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Opponent's report of the Masters thesis of Bc. Tereza Dolejšková entitled, "The role of Gluconate shunt in the immunity of *Drosophila melanogaster*.

The candidate's host group has a long-held research interest into the metabolic regulation of immune response, utilising the powerful molecular genetics afforded by the fruit fly model. The work is an extension on how crucial metabolic reserves are mobilised in response to immune challenge; in this case using the established model of parasitic wasp infection of fruit fly larvae. Specifically, how (or indeed if) elevated levels of mobilised glucose in the haemolymph can enter the pentose phosphate pathway, to produce the necessary intra-cellular electro-chemical reducing power (in the form of generated NADPH) required to sustain an effective immune response (*e.g.* including the sufficient/appropriate differentiation of lamellocytes that encapsulate in injected wasp egg), via a hypothesised insect specific extended "Gluconate shunt" pathway (rather than the more classical entry point, observed in other species, provided by the enzyme Gluconokinase, absent in insects). Thus, the thesis describes a host of metabolic tracing experiments (with and without wasp infection) of ^{13}C-labelled glucose, via a mass-spectrometry output, that confirms conversion of glucose to gluconate and further metabolites that ultimately lead to ribose-5-phosphate as an entry point to the PPP (which can be potentially cyclically metabolised to maximise NADPH generation); moreover that such metabolites are elevated after wasp infection. Collectively, these data (and other data describing the labelling of other related metabolites) provide strong evidence of the existence of such a hypothesised extended Gluconate shunt pathway. Moreover, the thesis describes the identification of candidate enzymes (based on homology to other species) that participate in such an extended Gluconate shunt pathway; settling on Regucalcin/Rgn, Had2 and RBKS (plus an unknown but required decarboxylase), the mRNA expression of which are all elevated after was infection. The functional effect of RNAi direct knock-down of these candidates (specifically in the hematopoietic lineage) was assessed against wildtype flies, using the same parasitic wasp infection assay (assaying lamellocyte production and fly infection resistance/survival rates). The results confirmed Rgn and Had2 knockdown resulted in the formation of less lamellocytes, whereas only Had2 knockdown reduced fly resistance/survival rates. The data and interpretations are of high quality and a number of related questions will follow below.

Overall the thesis follows a traditional format and is an extremely high-quality written piece of work at the Masters research degree level. Moreover, the central hypothesis and project aims are unambiguous and well-articulated. The writing is clear and concise and guides the reader through what are conceptually difficult to understand and unavoidably complicated metabolic pathways. This is particularly evident in the well-constructed Introduction and Discussion sections; the latter being especially noted for the fact the candidate has successfully assimilated her own data within the wider context (avoiding all too often encountered traps of repeating the results) – *i.e.* she has used the Discussion to actually discuss the results! Regarding the presentation of the Results themselves, the same concise style is in evidence and appreciated. The figures are of high quality and easy to interpret. This reviewer enjoyed reading the thesis and certainly learnt something useful and can appreciate the significance of the results and findings, that are undeniably of publication quality. The only slight criticism one could make is that in places the text is sometimes written in an overly familiar and colloquial style. For example, on page 46 of the Discussion, the candidate lists as bullet points, the evidence derived from her data for the existence of the "gluconate-ribose" pathway in *Drosophila* (itself a good innovation), with the introductory sentence "Let's summarise the arguments for the existence of the "gluconate-ribose" pathway"; perhaps it would have been more appropriate to alternatively state, "The evidence in support of the existence of the "gluconate-ribose" pathway" are summarised below. However, this is really a minor point. Equally a few times in the text the word Drosophila was not italicised and the word "through"

was repeatedly misspelt as “trough”; again these are nit-picking points!

To summarise, the thesis is high quality and fulfils, or even exceeds, the requirements to be favourably evaluated by the defence committee. Moreover, one is tempted to consider the thesis content on the level of Ph.D. rather than Masters degree. One commends the thesis favourably to the evaluating committee.

Questions:

1) Page 1: Introduction. Could the candidate please expand their statement regarding the evolutionary origin of the PPP being shaped by the general chemical origin of the Archean oceans? This reviewer found this statement rather vague and was not sure what point the candidate was trying to make.
2) Could the candidate speculate/expand further their understanding for the possible loss of the Gluconokinase gene/activity in Drosophila (and it seems insects in general) as an entry point for the Gluconate shunt to the PPP?
3) Relating to the metabolite tracing experiments, +/- parasitic wasp infection, using ^{13}C-glucose, whilst it seems metabolites of the extended Gluconate shunt are elevated after infection (*i.e.* gluconate, dehydrogluconate, ribose) are elevated, the amount of labelled metabolite remains constant (albeit appreciable; confirming its origin in the originally labelled glucose). One may have expected this labelled population to also increase. How does the candidate explain this result? Is it not evidence that the increased/excess gluconate and further derivatives are being sourced from reservoirs other than glucose?
4) Relating to the discrepancy in the abundance of the partially labelled $^{13}C_5$, and sequentially related, ribulose -5-phosphate and ribose-5-phosphate metabolites (that one would assume would be equal – as for the other partially labelled variants of the same metabolites) and the fact there was a greater abundance of the $^{13}C_5$ labelled product (*i.e.* ribose-5-phosphate > ribulose-5-phosphate), could the candidate provide a reasoned/expanded explanation?
5) How was the rp49 gene selected as a normalising/ house-keeping control transcript for the QRTPCR analyses confirming candidate Extended Gluconate shunt enzyme/gene RNAi mediated knockdown?
6) Regarding the functional wasp infection assays in the candidate enzyme RNAi knockdown experiments, could the student comment/expand why they think the knockdown of only Had2 produced both reduced lamellocyte and impaired resistance/survival rates compared to the theorised knockdown of enzymes in the preceding (Rgn) and succeeding (RBKS) steps of the Extended Gluconate shunt? Could this be related to the degree of observed/confirmed transcript knockdown (of which Had2 RNAi was most robust – Fig. 19)? Alternatively, if it is related to protein/enzyme half-live/turnover (or the enzymatic kinetics), how might this be experimentally addressed in future? Is it possible, the enzymatic activity of Had2 represents a crucial rate-limiting bottle neck? Is there any evidence for other enzymes with redundant function that may explain the comparatively lesser (Rgn) or absent (RBKS) phenotypes observed?

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