Summary:

Monika Zuberova's dissertation presents a strong body of work that has already resulted in the publication in a good journal (Disease Models and Mechanisms. Impact factor 4.5). I am extremely familiar with this paper as I was reviewer number 1 and felt that it was a thoughtful and important addition to the literature.

In this dissertation, Zuberova shows that circulating adenosine levels in Drosophila larval hemolymph regulate the homeostasis of energy stores. High levels of adenosine, as might be found in a stressed or infected animal, lead to rapid depletion of glycogen and are ultimately fatal. This study is useful because it provides a simple model system to monitor adenosine signaling. Adenosine is an important signaling molecule in humans and is implicated in a variety of pathological conditions. I think that this is an important new model and helps move the fly from being a tool for studying mostly developmental biology to a physiological tool.

Zuberova also performed a genetic screen to identify genes that might be implicated in adenosine signaling in the fly. The screen searched for mutations that could suppress a lethal phenotype of an adenosine deaminase mutant. Five loci were identified and one in particular, phosphorylase kinase, suggests a reasonable story that can explain the phenotype in terms of changes in energy stores. The work was complicated by the sensitivity of the phenotype to genetic background and environmental conditions like food quality. My experience is that this is a common problem but the literature doesn't show that these problems exist, perhaps because we tend to publish only those experiments that are highly reproducible. I have grown wary of papers in which the authors do not take care to isogenize their stocks or tightly control environmental conditions because we find that all phenotypes are dependent upon these variables.

I strongly support Monica Zubeerova's work in pursuit of her Ph.D.

Questions:

I would like to see more detail at all points in the thesis. When something is stated in a general way, it should then be backed up with specific details. Examples are the following: "During hypoxia, it is released from various tissues (what tissues?)" "Adenosine modifies hormone release (Nyce, 1999) (which hormones?)" "The concentration of extracellular adenosine is normally maintained at very low levels. (What are these levels in humans, flies and plants?)" These are only examples; it would be useful if you could add details whenever you find yourself writing something general.

Adenosine is said to be "homeostatic" but isn't every signaling molecule homeostatic? Both hormones that induce and reverse a reaction are required to stabilize a system. Immune effectors that kill microbes are homeostatic in that they reduce microbe numbers back to normal and the negative regulators of immune effectors are homeostatic because they return effector levels back to normal. Please define the word and how you mean to use it here.

I'm curious about the statistical power of the screen and the choices that were made in determining the number of larvae that would be tested. What sorts of changes in survival could be seen with the number of larvae tested and would the screen have been more effective if you changed the number of larvae tested?

"From the beginning of my work with the *adgf-a mutants*, I had problems with bacterial contamination in the vial. Sometimes food in vials was completely overgrown by bacteria which interfered with larval development." This is strange and suggests that the eggs were contaminated with something. I would like to know the identity of the microbes found on the eggs – how might that be determined? Does this only happen with the mutant or does it happen more often with the mutant? Is the mutant more sensitive to the bacterial growth or do the bacteria only grow on the mutant. Why does bacterial growth cause trouble? In the literature, are there other flies that are sensitive to native microbiota? Why are Toll and imd mutants viable if they are so critical to fighting infections but your mutant succumbs to bacterial growth? This said, there is a possible explanation for the microbial overgrowth that is caused by your choice of carbon source. Sucrose in fly food can lead to the growth of slimy capsule forming bacteria. This can be eliminated by feeding the flies glucose-based food. Apparently fructose is required for the capsule production.

"Of course, it is impossible to exactly determine the larval size and thus to collect the larvae of exactly the same physiological age." Why is this impossible? Can you suggest some methods that could be used to quantitate larval size?

"**1)** it could indicate that larvae eat more or **2)** that they have more effective absorption of fructose in the gut or **3)** that they have less effective uptake of fructose from hemolymph into target tissues (see Fig. S12)." This is testable. How would you measure the eating rate in larvae? How might you measure fructose movement through the larva?

"(I am not sure, whether only glucose or also glucose-(1)6-phosphate are measured by the GAGO kit. By the mass spectrometry, only glucose was measured.)" You should find the answer to this question.

"Fig. 7 shows results from my previous measurement of glycogen reserves on CM diet, together with the new measurement on yeast diets: The *adgf-a* pupae of genetic background 1 (red line) consumed their glycogen reserves more quickly in comparison with *wt* (blue line)" Please be quantitative – what are the slopes of these lines and do they differ by ANCOVA?

