodes ricinum* on haem proteins obtained from vertebrate hosts. Two papers, where Jan is the first author, were published in highly reputable journals, eLife and Scientific Reports, and they represent a core of the Thesis. In addition, two follow-up papers are presented in the form of "Manuscript in preparation", plus four another papers co-authored by Jan are included in Thesis. The number and quality of publication outputs exceeds the average in the Faculty of Science, University of South Bohemia.

Strengths:

Jan does an excellent job of thoroughly presenting haem biology in animals and, particularly, metazoan parasites in the Introduction (pp. 2 – 19). As non-specialist in the field, I really appreciated the logical structure and overall clarity of the text.

Formal graphic arrangement of all papers, and the Thesis as a whole, appears as clear strength. I especially liked nice figures, combining photographs, diagrams and graphs.

The major strength, however, is the strength of presented research. I understand that the high quality of research is a result of team effort and good leadership by experienced supervisor. Nevertheless, Jan's great contribution to overall excellent result is apparent from the introductory texts and from the "Authors contributions" listed in the papers. Overall, the research described in this Thesis:

- addresses socially important topic, and has high application potential,
- is based on wide array of classical and modern techniques,
- combines discovery-power of RNAseq with thorough validation and, mainly, with follow-up functional studies.

Weaknesses:

There isn't much I can say in this section, especially seeing that most of the papers have already been thoroughly peer-reviewed (as justified by one of the Appendixes), and were published in good journals. However, just a couple of minor formal suggestions:

1. I would prefer to see the chapter of "Thesis objectives" more briefly and succinctly written. As it is now, it rather resembles a continuation of Introduction. Typically, Figure 10 represents a nice graphical summary of Introduction. Thesis objectives should formulate clear questions, or aims and, perhaps, outline the basic methodological approaches, but not much more.
2. I missed a chapter of "Conclusions" in the Thesis. It would be nice to see how the author generally evaluates his own effort during PhD. training. What is the overall interpretation of obtained results? What are the future directions? I understand that similar information is spread throughout the text of Thesis and also presented in the "Summary" in the

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accompanying leaflet. But still, the chapter of Conclusion should be a standard part of PhD. Thesis.

Discussion Questions:

1. What is your explanation for the observation (Paper I and ms in preparation I) that dietary haemoglobin is dispensable for most part of the tick growth and ontogeny (with the exception of embryogenesis). Lacking the classical eukaryotic biosynthetic pathway for haem, the ticks must obtain dietary haem from other than haemoglobin proteins. Which proteins are the sources of haem? Or, do the ticks employ some alternative haem biosynthetic pathway (similar to archaean pathway, for instance)?

2. During tick egg development, the haem liberated from host's haemoglobin is delivered to ovaries. How do you explain that the haem specifically originating from haemoglobin is essential for embryogenesis, while the haem originating from other sources (see question 1) was OK for the rest of development? What makes the haem of haemoglobin so specific?

3. Distinguishing the subset of differentially expressed genes that play a direct regulatory role in haem trafficking from the great number of differentially regulated genes on a temporal basis (between days 3 to 8 of feeding) presents a major technical challenge ... at least in my view (?) (Paper II.). You end up with only 15 genes significantly affected by haem in diet. Isn't that amount surprisingly low? Is this a result of your careful/conservative approach to RNAseq data analysis (risking that some genes are missed) or do you offer some other explanation?

4. In the ms in preparation II: Please, explain why have you assessed the effects of *glutathion s transferase* dsRNA on growth of adult *I. ricinus*, rather than assessing its effect on embryogenesis, which is supposed to be the stage most sensitive to haem absence in the diet?

Conclusion:

I strongly recommend this Thesis as high quality basis for awarding the PhD degree to Jan Perner.

In České Budějovice, 3 March 2017

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Evaluation of the PhD thesis of Mgr. Jan Perner:
"Nutritional requirements of ticks. Biology of haem in the tick *Ixodes ricinus*"

I have had the pleasure to review this outstanding body of work presented by Jan Perner for his PhD. The thesis deals with the nutritional requirements of ticks, with a specific focus on haem biology.

