**Report on doctoral thesis**

Student: **Balraj Melepat**

Thesis: **Neuro-immune effects of peripheral inflammation in avian models**

The reviewed thesis focused on the area of evolutionary and ecological immunology is aimed to investigate the innate immune response of birds during inflammation, which is one of the main scientific topics of the team of prof. Vinkler. The thesis is composed of 6 papers, four of them published, one submitted and one presented in the form of manuscript draft. Mr. Melepat is the first author for one published paper, one submitted manuscript and one manuscript draft. He is coauthor of three papers published in scientific journals. Regarding the thesis, it is composed of comprehensive review paper focused on virus-sensing innate immune receptors in vertebrates (Mr. Melepat as the first author of the paper), and several experimental studies investigating the effects of viral or bacterial inflammation on host immune response of three selected species, the representatives of Passeriformes and Psittaciformes. In brief, the most significant objectives related to the thesis are 1) to reveal/suggest the molecular immunity-associated mechanisms that lead the neuroinflammation following peripheral inflammation, and (2) to reveal the potential immunity-related mechanisms of the bird host adaptation to bacterial infection and host-pathogen evolutionary responses.

I really appreciate the high scientific quality of published papers attached to the thesis clearly approving the scientific potential of Mr. Melepat. The published papers of Mr. Melepat and his coworkers document his broad knowledge of the scientific literature related to the topic of the presented thesis, his practical experiences in a molecular laboratory regarding especially qPCR analyses and his capacity to interpret the obtain results from the host immunology perspective.

However, the overall quality of thesis presentation is rather moderate indicating a lack of time for the finalization of doctoral thesis. I include the selected comments below. These comments are mostly relevant to the general parts of the thesis and manuscript draft. For some papers (specifically, paper submitted to Veterinary Research and the manuscript draft) I noticed that the discussion is rather descriptive in comparison with other papers and is limited regarding the evolutionary implications of the study.

**Specific comments**

**General parts of the thesis**

Some parts corresponding to the general chapters, specifically, general introduction, general methods and general results and discussion are not easily to be interpreted without careful reading of all published or submitted papers (and supplementary materials related to these papers). The references to 6 papers or manuscripts may help to readers with orientation in various studies briefly described in general chapters. Model organisms, sampling design and

experiments should be described separately for each study instead of combining the experiment design of all studies in the same text.

Some of the figures are not referred in the text, or the reference numbers of some figures in the text are incorrect (the numbers are likely copied from published papers). In general chapters, there are also some references to the figures and supplementary material, which are related to the published papers, however, this fact is not specified in the text. Some of the figures are invisible (small size and resolution) in contrast to all photographs.

Some sentences included in general parts are not scientifically correct, e.g., first sentence page 8 – population (ecological unit) and order or family (taxonomic units) are incorrectly applied.

Regarding the extensive list of papers being a part of the thesis, I would like to ask Mr. Melepat about his personal professional contribution to transcriptomic and proteomic studies (specifically, papers 3, 4 and 5) and his participation in the experimental studies, i.e., sampling which was performed in collaborating institutions in USA (paper 5 and draft 6) or Animal Facilities of Faculty of Science, Charles University (papers 2, 3 and 4).

**Paper 2 (submitted to Veterinary Research).** The study is focused on the analyses of target immune genes related to inflammatory response in parrots using qPCR. As the conclusion suggests that future research to analyze the effects of the poly(I:C) stimulation in parrots should apply transcriptomic approach, I would like to ask why this study was not started from transcriptomic analysis.

Based on the supplementary material to this paper, I have small reservation to sampling design applied. I guess that for some specific reason it was not easy to obtain the individuals with the same origin, however, the experimental individuals of four different origins, different coloration pattern (likely playing no role in this study) and also different sex of specimens (females and males being not equally distributed between the treatments and between sampling times 3, 6 and 24 hours) were used. A relatively small and heterogenous sampling also means that the interaction sex-treatment could not be included in the statistical models.

What is your explanation for the highest variability of the expression for all genes analyzed in the control group at the sampling point 24 hours? I am afraid that it could be generated by highly heterogeneous individuals in the experiment.

Results. I would recommend to specify the number of significant correlations between the expressions of different pairs of the genes in different tissues. I also suggest to avoid the multiple repetition of the same information regarding correlations (text, figure 1 and table S10).