"Ad 1) Feeding larvae are robots for eating, *wt* larvae probably eat as much as possible, thus it seems impossible that *adgf-a mutants* could increase their eating." What could be done to test this idea that larvae eat at their maximum rate all day long? If feeding is a homeostatically regulated phenomenon, why might it be bad for the larvae to eat at their maximal rate at all times?

"However, it is probably caused by the fact that there might had been more living (breathing) animals in the vial than I thought." Is the respiration rate of a recently dead larva different from that of a live larva? Why might this be different from mice and humans?

"This supports the possibility, that excessive load of nitrogen excretion pathway (uric acid synthesis) may interfere with impaired adenosine deamination and that the *adgf-a* larvae die in the time when they accumulate too much of these waste products from yeast's proteins or purines degradation." How could this idea be tested?

How does uric acid excretion differ in humans compared to mice and flies?

Knowing now that glycogen regulation is important, are there other mutants you could test to support this hypothesis?

Stanford University, January 4th, 2012

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Cologne, 03. 01. 2012

Evaluation of Ph.D. thesis by

Mgr. Monika Zuberova

entitled

"The role of adenosine signaling pathway in regulation of metabolic reserves"

The Ph.D. thesis submitted by Monika Zuberova focuses on the role of adenosine signaling in regulation of metabolic and energy homeostasis. She utilized the previously established adenosine deaminase deficiency model of *Drosophila melanogaster* that is based on deficiency of the *ADGF-A* gene (Dolezal et al. 2003, 2005). She showed that increased concentration of extracellular adenosine causes hyperglycemia and a decrease of glycogen stores, leading to death of the *Drosophila* larvae due to insufficient accumulation of energy reserves needed for further development. The disruption of glucose metabolism induced by *adgf-a* loss depends on the intact adenosine signaling pathway, as adenosine receptor (*adoR)* deficiency suppresses *adgf-a* mutant phenotypes including lethality. Death of flies can be partially overcome by feeding larvae on a high-sugar diet. These results are part of the manuscript published in 2010 in a peer-reviewed journal *Disease Models & Mechanisms* entitled "**Increased extracellular adenosine in *Drosophila* that are deficient in adenosine deaminase activates a release of energy stores leading to wasting and death.**" and Monika is the first author. The initial hint to explore the role of extracellular adenosine in regulation of carbohydrate metabolism came from a genetic screen aiming to find suppressors of *adgf-a* mutant phenotypes using a deficiency kit. The low- and high-resolution genetic screens identified several putative suppressors of *adgf-a* mutant phenotypes including *PhKgamma*, *knockdown* (*kdn*, citrate synthase), CG14478 (*monar*) and *invected* (*inv,* transcription factor). Nevertheless, the genetic screen pointed to necessity of cleaning the genetic background of *adgf-a* mutants. Upon backcrossing of *adgf-a* mutants for multiple generations, the suppressive effect of all identified candidates diminished indicating a presence of additional mutations in the background of the original *adgf-a* mutant stock. Fortunately, the sensitivity of cleaned *adgf-a* mutants to diet composition remained. Monika further investigated the relation of *adgf-a* and *cactus* mutant phenotypes with regards to adenosine and carbohydrate metabolism and determined the developmental profile of energetic reserves.

Formal aspect:

The introduction is concise, providing sufficient overview of studied topic. Objectives are clearly stated. The part containing Results and Discussion is not completely easy to follow mainly due to figure mislabeling (on page 18: "Fig. 3A", "Fig. 10B", page 19: "Fig.9A", page 23 "Figure 13A" do not exist), inconsistencies in Figure layouts (Charts in different format and colors: Fig. S6, S7, S9) and Figure legends (what represent error bars Fig. S7, Fig. S3B). The Figures carry an extensive amount of information, thus it would be wise placing them in the main text and not as Supplements, which requires constant page flipping. The data presented outside of the manuscript (survival and metabolite measurements) (Zuberova et al., 2010) are not statistically evaluated which raises the question as to their significance and relation to the published data (Zuberova et al., 2010). The methods described in Results and Discussion would deserve a separate chapter "Material and Methods". It is sufficient to mention a problem with food contamination only ones throughout the text as repetition leads to questioning the data quality. Monika offers a number of plausible explanations for most of her results, showing her thorough thinking about the data. However, the discussion lacks reflection of the published literature. The chapter containing Summary and Conclusions is well written and touches upon the most important findings. It would benefit further from incorporating the current state-of-art knowledge.

Established abbreviation should not be changed: "hours after egg laying (hal). Should be abbreviated "hours AEL" or " hr AEL". It is hard not to mention a few grammatical and spelling errors which appear throughout the text.

