October 19, 2015/ Ankara

Prof. RNDr. Jan Tachezy, Ph.D.
Chairman of the Committee


Student Name: Mgr. Jana HLAVACOVA
Supervisor Name: Prof. RNDr. Petr VOLF, CSc
Title of PhD Thesis: Effect of abiotic and biotic factors on Leishmania development in sand fly vectors

Dear Prof. Tachezy,

After evaluation of the thesis of Jana Hlavacova entitled “Effect of abiotic and biotic factors on Leishmania development in sand fly vectors ”, please find in attachment the assessment report prepared by me. I, with great pleasure, report that the thesis makes a significant contribution to knowledge, makes a significant contribution to the understanding of the subject with which deals, demonstrates the candidate’s capacity to carry out independent research, contains material worthy of publications, and that the format and literary presentation of the thesis are satisfactory.

I recommend that the candidate be awarded the degree of PhD.

I enclose a list of my overall assessment, comments, corrections, and questions according to the rules for PhD thesis at the Charles University.

Sincerely,

Prof. Dr. BüleNT ALTen
Evaluation Report of the PhD thesis entitled “Effects of abiotic and biotic factors on Leishmania development in sand fly vectors” by Bülent ALTEN

Overall Assessment:

This thesis is an outstanding piece of research and writing, distinguished by: through use of both primary and state-of-the art methods and materials; sophisticated specification of problems with higher-up experimental design; a deep and wide-ranging engagement with literature; clear demonstration of how environmental parameters (abiotic and biotic) effects on parasite development (Leishmania) in their proven and/or candidate vectors relation with current or possible climate change scenarios; fine critical skills; an ability to mount detailed and cogent arguments; and a real gift for lively and yet carefully nuanced writing.

I was very pleased to read and review Jana Hlavacova’s dissertation thesis. Its scientific merit is high reflecting Jana’s methodological approach to studying the process on the development of Leishmania parasites under different ambient temperatures, effects of several biotic factors such as Psychodiella sergenti and Leptomonas seymouri in sand fly species. It also reflects well upon Jana’s adaptability to contribute towards the development of new methodologies, acquiring new skills and performing meaningful experiments in laboratory. Throughout the studies, she published one paper as first author in Journal of Medical Entomology, one paper as second author in Journal of Medical Entomology and one paper as third in PloS Pathogens, in total of three papers, under following three different but interrelated specific objectives:

1) To evaluate effects of temperature on the development of Leishmania in sand flies naturally occurring in different habitats; Leishmania infantum in Phlebotomus perniciosus and Lutzomyia longipalpis, and Leishmania peruviana and Leishmania braziliensis in Lutzomyia longipalpis (Core of the thesis);

2) To elucidate effects of gregarine Psychodiella sergenti on Leishmania tropica development in Phlebotomus sergenti;

3) To test the capability of Leptomonas seymouri to develop in Phlebotomus orientalis and Phlebotomus argentipes in sugar- and blood-initiated infection, and compare its development with Leishmania donovani during co-infection.

For me, it is crucial to find out accurate result (s) and new areas/ future topics from a set of given studies during studies. This proves and shows the quality of the study and adaptation and concentration of the candidate to his/her own experiments. In this set of study, I determined that Jana has already reached a total of 13 exact results which were listed below and revealed four new/future study areas. The fact that each one contains very important criteria which can be titled for other PhD thesis and/or investigations.

Subject 1: She was interested in the effect of temperature which is one of the main environmental factors

Results:

1) Even though intensities of infection were lower at the lower temperature on day 2 post blood-meal (PBM), on day 8 PBM L. infantum developed comparably well with heavy late stage infections and colonization of the stomodeal valve frequently observed in all combinations tested.

2) The lower intensities of infection on day 2 PBM indicate that multiplication of promastigotes was slower at the lower temperature; however it did not have any negative impact on parasite establishment and further development in the vectors.

3) Slower multiplication of parasites, defecation of sand flies was also slower at the lower temperature in both species tested.

4) While more than 80% of Lu. longipalpis females digested and defecated blood meal remains by day 3 PBM, this process took approximately 6-7 days at 20°C.

5) Digestion and defecation of P. perniciosus was significantly slower; at 26°C, almost 90% of females were defecated on day 4 PBM and only two thirds of defecated females were observed by day 7 PBM at 20°C.

Subject 2: She was interested in the effect of gregarine on Leishmania parasite in sand fly species

Results:

1) After comparing the development of two Vianna species, even though both species are closely related, she found that they occur in different biotopes and a cause a different manifestation of the diseases.

