BIOLOGY CENTRE CAS
Institute of Parasitology
address: Branišovská 1160/31, 370 05 České Budějovice, Czech Republic
IBAN – CZ24 0800 0000 0000 0606 3942 | SWIFT CODE – GIBACZPX | VAT No.: CZ60077344
phone: +420 387 775 403 | fax: +420 385 310 388 | www.paru.cas.cz | e-mail: paru@paru.cas.cz

Evaluation of Barbora Vomáčková Kykalová's doctoral thesis entitled "Identification and functional studies of sand´s fly immune-related genes".

First of all, I would like to congratulate the student on successfully completing her doctoral thesis and submitting it at a time in her life when thoughts other than those of science are at the forefront of her mind. It is encouraging to have these successful cases as examples for other students (male or female) who are concerned with starting families and balancing work and family life.

To summarize the form of the thesis, it contains a nice introduction to the project, followed by four already accepted and therefore peer-reviewed research papers, a supplementary chapter describing efforts to introduce the CRISPR/Cas9 method in sand flies, and it concludes with a brief summary of all results.

In her experiments, the student worked with two sand fly species (*L. longipalpis* and *P. papatasi*) that were exposed to different challenges such as *Leishmania* infections, bacteria-rich food and pathogen-associated molecular patterns (PAMPs), followed by qPCR to detect changes in the expression of a small number of genes involved in the sand fly immune response to pathogens. In a further series of experiments, she investigated whether RNAi silencing of some of these selected genes would have an effect on infection with Leishmania parasites in the gut. It is important to appreciate how much work is involved in preparing the samples for a particular qPCR. This includes the care of the sandflies, their feeding, infection experiments, injection of dsRNA, treatment with antibiotics and dissection at short intervals. Not to mention the injection of thousands of eggs to create the CRISPR/Cas9 mutant.

In formal terms, the dissertation contains all the necessary requirements, and I have no objections to it.

From my personal perspective, I would welcome a broader view on the topic of pathogen-host interactions with defined knowledge gaps in the field and how further scientific knowledge will lead to new strategies in the fight against vector-borne diseases. A chapter on these new approaches and their implementation would also be very edifying. I would also have welcomed a preface before each chapter summarizing the rationale for the experiments, stating the hypotheses, and briefly summarizing whether or not the results obtained support the hypothesis. In the context of the

conclusions, which focused on the re-stating of the results, I would again welcome a more comprehensive presentation of the data obtained. I realize that this was not easy due to the somewhat limited methodological approach, where the qPCR method was only used for a relatively small group of genes, and therefore it was probably not possible to draw great conclusions. However, considering how much work went into obtaining the individual RNA samples, I find it rather unfortunate that the samples were not used more broadly to analyze a larger number of genes, e.g. using the BioMark High-throughput qPCR system (Fluidigm). Does the student think this method could be useful for her research? What other genes would she like to investigate? What other methods could be considered for deeper mapping of host-parasite interactions?

The work is mainly concerned with the investigation of three main signaling pathways, namely Toll, IMD and Jak-STAT. Their activation leads to increased transcription of e.g. antimicrobial peptides (AMPs). The student focused on defensin 1, 2 and attacin. Are there other AMPs? Why these?
Are another genes under the control of the transcription factors Dorsal and Relish known from other model insects?
The student has shown very nicely that the expression of defensin 1 is only increased in the gut after activation by the pathogen, while defensin 2 is expressed in the rest of the body (carcasses). How similar are these defensins? Where are they located in the genome and are their promoters known? What molecular mechanism could be responsible for this tissue-specific expression?

I was fascinated by the work in which the RNAi mutants were produced. I appreciate the scope of the work and the precise planning and timing to make the experiment work. I wonder how large (in bp) the dsRNA was that was injected into the sand fly? And how were the qPCR primers designed to check for successful silencing? If I imagine that the gene for defensin is quite small, it is difficult to design the region for the dsRNA and then for the qPCR analysis so that they do not overlap and give false negative results. How was this handled? Would it be possible to already directly inject siRNAs for a specific gene?

When I read a review on the topic of the thesis, I was intrigued by the concept of immune priming in mosquitoes. Has anything similar been demonstrated in sand flies in relation to Leishmania infections?

In the chapter dealing with the CRIPSR/Cas9 approach, it occurred to me that you are actually relying on the non-homologous end joining repair mechanism because you have not designed a template for homologous recombination. I was even more surprised that you expect deletions of up to 300 bp. My question is whether the NHEJ pathway is well described in sand flies, whether it is active and whether there is a way to activate it and thus increase the probability of success of genetic modification. And if you have not thought of injecting a homologous template and thus

activating homologous recombination, which could be more efficient in repairing ds-DNA breaks. Would there be a possibility of an antibiotic selection of the mutant and/or the introduction of a marker gene? And if so, which one?

The third publication deals with the effect of LPG and LPS on changes in the expression not only of immune genes, but also, for example, of gut surface proteins (galectin and mucin) and digestive enzymes trypsin and chymotrypsin. What role do these genes play in the gut of the infected sand fly? Did you expect an increased expression of these genes? And why? Are they involved in controlling the parasite load?

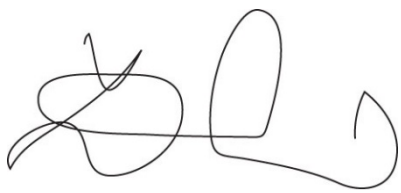
The student often writes that her studies could identify a promising molecular target for parasite control strategies? This is certainly true, but how exactly does she envision this. For example, if defensin were the promising molecule, what would a strategy that would lead to a decrease in leishmaniasis prevalence look like?

What do the students think about the release of genetically modified insect vectors? What effects can this have on the ecosystem? Have such experiments already been carried out and with what results?

Insecticide-treated bed nets are used to combat malaria in order to prevent the transmission of Plasmodium. Would something like this also work for Leishmania? For example, insecticide-treated clothing?

Last but not least, the Leishmania must also exploit the physiological environment of the blood-fed sand fly to develop and become transmissible. Is anything known about this "positive" side of the host-pathogen interactions?

I would like to note that all my questions have arisen out of pure curiosity and fascination for the system, for the student's excellent results and for the work itself. I look forward to the discussion. In my opinion, this dissertation meets the requirements for the doctoral degree and I recommend the work for defense.



In České Budějovice, 29.5.2024

doc. RNDr. Alena Panicucci Zíková, PhD