Example: page 42: Figure 10 legend "Toll pathway trigger",
Figure S3 legend "(of Exel6006;adgf-a larvaea)", "glycogen phosphorilase", similar mistake is repeated in TabS4A.
page 34: Figure 8 "Glykogen (ug/larva)"

Specific comments and questions:

I strongly object using a word "wild-type" when referring to a fly stock *of adgf-a/TM3 Act-GFP* genotype containing deletion of *adgf-a* gene balanced over *TM3* balancer with *Act-GFP* transgene. This is surely not a genotype that would fulfill textbook definition of "wild-type" as the typical form of an organism occurring in nature, as distinguished from

mutant forms that may result from selective breeding."

3. Part I. of results & discussion - Genetic screening

The final outcome of the genetic screen is unfortunate. However, it sends an extremely important message that should not be neglected. It clearly demonstrates that regulation of energy metabolism is amazingly complex, involving a multitude of genes and regulatory circuits. It highlights the need to unify genetic backgrounds and to use proper controls when investigating metabolism. Taking this information into account I find the results in chapter 4.1.2. Hemolymph metabolites in genetically rescued *adgf-a* larvae part "Rescue mutations identified in genetic screening" irrelevant.

Having such an experience, I am surprised that Monika used *adgf-a* heterozygous *TM3* balanced larvae for her experiments as *controls*.

Besides sugar sensitivity which of the listed *adgf-a* mutant phenotypes are retained upon backcrossing?

Page 18) The composition of CM30 and CM60 diets regarding the sugar and yeast concentrations is not common knowledge. It would help to include such info directly into the text rather than in Supplements.

4.1.1. Differences in hemolymph metabolites between *adgf-a* and *wt* larvae

Page 23) *"One possible explanation for this is that increased glucose damages tissues by glycosylation of membrane proteins resulting in ATP release from damaged cells. ATP is then dephosphorylated forming extracellular adenosine."*

I find Monika's explanation hard to reconcile as higher glucose concentration significantly increases adult eclosion rate of *adgf-a* mutants and as stated in the manuscript "it is not hyperglycemia (at this level) that kills the *adgf-a* mutants." (Zuberova et al., 2010, Fig. 2A). Could Monika comment on this and provide a reference to some publication in which hyperglycemia would lead to enhanced glycosylation of membrane proteins?

Page 24) *"The concentration of inosine is higher in the adgf-a larvae in comparison to wt and increases significantly on pure yeast diet (it is about 1.5-2 times higher on sucrose supplemented diets and about three times higher on pure yeast diet in adgf-a than in wt)."*

Data were not subjected to statistical test thus the word "significant" should be omitted.

4.1.2. Hemolymph metabolites in genetically rescued *adgf-a* larvae

Page 27) *"Concentrations of purines are shown in the same unit in both figures, concentrations of carbohydrates are in arbitrary units in Fig. S10."*

What do the arbitrary units represent? Were the values normalized to something (animal, weight)?

Page 27) *"I obtained only one or two replicates from some larvae (numbers of obtained replicates are given in the graph). However, because of small s.e.m., the number of replicates seems not to be so important."*

I completely disagree with the statement. The number of replicates is crucial for statistical evaluation of the dataset to show significance. How confident can one be that the observed differences are real and did not occur by chance?

4.1.3. Hemolymph metabolites in *cactus* larvae

Page 28 *"Moreover, they are normally delayed after wt larvae."* Referring to larvae carrying cactus null or hypomorphic alleles.

What does "*normally delayed*" mean?

4.2 Developmental profile of energetic reserves

I find data presented in this chapter rather confusing and misleading. Various explanations, speculations are offered however without referring to the published literature. The confusion comes mainly from presentation of metabolite measurements.

Was there any specific reason to present metabolite measurements in micrograms per animal (S11A-F)? How significant are these results?

The *adgf-a* mutants are developmentally delayed. Are they also smaller/lighter/growth retarded when compared to *controls* of similar developmental stage? If yes, it is not surprising that the total amount of proteins is reduced in *adgf-a* mutants. It would be helpful and avoid unnecessary speculations if the metabolite measurements were

expressed as micrograms of proteins, Glucose, Glycogen, etc. normalized to larval body weight. Evidently, the normalization to protein content erases the differences or even reverses the trends for certain metabolites.

Could Monika comment on differences in measurements of total protein content (large versus minimal changes) presented in Figure 4A,B (Zuberova et al., 2010) and S11D?

Page 31) *"So, the length of fatty acids could shorten during starvation/wandering period, although the total amount of triglyceride molecules remains about the same."*

Is Monika suggesting a new way of Triacylglycerol (TGA) hydrolysis?

