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Evaluation of PhD dissertation **Name of PhD Candidate:** Tomáš Štětina

Title of thesis: Low-temperature injury in insect tissues and mechanisms of its repair

The PhD work of Tomáš Štětina is a very detailed analysis of the physiological processes occurring after exposure to cold/freezing stress of varying intensity in the larvae of two model insects, *D. melanogaster* and *C. costata*. The thesis presents 115 pages, including 20 pages of bibliographic references (in the introduction). The manuscript is logically arranged, clear and well written, in very good English. The first part is an introduction of 50 pages.

The introduction presents the essential and important notions related to the biology of low temperature/cryobiology in insects, with a well balanced mix of old and recent relevant references. This part would have been even more enjoyable to read if it had included some representative schema, figures or other relevant illustrations. The introduction describes very well the scientific context, definitions, background and the reasons for choosing the particular subject. Yet, a section could have been included in introduction to clearly state the specific research hypotheses. The two first papers are more explorative than hypothesis-driven, so I concede that raising specific/functional hypotheses is not an easy task, but based on literature, I assume some physiological responses/processes were expected. Of course, the specific goals/hypotheses are indicated within each chapter, but it would have been useful to state and list at the end of the introduction both the general goal and also the specific working hypotheses based on identified gaps that were previously underscored. This would have given the opportunity to come back to these specific goals and hypotheses in the concluding part of the manuscript and discuss whether or not these were accepted or refuted.

The presentation of the results is well structured in three distinct parts. Paper 1) Recovery from cold/freezing stress in larvae Chymomyza costata, Paper 2) Recovery from cold/freezing stress in larvae Drosophila melanogaster and Paper 3) Mitochondria as targets of low temperature-induced injury. Each chapter is now published in top journals: Sci Rep, IBMB and Proc.B.

The general discussion, of only 2 pages, is probably a little light in terms of content. Perhaps in the discussion Tomáš could have underscored a bit more exhaustively the importance of his work in a global and integrated way. In addition, he could have explained how the outcomes of his work fit relative to the existing body of knowledge about the subject (there is no citation in the discussion). The motivation for this work was that cold-related literature has been primarily dealing with adaptations linked to gradual acquisition of cold resistance during entry into diapause and/or cold acclimation, while adaptation to post-stress recovery period was less described. I generally agree with this notion, yet, it should be noted that the mechanisms for repairing cold damage have also been explored (in different context) in the literature dealing with FTR (fluctuating thermal regimes) ; it could have been interesting to take a look this literature as well. Finally, given the similarity of the methods and questions in both biological models, I expected maybe a little discussion on the potential generality and/or divergence of the recovery/repair processes in the two models (a comparative discussion). That being said, these minor criticisms (mainly on discussion part) do not in any way detract from the merit and quality of the work that has been provided and presented in this thesis. It is clear that Tomáš has gathered an impressive and robust bunch of novel data and he has made great efforts to publish his results in international peer-reviewed journals. This is exactly what is expected of a PhD candidate, so congratulation for that.

I was also impressed by the diversity of experimental approaches that Tomáš used in the different parts and the number of techniques that he has learned and successfully used. This work is based on a large panel of cutting-edge ecophysiological (respirometry, mitochondrial physiology, biochemistry, imaging, etc) and Omics (metabolomics, genomics) approaches to explore physiological processes related to repair of cold/freeze injury. The methods used in the different studies were appropriate and the experiments appear to have been carried out with great care and precision, making the whole data set highly reliable.

With regard to the data produced, I want to underline the high quality of the research presented in this thesis. Tomáš and co-workers have been able to make significant breakthroughs that I consider as major: 1) the importance of considering delayed mortality effects, 2) the specific physiological adaptations (i.e. Omics responses) to various types/intensities of cold/freezing stress, and 3) the critical role of impaired mitochondrial functions in frozen larvae. As a result, Tomáš work has been already published in three top-quality peer-reviewed journals. My general opinion is that the PhD work of Tomáš brings a valuable contribution to our understanding of thermal/cold/freezing adaptation of two Drosophila species living in very contrasted thermal environments. The topic is timely with the increasing interest in the molecular and physiological basis of response to temperature. The nature of cold response/tolerance is highly complex which makes the effort to unravel it very difficult.

As examiner, I also have to comment the technical and scientific content of this dissertation and highlight weaknesses or issues. While a PhD dissertation usually contains parts or chapters in preparation for publication, here, Tomáš completed a work that is entirely published in excellent peer-reviewed journals. He also published other excellent papers that he did not even include. Therefore, considering that the technical and scientific aspects have already been evaluated by external referees, I have no criticism to raise on these aspects (or just a few minor questions). The technical and scientific aspects of this thesis are to my opinion of excellent quality.

