May 21, 2018

Evaluation of Master Thesis: Gabriela Krejčová

This thesis has clearly been written by an accomplished student, and it was my great pleasure to review it. I regret not being able to participate at the defense in person. Herewith please find my general comments and specific questions which I hope will get answered at the defense and perhaps in writing to my e-mail address (jindra@entu.cas.cz).

The thesis is well organized and complete with all its sections fulfilling their purpose. It is particularly notable that the Discussion does not repeat the Results, as is typical of student writing, but rather critically weighs the significance and limitations of the data. The thesis starts with 20 pages of an Introduction, rather an overview. I am impressed that Gabriela has attempted a synthesis of such a huge field of study ranging from metabolism and immunity to disease. While this Introduction aims to provide a broad background, it reads in some parts like a 'laundry list', itemizing individual processes without explaining their actual relevance to the project. This makes it a bit hard to follow. A clearer distinction whether a given description applies to mammals, insects, or both would also be helpful.

I commend Gabriela for writing her thesis in English. The logical composition of the text which flows smoothly from hypothesis to experimental rationale to data interpretation clearly shows solid understanding of the subject and good thinking. Of course, the English itself is not quite there yet – but soon could be. Frequent mistakes ("s" in plural, no "s" in third person, wrong form of adjective, wrong tense, articles) and 'czechisms' (reversed subject-object word order, comma before "that" or "whether") constantly reappear to compromise the readability of the text. I estimate it would only take one afternoon to remove these glitches from Gabriela's writing. Overall, her style is quite mature and rich in vocabulary.

The thesis presents an impressive amount and quality of experimental work; its main asset being its completeness. Gabriela exploits the *Drosophila* model and FACS sorting to analyze glucose levels and gene expression in the hemocytes (*Drosophila* macrophages) of infected control or genetically manipulated flies. She systematically follows through the experiments to test her hypotheses and finally arrives to a conclusive model, which can be summarized as follows:

Bacterial infection induces the hemocytes of adult flies to reprogram their metabolism towards aerobic glycolysis. This metabolic switch depends on increased availability of glucose to the hemocytes and its purpose is to mobilize quick energy to mount an acute immune response. The hemocytes uptake glucose and transiently upregulate expression of glycolytic enzymes while TCA enzymes decline later on. Gabriela's data suggest that the Hypoxia inducible factor (HIF) signaling pathway is a critical driver of the switch. Acting in the hemocytes, HIF activates expression of a secreted insulin decoy ImpL2, thus securing extra glucose for the hemocytes. HIF also induces at least some of the glycolytic enzyme genes such as lactate dehydrogenase.

Summary:
In my opinion this is an excellent thesis, borderline between masters and Ph.D. levels. Based on its written form, I strongly believe and recommend that this thesis be graded "excellent" (1).

Because the work is so good, I can perhaps raise a few critical questions and comments:

1. Increased uptake of labeled **deoxyglucose** is not obvious from Fig. 13. Given the strong background, panels B (control) and D (infected) are not well comparable. The uptake should have been compared at higher magnification as shown in Figures 14 and 15. Unfortunately, those two figures fail to show the situation in uninfected flies, which is a problem. What are the 2-NBDG positive structures in Fig. 15? Normal glucose would probably be metabolized but where do you expect the 2-NBDG homolog to end up?
2. Similarly, **uninfected controls** should have been included in Figures 16 and 17; in fact these do not even state whether or not the flies were challenged with bacteria. Even though the author explains that there was no appreciable increase in the LDH::mCherry signal upon infection (signal was already high to begin with), showing these controls is mandatory.
3. Looking at Figures 13-17 I could not stop thinking whether **hemocyte cell numbers** might have increased upon infection. And if so, how would the glucose level or mRNA expression data change if normalized to cell numbers. It is not until in the Discussion (p.62) that the author explains that hemocytes are born in embryos and larvae and thus their numbers in adults should be constant – a notion that she immediately challenges by referring to hematopoietic hubs (Ghosh et al., 2015). Hemocyte numbers should have been addressed in the Results, particularly as Fig. 13 might indicate an increase.
4. Can the author suggest an experiment to test for hemocyte proliferation directly?
5. Most of the work has been done on adult **males**, for a good reason of excluding some immunity and metabolism related processes such as mating or oogenesis. Why then was the in situ X-gal staining performed in **females**? – Fig. 18. Moreover, the non-infected female in Fig. 18B has a much darker eye than the infected fly. This could either mean **different genotypes or different age**. Was the infected female heterozygous and the control homozygous for the HRE-lacZ transgene? – or was the control older? The second possibility would be particularly problematic as hemocyte number and function are said to decline with age (p.62). Finally, it would be good to perform double labeling of beta-galactosidase with Hml>Gfp to make sure these cells are indeed hemocytes.
6. The author seems to be concerned that the transient upregulation of several glycolytic enzyme genes is not accompanied by concomitant **downregulation of TCA genes** (which occurs later and to a minor extent). I would not worry too much about that. The TCA activity is fueled by pyruvate, and if all glucose ends up converted to lactate in aerobic glycolysis, the TCA and OxPhos will likely slow down.
7. A more interesting question is which of the glycolysis genes upregulated upon infection contain **HREs**. The RNAi data show that GAPDH1 is induced by infection independently

of HIF1a (Fig. 19D). Does GAPDH1 lack a HRE? And do the HIF-dependent genes have it? Only on p.68 the author mentions that LDH, Eno, and ImpL2 genes contain HREs.

