gene amplification? Different sensitivities of the two PCRs or an effect of different gene copy numbers do not seem to be the reason, since at 2 DPD, higher detection of ama-1 than of 18S rRNA can be observed.

3. In the proteasome experiments:

i. How was the inhibitor dose used to mice calculated? Is there any relationship between the doses used *in vitro* and *in vivo*? Could a higher dose be used in mice? (Although the parasitemia has a significant decrease with the drug, the effect observed is only partial). Could a combination of drugs be useful?

ii. Was the cytotoxicity assay applied to *Babesia* parasites exposed to the inhibitors? It would be interesting to know if the inhibitors kill the parasites or only arrest their growth temporarily.

iii. How specific are the protease inhibitors used? Could the observed effects be due to the combined inhibition of proteasome proteases and other serine proteases (not part of the proteasome) with functions in other cell processes?

iv. How specific is the fluorogenic substrate used? Could you be detecting other serine proteases as well?

v. In the gametocytogenesis work you observed that the bdccp genes are down regulated after long term cultivation. Please speculate on which type of control of gene expression could be operative in this case? You could base your answer on available mechanisms of gene control found in other *Babesia* spp. or other Apicomplexans.

Final remarks:

I enjoyed reviewing this Thesis and consider that if fulfills the required conditions for its defense. I also had the opportunity to be present at a presentation by Marie at the 9th International Conference on Ticks and Tick-borne Pathogens (Sept. 2017) and could see her fluency in the subject, and her strong identification with her work. I have no doubt that she deserves her PhD degree, and will have a successful career as a researcher.

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Review of PhD Thesis: "Establishment of Babesia laboratory model and its experimental application" by Marie Jalovecka

General thoughts:

This Ph.D. Thesis presents a very comprehensive analysis of different aspects of two *Babesia* parasites that can cause human babesiosis: *B. divergens* and *B. microti*.

First, it includes valuable review information on the parasites' life cycles and their interactions with their tick vectors. Second, it deals with – either revising or setting up - several relevant techniques connected to these parasites. These studies include (i) analyzing the validity of microscopy to detect *Babesia* parasites in human blood as compared to PCR, (ii) the establishment of a method to induce and quantify gametocytogenesis of *B. divergens*, (iii) the setting up of *B. microti* infection of mice and (iv) the implementation and optimization of *B. microti* acquisition by ticks. Finally, this Thesis also provides novel information on tick molecules that regulate *Babesia* parasite acquisition, and on parasite protease activities connected to the proteasome, and their potential usefulness as therapeutic targets.

Part of the information presented in this Thesis has been included in 3 manuscripts that were published in important peer-reviewed scientific journals (one in 2013 and two in 2016), and 2 manuscripts in preparation. The Thesis also contains original, non published data. Marie Jalovecka is first author in three of the mentioned manuscripts, and corresponding author in two of them.

The Thesis is clearly written, well organized, and interesting to read. The Tables and Figures are of good quality, and are adequately described in appropriate legends. There has been a thorough and updated analysis of the bibliography. I found a few grammar mistakes in the unpublished parts, but they did not affect the clarity of the text. I consider it worthy that the experimental approaches that did not give the expected results are also mentioned and described. This type of information is very often not useful for publication but it is still very valuable for those researchers that work in the same field.

Specific Questions:

1. Did hematocrit decrease during *Babesia microti* infection of mice?

2. How do you explain the PCR results of Figure 18 (page 125) where amplification of both 18S rRNA gene and ama-1 gene is found in some ticks, while some only show 18S rRNA

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Reviewer's evaluation and comments on the PhD thesis entitled 'Establishment of *Babesia* laboratory model and its experimental application' submitted by Marie Jalovecka

The thesis describes a very interesting and substantial body of original work focused on

- The detection and quantification of gametogenesis in *B. divergens in vitro* cultures and the effects of various stressors on the development of gametocytes (excellent 1st author publication in *Parasites & Vectors*)
- The effect of several proteasome inhibitors on *B. divergens in vitro* and *B. microti in vivo* (manuscript in preparation)
- The role of various tick immune signaling pathways in the protection against *Babesia* infection (promising work in progress)

In addition the candidate took part in a laboratory ring trial aimed at determining the validity of a novel microscopic method for the detection of *Borrelia* and *Babesia* spp. (published in *Infectious Diseases*, co-author). Finally she participated in a very interesting review on the interactions of the tick immune system with transmitted pathogens (published *in Frontiers in Cellular and Infection Microbiology*, co-author), and has drafted a review paper entitled 'Life cycle of piroplasms: comprehensive analysis' (manuscript in preparation). In all collaborative aspects the role of the candidate is clearly defined.

Overall the work makes a significant contribution to what we know about the biology of the two parasite species in many instances using novel tools to address 'old problems'. This approach has not only given rise to new interesting information but has also opened up fresh research avenues which are currently being explored by the research team. During the course of her project the candidate also supervised a Master student. It is clear that Maruska has achieved the academic standard and independence expected of a PhD candidate.