Jan Perner's PhD thesis comprises 208 pages in total, with a general introduction followed by chapters composed almost entirely of published papers. The latter includes two first author papers published in eLife and Scientific Reports, a commentary as corresponding author in Current Biology, co-author of a Book Chapter, and two empirical papers published in Ticks and Tick-borne Diseases. Two additional manuscripts as first author are also in preparation. The thesis is composed in a logical manner and subdivided into sensible sections. Figures and diagrams are appropriate and very well crafted. Primary references that do not include those in published papers and reviews count up to 128 and include relevant and up to date literature. The standard of written English is very good and only few minor modifications are included at the end of the report. I overall enjoyed the reading of the thesis very much and concluded that this was a very professional and substantial body of work.

The introduction provides an extensive overview of haem biology in protozoans, nematodes, arthropods and finally ticks. It nicely works up to tick biology and always strive to present a comparative approach. It ends with problem statements and objectives for the thesis that clearly delineate the thesis structure and replace a concluding discussion (see Question 33). Since a large part of the thesis deals with subject matter different from haem itself, the introduction could have included sections on general blood digestion, tick biology or tick-host interaction. However, in current form its focus is appropriate.

Part I deals with the nutritional dependence of ticks on host haemoglobin. The candidate demonstrates the dependence of ticks on host-derived haem by feeding nymphs and adults on serum using an artificial feeding system. While nymphs can engorge and molt, adults can feed and lay eggs, no embryonic development can occur in the absence of haem. Embryogenesis can be rescued by supplementing the serum with haemoglobin. The author also shows that host-derived haem is not necessary for molting of nymphs.

Question 1 (p. 27): The candidate argue that the main role of haem in tick biology is its indispensable participation during embryogenesis, since serum-fed nymphs can molt into adults and when blood-fed in the subsequent adult stage can mate, engorge and lay viable eggs. This implies that dietary haem does not play a role in trans-stadial development, affect spermatogenesis in males or affect reproductive capability in females, but are crucial for embryogenesis. However, working on the assumption that the main role of haem in tick cell biology would be the canonical function of electron transport during oxidative phosphorylation, the above argument may be construed differently. Larvae that feed on blood incorporate enough haem to load their mitochondria and allow them to molt. This same haem is re-utilized in the nymphs in their mitochondria, implying that nymphs grow bigger without necessarily dividing into more cells. The same ratio of cells and mitochondria allows the nymph to feed on serum and molt to adults that would still possess the same amount of haem to load their mitochondria (or does mitochondria survive molting intact?). By blood-feeding as adults, a new source of haem can be incorporated into eggs that allow viable eggs to be laid. Adult ticks that feed on serum has no extra haem, since all is sequestered into mitochondria and none is bioavailable for incorporation into eggs. However, since these ticks have enough haem for their biological functioning, they can mate, engorge and oviposition. The role of haem in embryogenesis would still be its canonical function in oxidative phosphorylation and the reason why it is crucial is the high energy requirements of development and to supply the hundreds-thousands of larvae that will hatch and need to survive till their first blood-meal. Do the author propose another role for haem beyond this canonical one?

Question 2 (p. 33): The author indicated that as little as 0.1% haemoglobin supplemented could rescue embryogenesis of serum fed ticks. May this be expected if the majority of ingested haem is sequestered into hemosomes? Can it be estimated what amount of haem from the blood-meal becomes bioavailable to the tick and that this is a limiting step?

The author next shows that while haem is required for embryogenesis, it can be replaced as source of amino acids by other serum proteins, but that it is not a source of exogenous iron, since no haem oxygenase is present in chelicerates and haem can therefore not be degraded.

Question 3 (p. 35): If haem oxygenase is absent in all mites and chelicerates, and haem is generally the major source of iron for other animals, would it not argue for the presence of an alternative pathway to extract iron from haem without degradation? For example, it was shown that ferrochelatase could demetalize haem.

Question 4 (p.35, Fig. 3D): There is a general trend towards upregulation of αIrFer1 in all organs of SF ticks, indicating iron deficiency. However, the ion levels in ovaries and salivary glands are comparable between organs, BF and SF. Since elemental analysis measures elemental iron and haem may still be sequestered from the nymphal stage, it would have been of interest to measure these organs before feeding to determine baseline levels. If baseline levels were similar, it would argue against the use of host serum components for extra iron.