8. The author states on p.54 that "both LDH and Sima are essential for proper immune response to pathogenic bacteria" based on short survival times of ImpL3 (LDH) and Sima (HIF1a) RNAi adults challenged with bacterial infection (Figures 23 and 24). I disagree with this conclusion because Figures 23 and 24 fail to show survival of uninfected RNAi and control flies. It is possible that knockdown of either LDH or Sima/HIF1a alone compromises lifespan even in the absence of bacterial infection – after all both are essential genes. Does the author know the fate of the RNAi hemocytes? Technically speaking these experiments only show dependence of survival of infected flies on LDH and Sima, but fail to causally link the reduced survival with the infection. Consequently the author gets into trouble on p.57 (top paragraph) when trying to ascribe the slight increase in CFU to loss of LDH and Sima. If they had the missing RNAi data for uninfected controls, perhaps they could better argue that it is indeed the weakened antibacterial defense that kills the flies whose hemocytes lack LDH or Sima. By the way, the flies probably do not experience a 'faster death' but an early death; they die earlier than controls.
9. There are many ways to improve readability. For example, the nomenclature of genes could be unified (ImpL3 vs. LDH, HIF1a vs. Sima, etc), actual time points (rather than T1, T2, T3) given in text and figures, and abbreviated descriptions of the actual genotypes provided rather than references to a particular cross in the Methods.

The above comments are not meant to criticize the author. They aim to illustrate how a reviewer might search for weaknesses in the work if submitted to a journal. I hope that they might help in future preparation of the manuscript. Meanwhile, I would like to congratulate Gabriela to her great achievement.

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Evaluation of the the master thesis of Gabriela Krejcova

In her thesis, Gabriela Krejcova aimed to elucidate the metabolic characteristics of the adult Drosophila immune cells under the bacterial infection challenge and she also tried to reveal some of the molecular mechanisms behind the observed metabolic changes. She performed mRNA analysis of key genes involved in glycolysis and the TCA cycle as well as some of their putative regulators, she monitored metabolic parameters *in vivo* using lactate dehydrogenase reporter and the uptake of fluorescent glucose and she assessed the survival of infected flies. Her data suggest the role of Hif-1 as the master regulator of immune response, securing high glucose levels for immune cells via the secretion of Impl2 and increasing immune cells glucose metabolism via upregulation of lactate dehydrogenase (Impl3) and other enzymes of glycolysis. As Grabriela showed, the lack of Hif-1 as well as lack of Impl3 significantly impairs the fly survival after the bacterial infection. These data are new and important, not only in the fly model but also in the mammalian context.

The master thesis contains nearly 100 pages and it is very well written, to my opinion exceeding the standard in our faculty. The introduction occupying 40 pages comprehensively summarizes the current literature and questions in the field, the methods are detailed and figures properly described, leaving no space for me as the referee to pick any formal mistakes. The results are solid, mostly based on large amount of data and biological replicates. The asset of the thesis is however the discussion that clearly shows that Gabriela is able to critically analyze the outcomes of her experiment, set them in a wider context and importantly, suggest future experiments that may deal with some of the unanswered questions. In fact, majority of the questions I wrote down as I was reading the thesis were already raised in the discussion. Nevertheless, a few of them remained and Gabriela can discuss some of them during her defence:

1. Gabriela's results suggest that the plasmatocytes in the adult fly resemble M1 macrophages in vertebrates, based on their metabolic characteristics. Is there any evidence for M2 type in the fly? Not all Hml>GFP cells express Ldh based on the data in

Fig.16 and not all the Ldh expressing cells are Hml ositive. Could these subpopulations be FACS sorted and their expression characterized?

2. In Fig. 15 the 2-NBDG signal accumulates within large vesicles inside the cell and not uniformly in the cytoplasm, as one could expect. Do you have any idea what these structures are?

3. Although the sima-RNAi and Impl3-RNAi flies die sooner after the infection, they do not seem to have massively more living bacteria present in their bodies (Fig. 25, 26). Despite this observation Gabriela states twice in her thesis that 'we still believe the the immune cells exhibit impaired phahocytosis and they are dying due to deteriorated ability of bacterial killing'. What is the basis of this claim and what could be an alternative explanation to these observations?

4. A crucial question is raised in the discussion: 'What leads the immune cells to increase the glycolytic pathway, could they be limited by oxygen?'. My question is - could it be the proliferating / quickly metabolizing bacteria that take away the oxygen from the fly? Woudl Hif-1 based response be still triggered if you inject heat inactivated dead bacteria, instead of alive ones? Would Hif-1 respone be triggered after wasp infection of larvae?

5. As Gabriela points out, maybe the cells just die at the later stages of infection and new cells are not formed (as current model suggests). Did anybody performed repeated fly infections on flies that survived previous milder infection? Would you expect better or worse response? What would happen if you block cell death by expressing Hem-Gal4, UAS-p35 or if you block cell proliferation by Hem-Gal4, myc-RNAi (or some other cell cycle/proliferation promoting gene)?

The thesis is of outstanding quality and I do not hesitate to evaluate it as 'excellent'.

Ales Kreji