My only reservation is the highly unconventional format of this thesis, which reads more like a patchwork than a coherent whole. While the two review papers are interesting and touch on most of the relevant aspects of the work, I would have liked to have seen a short overall introduction that outlines the current state of knowledge and the gaps to be addressed as well as a clear description of the research objectives. The chosen format also makes it difficult at times to track down methods, figures, tables or numbered references. If the thesis is to be made more widely available on-line the candidate may need to obtain permission from the various copyright holders of the papers that are bound into it.

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Specific comments and questions
Part 1:

Comments/queries:

- When discussing human babesiosis it is important to distinguish between *B. divergens* and *B. microti*, as they are two very different pathogens! Considering the extremely low incidence of *B. divergens* cases in Europe I do not think it is justified to describe it as an 'emerging human pathogen'.
- According to some authors (e.g. Bock et al 2004; Gough et al 1998) a round of schizogony occurs *before* fully differentiated 'Strahlenkoerper' are formed (p. 29).

Possible question for discussion during the *viva voce* examination:
How does blood digestion in ticks differ from that in haematophagous insects and how may this affect their capacity to act as vectors?

Part 2:
Comments/queries

- Considering that this is the only section in the thesis where Materials and Methods are described, it lacks a lot of detail. For example, in our experience % PVC in *in vitro* culture is critical. This should be specified. Also how were ticks fixed onto mice during feeding and how were they recovered? How were male and female nymphs differentiated? For the RNA interference assay what was the dsRNA suspended in? The fluorescent in situ hybridization assay mentions two dyes (ALEXA Fluor and TAMRA) but does not specify which one was used and when.
- Why were such unusually high cycle numbers used in the qPCR (p. 47)?
- Please specify animal license numbers for all experiments using animals!

Possible question for discussion during the *viva voce* examination:
You suggest that the incidence of asymptomatic human babesiosis may be quite high. What do you think the characteristics of these asymptomatic infections would be e.g. level and duration of parasitaemia, immune response? What is the evidence? Also can you hypothesise on why are there so few endemic clinical *B. microti* cases in Europe?

Part 3
Comments/queries:

- *B. divergens* is a bovine parasite! It is misleading to describe cattle as 'reservoir hosts' (p.66).
- Maltese cross formation is typical of many *Theileria* spp (p.67)

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- I would not say that *B. divergens* was ever successfully adapted for culture in horse erythrocytes (p.68). No long-term cultures could be established.
- Again please be careful not to overstate the clinical importance of human babesiosis outside the US (p. 94).
- Could the failure to infect gerbils have been due to the *Babesia* lab strain rather than the gerbils? I consulted Jeremy Gray on this and he assures me that he isolated more than 30 *B. divergens* 'strains' straight from the field into gerbils (bought from 2 different suppliers) with no difficulty whatsoever. He says the biggest problem was that the parasite always grew too fast!
- How do you explain the significant number of dead PMJ2R cells in untreated cultures (~30%!) (p. 107)?

Possible question for discussion during the *viva voce* examination:
Do you have any explanation for the apparent inability of larval/nymphal ticks to acquire *B. divergens* infections?

Part 4, chapter 1
Comments/queries:

- Lack of a more conventional structure make this chapter difficult to follow. It is difficult to see the rational behind using an unspecified (but apparently substantial) number of animals in experiments that yielded little new information with regard to transmission routes, levels of parasitaemia, parasite morphology or host immune response. The omission of a discrete M&M's section means that the number of animals/ticks/replicates per experiment is unclear. Moreover the methodology of the FISH method, the most important outcome of this chapter, is not clearly described.
- Considering that it has been possible to identify previous hosts from questing ticks (i.e. once they had molted) using PCR (Pichon et al 2003. Journal of Medical Entomology 40, 723–731), it is highly unlikely that after only 4 days post detachment the mouse DNA would have been degraded to such an extent that it could not be amplified (p. 124).

Possible question for discussion during the *viva voce* examination:
What possible clues could the ticks have had from their lab environment to produce the seasonal effects you describe? Were temperature and photoperiod tightly controlled? How recently had the ticks been collected in the field?

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Part 4, chapter 2:

Comments/queries:

- You state that 'silencing of Rel1, Rel2, NF-kb, Imd had no effect' (p. 135) yet Fig 22B indicates that silencing of Rel1 and STAT caused a *decrease* in *Babesi*a load even though the difference to the GFP control may have just failed to be significant at the (arbitrary) 0.05 confidence level. The same comment applies to your interpretation of Fig. 24.
- Parasite quantification seems to me to be relative to the tick genome rather than absolute as stated on page 133.

Possible question for discussion during the *viva voce* examination:

- In your opinion, do vector-borne pathogens represent a significant strain on the health/fitness of tick host?

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Overall I have no hesitation in recommending award of the title of Ph.D. to the candidate.