Question 5: It was nicely demonstrated that haem present in the hemolymph only derives from the current blood meal. This may be expected if haem from the previous

nymphal stage is sequestered in mitochondria. It also implies that once sequestered into mitochondria, the haem is not bioavailable anymore, but does still function. Do the author think that the same may be true for argasids, in particular the *Antricola*, *Nothoaspis* genera, where only larvae and possibly early stage nymphs feed on blood, while late stage and adults do not. How do *Antricola* adults deal with haem deficiency problems if ixodid biology is considered?

Question 6 (p. 39): The author indicated that the last three mitochondrial localized enzymes involved in haem biosynthesis are conserved in ticks. What role could these play in tick biology or mitochondrial function beyond being remnant functions still maintained by neutral evolution and genetic drift?

The author optimized artificial feeding for *I. ricinus* nymphs, something that has not been successful previously and therefore a worthy of accomplishment.

Question 7 (p. 71-74): This section is presented as results and discussion. The discussion presented has no depth or references, making the manuscript seem to be a work in progress.

Question 8 (p. 71): A significant difference in artificial nymphal feeding can be observed between blood and serum meals, with serum fed nymphs feeding faster and with better success. Should a comparison be made with adults?

Part II deals with responses of ticks to haemoglobin digestion and describes midgut transcriptome analysis for ticks fed on blood and serum at an early (Day 3) and replete (Day 8) stage. The integration of the transcriptome data into a metabolic narrative aimed at explaining functionalities in regard to tick feeding is impressive and should be a treasure trove for future discovery and hypothesis testing.

Question 9 (p. 85): It was hypothesized that depletion of RBC (disruption of normal feeding of ticks) could reveal adaptive traits that enable ticks to digest haemoglobin intracellularly, with haem acquisition and transport to haemocoel. Any perturbation to a system never encountered before (e.g. lack of RBCs) is bound to elicit secondary responses not necessarily related to the primary triat. How would adaptive traits be distinguished from stress/secondary response due to disruption? Alternatively, given that serum-fed ticks are able to function normally up to the point of oviposition, is it possible that feeding and digestion of serum proteins induced a standard feeding response that would include the specific adaptations for haemoglobin digestion. In this case, the adaptive traits could still be hidden in the data. One possible example of this may be the very similar expression patterns of the digestive enzymes observed in this study.

Question 10 (p. 87): Midgut transcriptomes was analysed with a low number of genes identified to be up or down-regulated. Could it be possible that potential haem transporters could be trafficked from other tissues (fat body) and therefore missed in the current experiment?

Question 11 (p. 87): Figure 2 indicate broken caeca. The figures shown indicate round bodies interpreted as large and small endosomes. How sure are the authors that

this represents digestive cells in the absence of TEM studies? Could this be digestive cells and the gut lumen? For the naïve reader not well versed in midgut morphology, this figure will be difficult to interpret.

Question 12 (p. 90): Gluthatione S-transferase was upregulated in blood-fed females. It should be noted that Dreher-Lesnick et al. 2006 observed upregulation of GST in the midguts of *Dermacentor variabilis* and specifically commented on its possible role as antioxidant or detoxifying agent during feeding to counteract oxidative stress induced by the haem rich blood meal.

Question 13 (p. 90): Are there evidence that haem may be modified by sulfation and why this would occur? What about sequestration in the hemosome as insoluble aggregate as main mechanism of detoxification?

Question 14 (p. 91): It is stated that new data analysis supports a much better correlation between transcriptome and protein data. Is this true for ticks in general, or may this only be accurate for specific organs? In this regard, was any post-analysis done using these proposed normalization methods, on the previous transcriptome/proteome studies of *I. ricinus* that showed little correlation (Schwarz et al. 2014)?

Question 15 (p. 93): Is there any microscopic evidence of secretion of proteins into the midgut lumen, such as secretory vesicles/secretory pathway that buds into the lumen that would support active secretion to target host proteases?

Question 16 (p. 91-98): An impressive synthesis of the transcriptome of midgut expression during tick feeding is presented that paints a story of blood-meal specific adaptation in ticks. Are the functionalities described considered to be tick specific or will we find them in the midguts of non-hematophagous mites as well, that also need to digest meals?

The author next investigate the implications from the NGS data by characterizing the interaction of GST and haem.