2) Whereas L. braziliensis (low land species) developed well in both temperatures tested causing heavy late stage infections, L. peruviana (mountain species) did well only at 20°C, while at 26°C nearly all infections were lost between days 2 and 8, i.e. the period of blood meal defecation. She supposes that this mountain species is adapted to sand flies living at
lower ambient temperatures and that delayed defecation gives promastigotes more time for establishment in the vector’s digestive tract.

3) In the study she compared *L. tropica* development in two colonies of *Phlebotomus Sergenti* originated from Israel, one infected and the other non-infected by gregarine *Psychodiella Sergenti*. She did not find any significant difference in *L. tropica* development neither in percentages of infected females nor in intensities of infection in any day tested between gregarine infected and non-infected groups of females.

4) According to her results, there is no apparent effect of gregarines on *L. tropica* development in its specific vector *P. Sergenti* tested in our experimental setting.

**Subject 3:** She was interested in to test the capability of *Leptomonas Seymouri* to develop in *Phlebotomus orientalis* and *Phlebotomus Argentipes* in sugar- and blood-initiated infection, and compare its development with *Leishmania Donovani* during co-infection.

**Results:**

1) In sugar-fed females, parasite numbers as well as rates of infection were gradually decreasing in time. On day 2 post sugar meal, 100% of *P. orientalis* and 59% of *P. argentipes* females were infected; however, until day 9 only a third of females remained infected, usually with just a few persisted parasites.

2) Additionally, she established a set of experiments on: Female sand flies were membrane-fed on blood meal containing mixture of mCherry-expressing *L. Seymouri* and GFP-expressing *L. Donovani*. Results were similar to the previous experiments, only few females were found infected with low numbers of *L. Seymouri* on day 5 post feeding. On the other hand, *L. Donovani* developed well and thriving heavy late-stage infections were observed.

3) She showed that *L. Seymouri* is capable to persist for some time in the digestive tract of the natural vectors of *L. Donovani*. However, the infection falls gradually down in all experimental settings tested and it appears plausible that parasites may be defecated with meal remnants.

4) Role of sand flies in *L. Seymouri* transmission to human seems to be unlikely, and other possible routes of infection should be further investigated.

**CONCLUSION of findings:**

She demonstrated that temperature can be a limiting factor for Leishmania development in sand flies. Further, she detected that gregarine *Psychodiella Sergenti* does not have any negative effect on *P. Sergenti* competence to *L. tropica*. Finally, she showed that monoxenous trypanosomatid *Leptomonas Seymouri* can for some time persist in the digestive tract of two vectors of *L. Donovani*, either alone or in co-infection with *L. Donovani*, but it is not capable to produce late-stage infection. The association of monoxenous trypanosomatids with “leishmaniasis like” diseases in immunodeficient and visceral leishmaniasis patients raise a question about their clinical relevance, which remains to be learned about in the future.

As we know that the study performing under laboratory conditions must be as much as high quality that can be mimic natural conditions. This study, on one side, demonstrated the high resolution set of studies under laboratory conditions, and on the other side, it showed the possible transmission rate under suppression of biotic and abiotic conditions and the number of promastigotes in sand fly females. It is quite important finding which emphasizes the importance of determining the natural parameters for the development of leishmania parasite. Because of particularly with this result and altogether the findings from other objectives of the thesis, I found Jana’s research to be on the whole original.

Overall, no important mistake in experimental design of this thesis has been determined. But, its related studies, it would be good if using sophisticated method/design such as “geometric morphometrics” with size and shape analyses has done morphological studies. With this way, the possible morphological differences between different conditions could be easily determined.

Jana has shown appropriateness for a diverse set of methodologies, ranging from the biological (sand fly and parasite culture, sand fly and parasite infection, inoculation etc.), to molecular (RAPD, PCR, QPCR), to ecological (life-cycle parameters, effect of constant temperature on species), and to the statistic. Each appears to be conducted with considerable expertise to obtain high quality, publishable results.

Jana’s interpretation of her results is comprehensive, comparable and well-balanced-taking care to not over interpret her data but state where it makes an important contribution to the field.

I found Jana’s thesis to be well set out and easily digested whilst presenting an up to date and scientifically accurate synthesis of the topic of parasite-v-biota interactions. Jana has written a very comprehensive introduction and review. Information on the
subject of her subjects has been still accumulating, sometimes it seems instead of things getting clearer they are getting more confused. We know that in many instances, previous results are being challenged by new findings. In this study, Jana has offered more new challenges with her cogent arguments, and she interpreted them very well.

**Specific critical comments:**

There are very less misspellings found throughout the thesis, which I have carefully checked for.

**Page 18:** “During their life cycle, sand flies live in different environments and feed on different meal. Larvae develop in soil rich in organic material, for example in animal shelters and burrows, caves, or termite hills. Adults of both sexes feed on a sugar meal and females also on a blood meal (Feliciangeli, 2004; Maroli et al., 2013).”