It is well established that TGA levels decrease during starvation. TGA is an ester composed of glycerol and three fatty acid chains stored in lipid droplets, which are hydrolyzed to fatty acids and glycerol by hormone sensitive lipases. Glycerol is phosphorylated and serves as a substrate for glycolysis. Fatty acids become activated (Fatty acyl-CoA) and enter mitochondria through carnitine shuttle enzymatic system to be oxidized or converted to ketone bodies.

Page 30) According to data presented in Figure S11D the protein concentration raises steadily in both *control* and *adgf-a* mutant larvae during third instar and wandering phase. *"This could be explained by conversion of water insoluble proteins (e.g. bound to cuticle) to water soluble ones."*

This is an interesting thought. Could Monika substantiate her explanation by citing some published data?

It has been well documented that protein synthesis in a fat body dramatically increases during third instar in response to 20HE. For example hexamerins such as LSP1 are synthesized by fat body cells and secreted into the hemolymph, attaining peak levels at the white prepupal stage (Burmester et al., 1999; Deutsch et al., 1989). They are reabsorbed back into storage granules and utilized during metamorphosis (Levenbook and Bauer 1984).

5. Summary and conclusions

Page 39) It has been suggested that extracellular adenosine serves as an anti-insulin hormone parallel to AKH, keeping hemolymph glucose levels high possibly causing

insulin resistance via desensitizing cells to dILPs. Can you speculate on the plausible molecular mechanism leading to insulin resistance? Did you probe dILP, InR expression or FOXO activity in *adgf-a* mutants or tested genetic interaction with the Insulin signaling pathway components?

Page 39) *"High sugar diet probably facilitates passive (insulin independent) transport of glucose into cells (because of higher concentration gradient in system gut-hemolymph-target tissue)."* This speculation would again deserve an example from literature.

The *adgf-a* mutants display multiple phenotypes akin to insulin resistance provoked by feeding larvae a high-sugar diet (Musselman et al., 2011): developmental delay, high carbohydrate levels in hemolymph, decreased glycogen reserves. The high-sugar diet shifts balance towards TGA storage. If the *adgf-a* larvae were indeed insulin resistant one would predict that 10%-sucrose diet would worsen the mutant phenotype rather than suppress it?

Data presented in Figure S11I suggest that *adgf-a* mutants store more TGA compared to *controls,* which is reminiscent of larvae fed on high-sugar diet diet (Musselman et al., 2011) and insulin pathway mutants (Bohni et al., 1999, Abe, 1998, Kimura et al., 1997).

Is it possible that the observed rescue by high sugar diet results from utilizing extra TGA reserves? Did you measure TGA content in *adgf-a* mutants fed on high-sugar diet?

8. Supplements

Page 57) Figure S8 panel C does not show wt pharate adult (eye color is not visible).

Out of curiosity

Was there any specific reason to use 95% EtOH for larvae sterilization when 75% EtOH is known to be a better disinfectant and more efficient in killing bacteria?

Besides ADGF-A expression regulated by *hs-Gal4,* were other Gal4 drivers (fat body, hemocyte specific) tested for rescue of *cactus* null or hypomorphic mutants? Would you expect suppression and different extend of rescue of *cactus* mutant phenotypes/lethality based on the driver used?

Did you notice any changes in developmental timing growing *control* and *adgf-a* mutant larvae on food containing antibiotics?

The animals homozygous null for *adoR* receptor are fully viable suggesting that regulation of glucose levels via adenosine is not required for normal development under physiological conditions. How are *adoR* mutants doing under stress such as immune challenge?

Can you separate the two phenotypes of *adgf-a* mutants: the exaggerated reaction of immune system and fat body disintegration, by tissue specific $adgf\text{-}a^{RNAi}$ or interfering with AdoR function in *adgf-a* mutant background using $adoR^{RNAi}$ targeted to either hemocytes or fat body?

Conclusion

Taken together it seems that writing the Ph.D. thesis was a work against the clock. Considering the Ph.D. thesis as an exercise of rigorous scientific writing the text contains a bit to many unfounded speculations and feelings that undermine the overall impression from a presented work. However, Monika clearly showed that she has comprehended the studied topic, mastered numerous techniques of cell and molecular biology, and learned her unforgettable lesson from fly genetics. Her work provides important novel insights into regulation of energy metabolism via extracellular adenosine in *Drosophila* with relevance to human health and establishes a foundation for future research. The questions and comments that are mentioned above should be discussed during the oral presentation. I suggest to correct references to Figures and spelling errors in the text. I recommend awarding Mgr. Monika Zuberova a Ph.D. degree.

With best regards,

Uhlířová

Mirka Uhlirova, Ph.D.