Question 17 (p. 106-111): This section is presented as results and discussion. The discussion presented has no depth or references, making the manuscript seem to be a work in progress.

Question 18 (p. 110): How is the inability to bind to GSH affinity columns explained given the intact enzymatic activity of the enzyme?

Question 19 (p. 110): Haemin-agarose pull-down indicate a loss of 40% of the protein, while 100% activity was lost (bound?). Does this indicate that 60% of the rGST is inactive? How would this affect the biochemical parameters such as stoichiometry and Ki determinations? It was also unclear what concentration of rGST was used in the various experiments, to get a feel for the ratio's used and whether it represent 100% active sites.

Question 20: GST presumably play a role in haem detoxification, perhaps as haem scavenger. It would be interesting to know whether post-feeding events up to

oviposition and embryogenesis would be affected, since oxidative damage may not have an immediate phenotype. This would also hold if GST may play a role in haem transport.

Question 21: GST generally play a role in conjugation. Is there evidence that haem modification occurred during GST-haem interaction? If not, then what role would GST play given its other roles in detoxification and the apparently intact active site? If it is a scavenger of haem (presumably irreversible to remove haem from the system), then the question arise whether it would be able to perform its other functions effectively? It would be interesting to know whether GST can bind haem and perform enzymatic functions. I.e. Does haem bind in the GST active site?

Part III deals with the possibility of targeting aspects of the digestive system as means to control ticks. The candidate is a co-author on four papers presented in this section, which indicates his ability to contribute and collaborate to the work of others, both in intellectual and experimental capacity.

The first paper is a review dealing with advances in tick vaccines, specifically focusing on targets involved in blood-meal processing (Kopáček et al., in press). The second investigated vaccination of a variety of proteins identified to be involved in iron and haem metabolism (Hajdušek et al. 2016).

Question 22 (p. 122): It is indicated that the digestive system of ticks differ significantly from that of blood-feeding insects which use extracellular neutral or alkaline proteases vs the intracellular acidic aspartic and cysteine proteases of the endosomal system used in ticks, similar to protozoan blood-borne parasites. This may be a specific adaptation to blood-feeding, or may be more ancient. What is known for the digestive system of other chelicerates or mites?

Question 23 (p. 128, 161): Large and small endosomal vesicles seem to both derive from primary lysosomes. Are these different sub-cellular organelles, i.e. are coated pits targeted specifically to large endosomes? If so, do both large and small vesicles derive from primary lysosomes?

Question 24 (p. 129): The lysosomal system also functions in general protein turnover biology (autophagy) and this system can possibly be traced back to the last common eukaryotic ancestor. Would you consider the conservation of the lysosomal digestive system between ticks and protozoans to be continuous (digestive system derived from a common ancestor), or independent exaptation of the normal lysosomal system, in which case this may only be as recent as the origin of chelicerates, Acari or ticks?

Question 25 (p. 130): A shift in pH optimum in the endosomes is observed during feeding when initial proteolysis occurs at low pH and then shift to higher pH. Is this change considered an active process linked for example with a proton transporter (how would this be regulated?) or related to catabolic chemistry?

Question 26 (p. 132, 153): A function for HRG-1 as transporter of haem from the midgut endosomes was proposed based on a similar role in the orthologs from

vertebrates. However, expression is highest in ovaries. A similar argument may be made for FLVCR function in transport from digestive cells, since expression seems to be negligible in midguts. Have localization experiments been contemplated to confirm the localization to endosomes and cell membranes? Similarly, export mechanisms for haem and Fe are proposed, but no import mechanisms into cells. Is this considered to be the same?

Question 27 (p. 138): It is proposed that a chemically defined diet may be the only way to elucidate the triggering mechanisms involved in upregulation of the digestive enzymes during feeding and then as central theme of the thesis, the use of an artificial feeding system. It is acknowledged that mating is an important non-dietary trigger in adults. Could other non-dietary triggers exist which may be more mechanical, such as hydrostatic pressure or conversely colloidal osmotic pressure? Other sensory receptors such as gustatory, mechano or olfactory receptors in the chelicerae may also be considered.