*Reference is not necessary here, because the information is quite common.*

**Page 29:** “Sadlova and Volf (2009) demonstrated that the break-down of peritrophic matrix enables the transformation of procyclic promastigotes to long nectomonads (Sadlova and Volf, 2009) and….”

To write two times references in same sentence is not necessary. Please remove last one at the end of sentence.

**Page 30:** No parasite’s binding to the gut was observed, conversely to what was found in some monoxenous trypanosomatids in mosquitoes (Fampa et al., 2006)2003; Corrêa-da-Silva et al., ??

I don’t understand format of references. Maybe it is typing mistake.

**General Questions and comments:**

First, I frankly say that I learnt many new hypotheses, methods, concepts etc. when I read this thesis. It is important for me even if I am a member of Jury. Just in this point I would like to thanks to Jana. The thesis is well designed, it answered many questions, and more important it opened new gates and served many new research topics to biological science.

I have 9 questions and/or comments and/or suggestions for learning much more. The questions are not ordered according to consecutive sections.

1: *(ABSTRACT)* It is obseletely correct assessment that adaptation to slower metabolism is completely contact with lower temperatures. But 20 °C how much lower temperature? If we consider that the model vector is *Lu. longipalpis* which distribute relatively tropical climate; in this case, how this evaluation match realities in natural conditions? Please read the papers attached in the pdf.

2: *(PAGE 15)* “Many authors recorded that time of the development, i.e. oviposition time, incubation period or mean generation time negatively correlate with the rising temperature (Endris et al., 1984; Guzmán and Tesh, 2000; Kasap and Alten, 2005; Kasap and Alten, 2006; Chelbi nad Zhioua, 2007).”

Question is that, is it suitable to say negatively correlate with rising temperature here? Here is a curve with correlation between temperature and development of sand flies which started from lower temperatures to optimum and then decreasing after optimum according to increasing temperature (Shelford rule). What do you think?

3: What is lower temperature for sand flies? 15 °C, or 20 °C, or 23 °C ?, what is optimum 28 °C? what is highest temperature 32? Do you know what temperature is “ecological zero for sand flies, in general?”

4: Do you know Van Hoff rule? If we increase the temperature +10, metabolic reactions also increase 2 or 3 times. It is Q10 rule. Krogh says it is not correct (or partly correct). This works like Shelford rule that if we increase temperature continuously, there is optimum level for metabolic reactions, then metabolic reactions such as development time or incubation period decrease in contrast to increasing temperature. What do you think? If you tried a temperature scale from ecological zero to maximum, do you think it would be good to see which temperature is optimum, and which one minimum or maximum?

5: According to you which species can be more adaptive at northern latitudes if we consider the climate change effect, *P. perniciosus* or *Lu. longipalpis*?
Leishmania promastigotes develop in the sand fly gut, and therefore it is obvious to suppose that they may be affected by changes caused by altered ambient temperatures during life of their vectors. Positive correlation of rising temperature (10-25°C) with a proportion of infected females of *P. ariasi* and speed of *L. infantum* multiplication was detected by Rioux et al. (1985).

Question is that is it endless? If not which temperature is the upper limit? (For example: 24 °C is the upper limit of *Plasmodium vivax*).

Does it means that if mean ambient temperature increase above optimum (28) because of climate change, there will not be parasite and leishmaniasis?

A negative effect of faster digestion and defecation on *L. amazonensis* development at higher temperature (28°C) observed Leaney (1977). He presumed that parasites do not have enough time to escape from the endoperitrophic space and establish infection in *Lu. longipalpis* due to the faster digestion at higher temperature.

We suppose that this mountain species is adapted to sand flies living at lower ambient temperatures and that delayed defecation gives promastigotes more time for establishment in the vector’s digestive tract.

Good results. The result shows species isolation according to altitude and biotopes; on the other hand species overlap and adaptation level (*L. braziliensis*). So Do you think *L. braziliensis* should be more distributed species in Brazil?

Do you think both *Psychodiella sergenti* and *Leptomonas seymouri* are not good agents to control the leishmaniasis?

**Statement:**

The reviewer believes that the PhD candidate, Jana Hlavacova, has demonstrated her qualification for scientific work at the PhD level. The thesis fulfills qualitative requirements for a PhD dissertation and fulfills also the formal rule as far as the number of published papers in peer-reviewed and impacted journals is concerned. Moreover, the candidate published (3) papers to be needed.

In conclusion, I want to congratulate Jana and her supervisor Prof. Peter Volf for the excellent study. I find the study of Jana Hlavacova and her thesis fully deserving of a PhD. I recommend that the candidate be awarded the degree of Doctor of Philosophy.

Sincerely yours,

Prof. Dr. Bülent ALTEN
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