Figure 3 and corresponding text – I can guess what the author wanted to say. However, the sentences are not clearly formulated. For example: Line 229: how do you know that IL1B

response to high poly(I:C) started at 3 hours? You measured IL1B expression at 3, 6 and 24 hours, but you cannot exclude the possibility that IL1B response started before 3 hours. Line 230: IL1B response late decreased to a non-significant difference. To my opinion, IL1B response cannot decrease to any significant difference between the expressions of a gene measured in two treatments.

The significant differences within time points and treatment were tested statistically, however, without deep control in supplementary material, the graphical representation does not permit to recognize whether the difference was found between control and low poly(I:C), or control and high poly(I:C), or between control and both poly(I:C) or between low and high poly(I:C).

Figure 2. Treatment groups in three sampling time points are plotted on the x-axis. Control in Figure 2 is in green and not in blue. These mistakes should be corrected when resubmitting the manuscript.

**Paper 3.** Figure 3 - differential protein expression analysis in plasma. Is there any explanation for the similarity between samples C_C_1 (control) and DSS__LPS_6 (double treatment)? The same question for Figure 5 (differential protein expression in cerebrospinal fluid) - sample DSS_1 seems to be similar to the samples LPS and DSS_LPS treatments? As only 3 DSS samples were included in the analyses and one seems to be outlier, it is not obvious to interpret this heatmap correctly. In contrast, one LPS and one DSS_LPS has the expression pattern more similar to control, however, here the majority of samples within treatment showed similar pattern of protein expression.

**Paper 4**. This is very nice study investigating the associations between peripheral and brain inflammation linked to bacterial LPS. I have a methodological comment. For future studies it is very positive information that less costly QuantSeq with single-end reads is applicable to transcriptomics of immune gene profiles, however, the incompatibility between RNAseq and qPCR outputs is unfavorable information as many researches still apply high costly RNAseq with paired-end reads. Do you have some idea (or your colleagues) how to explain the discrepancy of the results between these two sequencing approaches? Did you observe the discrepancy between RNAseq and QuantSeq indicated in Figure 5 also for other four immune genes for which the expression pattern was validated by qPCR? The question just for curiosity: why did you apply the sequencing using short 80 bp reads?

**Paper 5** published in Frontiers in Immunology 2024, 15: 1250818. This is very interesting study indicating immune adaptation of house finch populations to a rapidly evolving bacterial pathogen. However, I was surprised by methodological approach. The transcriptomic study in paper 5, i.e., overexpression or underexpression of the selected gene set was not validated by qPCR, this validation is considered as a common approach and is currently also required by Frontiers in Immunology. Surprisingly, the same data set was used for **paper 6** quantifying the selected genes encoding the inflammatory cytokines and signaling modulators with the following justification: “the sixth paper extended our previous work presented in the fifth

paper, by performing RT-qPCR analyses to gain a deeper understanding of the gene expression patterns identified as differentially expressed in our transcriptomics study." Could you please, provide the explanation?

**Paper 5**. House finch were captured in nature and tested as negative for Mycoplasma gallisepticum infection. Could you exclude the possibility that the specimens in this study were not infected by other pathogens? This question is because of some outliers in heatmap of gene expression. Surprisingly the same figure is shown in general part of the thesis (Figure 11, page 22) using the clustering, and in this tree, the control samples are included in one of two main clusters together with some infected samples (infected by the old and more recent MG strains) whilst two other control samples clustered with infected ones.

**Paper 6**. I suppose this is a working version and the manuscript draft will be improved prior to submission. Again, the presentation of correlations should avoid the repetition in the text, Figure 1 and Table 1. I can see no red color or ellipse titled to the left that should indicate negative correlation in Figure 1. This figure should be excluded from the draft, and insignificant correlations should be also excluded from the Table 1 to simplify the orientation. However, this table suggests the similar problem for QuantSeq as shown for RNAseq in paper 5, specifically, the qPCR validation for BCL10 expression revealed by QuantSeq was not successful, which means this gene should be omitted from the manuscript.

Final question: What are the next perspectives regarding the research of the inflammatory response investigation in the three target bird species or other bird species? Do Mr. Melepat plan to start his own research built on the knowledge obtained from his doctoral study?

To conclude, the thesis of Mr. Melepat fulfills all requirements necessary for doctoral theses. I appreciate the quality and quantity of scientific work given by Mr. Melepat, and I have a pleasure to recommend his thesis for the defense.

September 9, 2024

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