Report on the dissertation thesis of the student Barbora Vomackova Kykalova

Summary
Leishmania parasites, the causative agents of leishmaniases, are transmitted to their mammalian hosts through the bite of insect vectors termed sand flies. Vector/pathogen interactions are a crucial aspect of the epidemiology of vector-borne diseases, yet little is known about the parameters of the sand fly/*Leishmania* interaction. During her PhD, Barbora Kykalova examined the role of the sand flies immunity on their biology in different conditions including infection with *Leishmania* parasites. More specifically, she described the expression profiles and investigated the role of diverse effectors and signalling pathways well-known for their role in the humoral immune response of other insects (namely AMPs, the Toll, the JAK-STAT and the Imd pathways). This work gave rise to 4 publications included in her thesis manuscript and is completed with the description of attempts of mutagenesis of the immune-related gene Caspar in the sand fly, using CRISPR/Cas9 genome editing.

General comments:

Overview:
The scientific approach undergone by the student is quite coherent and allows to tackle different aspects of the question of sand fly immunity. The techniques used (sand fly infection and dissection, RNA silencing, RT-qPCR) and the experimental workflow are sometimes challenging and seem well mastered. These investigations led to 4 published manuscripts, which is a good publication record for a PhD student. Even if unsuccessful, the last part on the CRISPR/Cas9 attempts is interesting and show that the student has a good understanding of the scientific approach. I will add that sand fly genome editing is particularly challenging to achieve. My main issue is the lack of questioning of the limitations and advantages of the tools and techniques used during the PhD. This is true mainly in the discussion part, but also to a lesser extend in the publications themselves. I also think that the introduction would need a few paragraphs and figures more, to describe the normal process of sand fly infection by *Leishmania* and also the technical aspects of reverse genetics in insects.

Language:
- The English level is very variable and sometimes insufficient depending on the parts. I understand that English is not the primary language of the student, but the text needs to be corrected to eliminate obvious English mistakes and problems of formulations, because they render the text difficult to read and sometimes even alter the proper meaning of the sentence. I did not highlight the incorrect sentences and strongly encourage the student to have her manuscript verified for language errors.
- A very frequent mistake: when reporting an observation made in a publication, the present time should be used, not the past. Ex: “the Toll pathway was active in Anophles sp. mosquitoes…” (P10): it should be “the Toll pathway is active in Anopheles…”, because it is always true.
- Another language mistake: the expression “gene level” or “gene profile” doesn’t mean anything. You can use “gene expression level”, even if it we don’t know if you are talking about the RNA or the protein level. The best would be to talk about mRNA expression level.

Thesis manuscript:
-The general organization of the thesis manuscript is good and makes a lot of sense.
-The introduction, even if correctly organized and consistent, is a bit short and lacks some essential parts to my opinion (see detailed comments below). Some paragraphs need writing improvement.
- The objectives of the thesis are clearly explained and well detailed. I did appreciate that the student clearly stated her contribution to the different publications that she signed as a co-author.
- The results section of the thesis manuscript consists in 4 publications and an unpublished section on sand fly mutagenesis. Their order is logical and the part on CRISPR is nice.
-The summary/discussion is interesting, even if it is too centered on the results description for me. I would appreciate a bit more “discussion” *per se*. In particular I would like more informations on the choice of RT-qPCR as the only readout. What are the limitations of this technique? Can you comment on the mRNA stability regulation? the post-traductional regulations (activation of proteins by phosphorylation or cleavage…)? Also, I think that this part lacks a bit of comparison with other studies, in particular involving transcriptomic, and other organisms.

Specific comments and questions:

Introduction part I
-A description of *Leishmania* development in the gut is needed, with a figure. This is important for the later discussion about the timing of dsRNA injection of relative to the infection stage and the dissection.

-The all paragraph on Jak-Stat at the end of P13 and beginning of P14 is confusing and needs to be re-worked. In particular, all affirmations must be followed by the appropriate reference ("SOCS… pseudo-JAK substrate", "three of these molecules… regulatory function.", "Its abundance…in *Drosophila*."…
- The section on AMPs is nice
-What is known about the expression of your genes of interest in transcriptomes made in sand flies?

Part II
- After the description of the RNAi pathway it is clearly missing more methodological explanations on the use of siRNA for doing knock downs in different organisms, especially insects, and a few comments on the limitations of the techniques. Can you comment on the mode of administration of the siRNA (injection? transfection? genetic integration?...). What is the duration of the silencing?
-The part on CRISPR/Cas9 also misses some technical details (at least a bit) on insect/sand fly genome editing: specificities about the administration by egg injection, mosaicism, isolation of mutations by crossing etc…You could even add a figure here.
- Page 21:"It ought to be mentioned that relish was the only successful gene knockout out of more than 5 targets they aimed": actually it was not 5 different targets but 5 series of injections for the same target.

Results: publications
-The main part of the result section of this PhD thesis is composed of 4 publications, describing the investigation of the mRNA expression level of different genes of interest linked to insect immunity and their role in sand flies. The work is well articulated.

Results: CRISPR
-This part presents the efforts made by the student to perform a knock-out of the gene Caspar in *P.papatasi*, using a CRISPR/Cas9 method. The attempts were unsuccessful, but the approach is correctly detailed.
-Page 8, the data about egg hatching, larval and adult survival rates should be presented as a figure instead of simply enumerated.
-On Figure 10, the sequencing plot in panel A is really not convincing. In panel B, F and G, we can clearly see a A/G heterozygote nucleotide, which makes me think that both alleles could actually be present in the initial population. Did you check several wt flies for this position?
-Page 11: "none of the eggs laid by the five G0 females hatched, which made us think that the reduction/inhibition of caspar may lead to lethality of offspring. Unfortunately, this hypothesis could not be verified": this is interesting! Maybe it actually worked then?
-You seem to have issue with the DNA isolation from the females post egg-laying (degraded DNA, fungal contamination): how was it done?
-Page 21: "We were searching for gene-segment cut offs similar to those obtained in relish-mutant flies which had size around 300bp (Louradour et al., 2019)": I am not sure that you should expect this in your case. Deletions could be longer/shorter depending on the distance between your predicted cleavage sites. How far away from each other are your sgRNAs?

Summary and conclusion
-As mentioned before, I think it would be nice to discuss a bit the limitations of looking only at mRNA levels. Otherwise the discussion is great.
-Are there potential fixation sites of Rel or Stat in the promotor region of the attacin gene?
-Could you consider alternate methods of genome editing?
-P107: "We used RNAi-mediated gene silencing of relish in carcasses and guts and follow the consequent expression of AMPs": I am not sure I understand: you did the same injection of dsRNA in the abdomen and then checked if the silencing was effective in the gut and the rest of the carcasse? Is it what you mean?
-P109: "Nitric oxide has antileishmanial activity and <u>therefore</u> parasites developed mechanism how to supress iNOS expression in infected macrophages in 33 vertebrate hosts (Orsini et al., 2016).": this is a very finalist sentence.

<u>Final evaluation:</u>
Given the different elements explained before, I, Isabelle Louradour, recommend the thesis for the defence. I declare that I completed this report on 24/05/2024, in Paris, France.