DEPARTMENT OF BIOLOGY
AARHUS UNIVERSITY

Johannes Overgaard, PhD
johannes.overgaard@biology.au.dk
AFDELING FOR ZOOFYSIOLOGI
Universitetsparken, Bygning 131
8000 Aarhus C
DANMARK
Telefon: (+45) 8942 - 2648
Fax: (+45) 8942 - 2586
http://www.ecophys.dk/

31/08/20

To whom it may concern

Evaluation of thesis by Tomáš Štětina
The thesis "Low-temperature injury in insect tissues and mechanisms of its repair" by Tomáš Štětina contains a general introductory review of insect cold tolerance strategies, causes of cold injury and a discussion of some of the putative physiological adaptations that are associated with variation in cold sensitivity of insects. The experimental work in the thesis is focussed on measuring the effects of cold stress after the cold exposure and therefore there is a considerable focus on putative mechanisms of repair. The bulk of the new scientific findings is presented in three peer review papers (In the thesis the third paper is listed as "in preparation", but a final version has since been accepted in Proceedings of the Royal Society B). All three papers have therefore been accepted in very well established journals where they are already being cited regularly. Below I comment on the 4 elements of the thesis (introductory chapter and paper 1-3), but the overall impression of the thesis is that it is well written, that there is a good and transparent link between the different studies included, that the data are presented intelligently and appropriately and that the studies cover a broad range of experimental and analytical tools. Three accepted papers in high quality journals and a comprehensive review does, in my view, represent a considerable contribution to the scientific field. This is even more impressive considering that the candidate has been associated with the production of several other studies which were not included in the thesis.
Introductory chapter: The introductory review gives a nice presentation of insect cold biology in general and links this to our general understanding of seasonal challenges for ectotherms. The review defines and explains central concepts in the measurement of "cold tolerance" and "cold hardiness" and discusses how cold hardiness is often linked to periods of quiescence and diapause. The review defines and discusses a range of the central adaptations that are associated with insect cold hardiness including precise and appropriate discussions of adaptations associated with freezing and supercoiling. In doing so the introductory review also explains the role of central physiological adaptations in insect cold biology such as membrane adaptation, cryoprotectant accumulation, antifreeze proteins, hsp expression, ice-nucleation proteins etc. The review also has some description of the physiological processes that occur subsequent to cold stress, but despite the inclusion of "repair" in the title of the thesis there is relatively little focus on how repair can and/or should be assessed experimentally (See list of questions below). Overall, I find the review to be very well written, it includes many appropriate references to the literature and clearly demonstrates that the candidate is well founded in the field. If the candidate wishes to edit the introductory chapter into a review for

publication, then I would recommend and increased focus on the novel aspects (repair/post stress responses) and less on description of the classical cold adaptations which are already reviewed comprehensively in the existing literature. Another advice would be to include a few more figures/tables to graphically present a summary of the central points. In relation to this I find that the figures that are included presently have a rather limited figure legend which make them harder to interpret.

Paper 1: The paper "Recovery from supercooling, freezing and cryopreservation stress in larvae of the drosophilid fly, *Chymomyza costata*" evaluates the consequences of three cold treatments (freezing (-30°C), supercooling (-10°C) and cryopreservation (-196°C)) against a control condition where the animals are simply placed at benign temperature. The focus is to evaluate these effects in terms of survival (data from another paper), overall metabolic costs of recovery (metabolic rate) and disturbance of organismal homeostasis (evaluated from measurement of extracellular ion concentrations, metabolomic profile and gene transcription). The data on metabolic rate (CO_2 production rate) suggests some energy costs (10-20% increase) associated with all three cold treatments. The study also shows that particularly freezing is linked extracellular hyperkalemia, but that recovery of ion balance is fast and probably relatively cheap energetically (when data are related to the measurements of CO_2 production rate). In the final experiments the paper examines the metabolic and transcriptomic changes following the cold treatment and based on these findings thay suggest that particularly cryopreserved larvae are unable to restore metabolic homeostasis (i.e. recover from an anaerobic event) which at least in some respects is also reflected in the transcriptomic data where several genes associated with energy metabolism remains differentially expressed in this experimental group. Overall I find the paper to be well written and to have a balanced and appropriate (and critical) discussion of the data, which both points to new interesting aspects of repair (or lack thereof), but also discusses some of the challenges associated with the correlative analysis of metabolomic and transcriptomic data. In the "question" section below I have a range of specific questions which deals with methodology and interpretation of data.