Question 28 (p. 153): It is accepted that concealed antigens have potential for vaccine candidates as evident for Bm-86. Bm-86 is a gut membrane protein and the mechanism of protection is presumably due to antibody binding, recognition by the host-derived complement system in the gut lumen, followed by membrane attack, resulting in gut rupture. The mechanism is therefore conceptually very strait forward. How is it foreseen that the mechanism of protection will work with proteins restricted to intra-cellular organelles such as mitochondria, the cytoplasm or even the hemolymph? Is this purely antibody-mediated (neutralizing blocking antibodies), or is a cellular or complement-based system envisaged at post-gut lumen levels? If neutralizing antibodies are the mechanism, then these would have to present at levels of similar concentration or higher to be effective in the hemolymph or non-gut organelles.

The third paper deals with digestion of serum albumin and how this compares to haemoglobin digestion (Sojka et al. 2016). Its shows that the general digestion mechanisms for both are similar even if they occur in different cellular compartments.

Question 29 (p. 164): Given that the dynamics of expression is similar for the majority of the peptidases except for IrCB, the difference in IrCB expression seem significant and possibly linked to haemoglobin uptake or digestion. Are there any evidence that specific localization of these digestive peptidases occur in the large and small endosomal vesicles?

The fourth paper is a commentary on and endorsement off a recently published paper dealing with tyrosine degradation. It discuss the interesting observation that the inability to remove excess tyrosine from the blood meal results in death of blood-feeding arthropods (Kopáček et al. 2016).

Question 30 (p. 168): Inclusion of this paper in the thesis seems peculiar, since it does not really integrate with the previous work related to haem biology. Could the authors confirm that there was an upregulation of the tyrosine degradation pathways in their own midgut transcriptome data, since this would provide linkage to this paper.

Part IV further investigate to use of artificial feeding to study different aspects of tick biology, notably the role of iron in tick feeding, the nutritional requirements of ticks with regard to serum, investigation of salivary secretion and anti-tick responses by the host.

Question 31 (p. 173): When the low molecular weight serum fraction (<10 kDa) is fed to ticks, they initiate feeding, but fail to progress to the rapid phase. Could the hydrostatic or colloidal osmotic pressure in general play a role in this, since this is in part the role of serum albumin in blood? In this scenario, sensing of blood volume initiate further feeding and development of cuticle, upregulation of midgut proteases etc. In this regard, ticks generate higher internal hydrostatic pressure during engorgement than any other terrestrial arthropod (Kaufman et al. 2016).

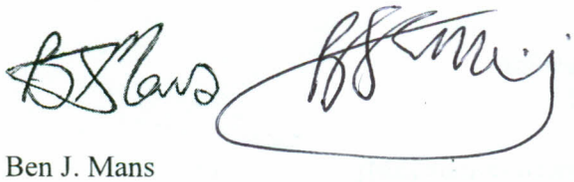
Question 32 (p. 175): A reduction in fed weight is observed for ticks fed repeatedly-infested rabbits. This is taken as evidence for a host defence response and impaired feeding. Is there evidence of tick rejection at the feeding sites? No effect could be seen when blood from these rabbits were used in artificial feeding, suggesting the "protecting" factors are not present, presumably due to defibrination. What effect does defibrination have on blood beyond removal of white cells and could this explain possible defence responses from the host at the feeding site, since the immune response is not the only defence targeted by ticks?

Question 33: A final concluding discussion would have been useful at the end of the thesis (after part IV) to tie the various chapters together, especially the manuscripts in preparation, unpublished data and the four papers presented in part III and integrate them with the published papers (Perner et al. 2016; 2016b). However, this omission does not seriously detract from the quality of work presented.

Conclusion

The body of work presented by the author is impressive. The methodologies employed range over a large subject area and indicate that the group he works in is truly multi-disciplinary. Techniques range from artificial feeding, bioinformatics, recombinant protein expression and purification, differential expression analysis using real-time PCR, siRNA, western blot, electrophoresis, HPLC, graphite furnace atomic absorption spectroscopy, next-generation sequencing and analysis, scanning electron microscopy, vaccination. The scientific arguments are strong and current work is integrated well with previous literature. I have no doubt that the candidate is ready to defend the thesis and recommend this whole-heartedly. Minor modifications follow on the next pages that does not impact on the quality of the science presented in this thesis.

In Pretoria, 4 March 2017



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