As said above, my only minor reservation about this PhD dissertation is about the content/length of the final discussion that could have included an integrative/comparative general conclusion. Tomáš could have attempt to answer how his work have contributed to advance knowledge in the area and what still needs to be explore? The discussion is where it should be demonstrated that the candidate is thinking about the big picture. Because the thesis is really focused on mechanisms, I think there was also opportunity to provide a sort of schematic representation integrating already-known and novel aspects of repair mechanisms. This is of course just a suggestion.

In summary, my general appreciation about the scientific and technical aspects of the work is excellent. Based on that I would rank Tomáš scientific work highly, in the top 25% of the PhDs I previously evaluated.

Questions:

Q1: Diapause is presented as a different process than acquisition of cold resistance (in the present work, diapause comes first then comes cold acclimatisation). P6 it is mentioned that the question of whether or not diapause is associated with the acquisition of cold hardiness remains a subject of debate. On the basis of your data, can you provide some elements to feed or clarify this debate?

Q2: P12: concerning proteostasis and the role of Hsp at cold, you mention p12 that cold-induced up-regulation of Hsp is not necessarily needed for repair of cold injury. Can you provide an alternative explanation for Hsp expression during recovery from cold (even after chilling)? In paper 1, you found hsp expression (Hsp22 and Hsp26) only in LN larvae (cryopreserved) not in other treatments. What is your general opinion about cold-denaturation - does it occur only at very extreme temperatures?

Q3: P17, you say that the term 'diapause' is used incorrectly in *D. melanogaster*. Why? can you explain - In fact the literature speaks about 'reproductive diapause'. Can you explain what you think a 'true reproductive diapause' should be? In *D. suzukii* literature uses the term 'winter morph' to characterize flies in reproductive diapause, more resistant to cold and morphologically distinct (bigger/darker). What is your opinion about this terminology?

Q4: *Chymomyza costata* enters larval diapause at short daylengths. So, it makes sense to study larvae. *D. melanogaster* does not have true diapause and overwintering stage is adult. Why did you chose larvae in *D. melanogaster*? - Working with larvae is more challenging as many genes may show expression related to developmental process rather than cold/recovery. For instance, proteolysis, lysosomal degradation, autophagy (see paper 2) … many of these are linked to degeneration of the larval tissues/cells during development. The developmental autophagy is known to be induced during the wandering larval stage and early metamorphosis at times when the animal is not feeding. Because autophagy plays roles in survival and also in tissue growth during nonfeeding periods. How did you account fort this? How would you separate both effects?

Q5: in both species you compared 3 levels/intensities of stress on Omics responses. In *C. costata* you found Freezing and Supercooling to be rather similar, while in *D. melanogaster*, both were stingingly different. Can you provide some putative explanation?

Q6: A question about the time scale of the measures. It is always very tricky to choose the right time-window of observation. In *C. costata* you chose up to 72h and in *D. melanogaste*r you chose up to 48h. Can you explain the rational? Does this correspond to some specific moments in the expression of delayed mortality in both species? If you had unlimited funds, would you change/adjust/increase the number of sampling times? How?

Q7: in both studies, very tricky to differentiate genes/metabolites that are involved in degenerative rather than repairing/adaptive processes (especially at extreme conditions). How to deal with this issue?

Q8: Intro p7 you mention that cuticle thickness changes with diapause, ok, but it also gets darker in acclimated/diapaused flies with increased melanin *via* the phenoloxidase cascade. This is supposed to improve thermoregulation in insects, but the phenoloxidase cascade also plays roles in insect immunity. What is your opinion of this? Is immunity a side effect (pleiotropy) or really functionally important for cold resistance (preparation) and tolerance (repair)?

--------- more specific questions/comments, if time allows -----------

- Intro: p3: LLT is time and temperature dependent. What do you think about thermal landscape approaches?
- paper 1, you assumed profiles will be similar between C (18°C) and S (-10°C). Yet, there is a shift of 28°C between both conditions. Why this assumption?
- paper 1: table 1: was there any delayed development? why development times were not assessed?
- paper 2: I could not find PCR validations?
- paper 2: (p68) I did not understand how control larvae also received FTR, please explain.
- paper 2: as many authors, you separate up and down regulated DE genes in functional analyses. What is the rational?
- paper 3 and conclusion p114: it is mentioned that mitochondrial functions (enzymes from electron transport chain and coupling) were assessed and still functional. Yet, I could not find these results in the published paper – why? Also, you could have cited a relevant paper Colinet et al 2017 in IBMB that actually found similar results on isolated mitochondria functions of cold exposed acclimated D. melanogaster.
- paper 3: you measured oxygen consumption of fat body tissue with PreSens system. This does not account for non-mitochondrial respiration and it is not done on isolated mitochondria. What is your opinion about PreSens system? is this good instrument? Did you try to see if PreSens measures are nicely correlated with mitochondrial ATP production or mitochondrial O2 production?

Hervé COLINET, Rennes 02/09/2020

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