Paper 2: The paper "Larvae of *Drosophila melanogaster* exhibit transcriptional activation of immune response pathways and antimicrobial peptides during recovery from supercooling stress" examines the metabolomics and transcriptomic changes occurring during recovery from two cold stress exposures (freezing and supercooling) in larvae from the moderately tolerant species *D. melanogaster*. In many ways the experimental approach in this paper is parallel to that of paper 1. It uses state of the art metabolomics and transcriptomic platforms to describe changes following a stress and through subsequent multivariate analysis the paper suggests a number of putative physiological/biochemical processes that are affected. The presentation of the findings are clear and I find that the paper has an appropriate balance between a "positive" and "negative" interpretation of the data. Thus, the data from "omics" analysis can point to process of important relevance to repair, but they may be confounded by methodological problems (i.e. are there moribund larvae in treatment groups) and analytical problems (i.e. how should an increase in gene transcription or metabolites be evaluated in terms of flux through the system)". While considering these problems the paper arrives at an appropriate conclusion: Freezing is associated a much more invasive cold treatment than super cooling characterised by a more persistent disruption of the metabolomics profile and upregulation of genes associated with cell death. In contrast the metabolomics disruption after supercooling is more transient and the differential gene regulation points to an interesting upregulation of genes associated with immune function.

Paper 3: The paper "insect mitochondria as targets of freezing induced injury" uses a range of techniques including electron microscope, enzymatic assays and respiration measurements to evaluate the structural and functional integrity of mitochondria following different cold exposures (supercooling, freezing and cryopreservation) in cold-hardy and cold-sensitive phenotypes of *C. costata*. As mentioned above paper 3 is now published in a fully accepted and peer reviewed version which I had already read carefully earlier. I have evaluated this paper from the final version published in Proc. Roy. Soc. B. (The main difference between the final version and the one included in the PhD thesis is the omission of mitochondrial respiration measurements performed on muscle tissue). The paper investigates mitochondrial adaptations along two axis – one is the difference between diapausing and coldhardy phenotypes against nondiapausing larvae. The other axis is to compare the effects of different cold exposures (akin to paper 1-2). The experiments firstly shows that there are limited differences in mitochondrial number between diapausing and nondiapausing phenotypes but the main finding (in my view) is the experimental demonstration that stressful cold treatments leads to the loss of structural integrity in mitochondria. Thus, Mitochondria swell and loose their characteristic membrane organisation following freezing of the non-diapausing animals. In contrast the evidence shows that short day diapausing larvae are able to retain mitochondrial structure and function. These results are discussed in relation to a putative mechanism that involves disruption of the selective properties of the inner mitochondrial membrane and an interesting element of the paper is the demonstration of how proline (a well known cryoprotectant) is able to partially defend mitochondrial integrity following freezing in the cold sensitive animals. To my knowledge this is one of the fist papers to demonstrate structural kolapse of mitochondria in relation to freezing stress and in addition to the novelty of the data this study will likely inspire many future studies of how the actual function of mitochondria is protected by acclimation.

From my evaluation above it is clear that all 4 elements in the thesis (introductory review and the three papers) are of high scientific quality and relevance. All good studies will give answers to some questions and open up for new ones and below I have listed some of the questions that I find interesting after reading this thesis.

Specific questions.

Introductory review:

Discuss the definition of cold-tolerance and cold-hardiness, comment on how you use them in the very first sentences of paper 1 and 2 respectively and comment on why it is important to separate these two terms. This discussion could also reflect on some of the similarities and differences between the physiological responses associated with cold-tolerance and cold-hardiness and how they may be connected evolutionary.

Discuss and define cold-resistance relative to cold-tolerance and discuss how these responses could/should be evaluated experimentally.

Discuss the choice of life stage and cold treatments used in the different studies

Paper 1+2+3:

Discuss the methodology used to assess metabolic rate and reflect on the strengths and

weaknesses of this method.
Discuss the methodology used to measure ion concentration and reflect on how these measurements relate directly/indirectly to the variable of interest (the electrical gradient over the membrane)
Discuss when, if and how the use of multivariate analysis is appropriate
Discuss from a specific and general perspective the pro's and cons of using omics. What is the experimental strength, what is the analytical weakness and how can the analytical weakness be confronted
Discuss the experimental design in which moribund animals may be included in the samples – when and why is this relevant and what are the problems with this issue. How can one experimentally separate results from animals destined for survival.
All three papers presented very interesting results that will stimulate new and interesting research –reflect on how you would concretely design future experiments to address the most central questions arising from the studies in paper 1-3.

OVERALL EVALUATION:

As can be seen from my comments above I find the thesis well written, it is relevant for the field and clearly demonstrates the breath of the candidates knowledge and experimental proficiency. I therefore recommend that the candidate is allowed to defend his thesis and look forward to an interesting discussion on insect cold biology.

Johannes Overgaard

Professor at section for Zoophysiology,
Department of Biology, Aarhus University