Dublin, 29 September 2017

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Evaluation of the Ph.D. thesis by Marie Jalovecká entitled "Establishment of *Babesia* laboratory model and its experimental application", presented to obtain the PhD grade from the University of South Bohemia and the University of Bretagne-Loirs

The thesis by Marie Jalovecká describes the work which she performed as part of a dual Czech-French Ph.D. Program within the University of Bretagne-Loire, ONIRIS, Nantes, France and the University of South Bohemua, Ceske Budejovice, Czech Republic from 2012 to 2017. It contains three published papers in international peer-reviewed journals. Ms Jalovecka was first author on one of these papers and a co-author of the other two. Two unpublished first-author manuscripts in preparation are also included in the thesis and these are, in the opinion of the reviewer, of a sufficiently high standard to warrant publication. The thesis' main focus lies on the development and application of laboratory models for *Babesia divergens* and *Babesia microti*, two apicomplexan parasites of medical and veterinary relevance. In general, the thesis is well written although there are still a number of grammatical and typographical errors, and it is clear that it embodies a large amount of original research data and experiments that shed light on an often enigmatic subject.

Part I, the general introduction, gives concise background information on piroplasms and on babesiosis and its transmission by *Ixodes ricinus* ticks in particular. It continues with two reviews, a published and highly cited review from 2013 on the interaction of the tick immune system with transmitted pathogens and a second unpublished review summarizing knowledge on the lifecycle of piroplasms.

I have a few questions and minor comments regarding the first part of the thesis:

Q1: on page 5, it is mentioned that the disease manifestation of red water fever in cattle may vary, depending on animal immune status, the number of questing ticks and parasite species or strain virulence. From a veterinary point of view, I missed the explicit reference to one major factor, related to the animal immune status and of importance in the endemic stability concept, here. Which factor is this?

On page 6, it is mentioned that there are seven genera of hard ticks, but there are actually 11 extant ixodid tick genera: *Amblyomma, Anomalohimalaya, Bothriocroton, Cosmiomma, Dermacentor, Haemaphysalis, Hyalomma, Margaropus, Nosomma, Rhipicentor* and *Rhipicephalus*.

Q2: in Table 3 of the review on tick innate immunity and tick-borne pathogens (page 18 of the thesis), examples are given of tick proteins implicated in tick-*Babesia* interactions, such as longicin and longipain. Are homologues of these proteins present in *Ixodes* ticks and if so, is anything known about their function?

In page 26, reference is made to the transforming *Theileria* species, but *Theileria* sp. (buffalo) is missing from the listed species. Evidence has been presented that this species is also capable of immortalizing its host cell (Zweygarth et al., 2009. Parasitol Res 105: 579-581; Bishop et al., 2015. Int J Parasitol Parasites Wildl 4: 333-342).

Q3: Regarding the manuscript in preparation, 'Life cycle of piroplasms: comprehensive analysis' and in the light of the previous review on tick innate immunity and tick-pathogen interactions, I would like to know which strategies piroplasms employ to evade the vertebrate host's immune system and if these strategies are shared between the different piroplasms?

In Part II of the thesis, the research methods used for the unpublished manuscripts are summarized. It also includes one methodological publication.

Q4: In the legend of figure 2 on page 48, which shows the transmission model for *B. microti*, it is mentioned that larvae were fed in cylinders fixed on the dorsal part of the mouse. A description of the exact procedure hereto appears to be missing from the thesis. What was the reason for feeding larvae to engorgement in cylinders instead of whole body feeding?

Part III describes efforts to establish a *Babesia divergens* laboratory model and its experimental application. It includes two manuscripts, one of which is unpublished.

Comment re page 67: the Maltese cross is characteristic, but not unique to *Babesia*, it is for instance also formed by *Theileria equi* (Mehlhorn & Schein, 1998. Parasitol Res 84: 467-475)

Q5: if nymphal ticks are indeed not competent to acquire and transmit *B. divergens* (page 70), which *I. ricinus* tick life stage would then mainly be responsible for transmission of *B. divergens* to cattle under natural conditions?

Q6: are homologues of the bdccp1, bdccp2 and/or bdccp3 genes present in the *B. microti* genome?

The focus in part IV of the thesis is on the development of a *B. microti* laboratory model and its use to examine the immune interactions between *B. microti* and *I. ricinus*.

Q7: field surveys from the USA report an infection rate of ~40% in the white-footed mouse (*Peromyscus leucopus*, page 110). How does this compare to the infection rate in reservoir hosts in Europe?

Q8: what was the rationale for selecting the PRA-99 strain Peabody for the development of the *B. microti* laboratory model? Would it be possible that other *B. microti* strains (e.g. the Munich strain with a reported parasitemia of up to 80%, page 114) or recently isolated strains, similar to the situation for *B. divergens*, display a stronger competence to produce gametocytes and may therefore be more suitable for use for the larval acquisition model?

Q9: was a statistical analysis performed for the data presented in Figure 14?

Q10: in the discussion and future perspectives, the possibility of parasite seasonality is mentioned (page 143). Which additional experiments do you envisage to elucidate this phenomenon and its potential role in the epidemiology of *B. microti* under natural conditions?

In conclusion, I am convinced that the extensive work described and discussed in this thesis demonstrates that Ms Marie Jalovecka fulfils all the criteria of a successful Ph.D. student and forms a significant contribution to our knowledge on *Babesia* parasite biology. I therefore recommend the board to award her the Ph.D. title.

Yours sincerely,

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