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Review of doctoral thesis Insect overwintering: physiological and biochemical adaptations to low temperatures by Jan Rozsypal

The request to be the reviewer of this thesis forced me finally to read thoroughly several articles written by my boss, and enabled me to criticize them. Nevertheless, as the role of the PhD. candidate in preparation of the articles was clearly stated, I may focus on the strengths and weaknesses of the thesis related to his contribution. Although the four papers were published or accepted for publication in high rated journals, it is often possible to find some errors overlooked by the authors, reviewers and editors, and it was.

First, the thesis begins with very well readable, comprehensive and well balanced introduction to insect diapause and cold hardiness written by the candidate himself (at least I hope so), which deserves a praise, except for three minor points. In the diapause chapter (p. 2–3), he ignores work by the supervisor of his supervisor, Dr. Hodek (e.g. 1996), in the point that the candidate insists on action of environmental cues for diapause termination. **(1)** What is instead the mechanism of diapause termination in many insects including some of his model species? In the classes of cold hardiness chapter he ignores my contribution to the classification which is in other way repeated in the chapter on the effects of low temperatures. Why to use the category chill injury (as opposed to freeze injury) when it is subsequently divided into two completely different injury mechanisms that have nothing in common? Try to think about the complex problem as when the temperature decreases, first , cold shock occurs or not, if not then freezing occurs of not and if not, cumulative chill injury occurs or not. On page 10 below, the candidate mentions multiple cases with no correlation between cryoprotectant concentration and cold hardiness. **(2)** Why is it so?

All the papers combine several methodical approaches that allow complex understanding of ecophysiology of overwintering of studied insects. It is understandable that such variable studies must be a result of co-operation of several specialists. Our candidate focused in measurements of supercooling point, which is easy, osmolality, which requires some more skill, and thermal hysteresis, which I hated for its very laborious nature when I studied it many years ago. The numbers of individuals measured are often rather small but understandable due to the laboriousness and sufficient for conclusions of the studies, except for study on codling moth with a single measurement of a mixed sample, which is unpardonable. Most data received on these above listed variables seem precise and useful.

All the papers published are equipped with self-explanatory, well readable coloured graphs and tables that - if I remember well - were just opposite in the candidate's master thesis. Because I should concentrate to the activities our candidate contributed to the studies, I will mention the method of measuring osmolality. The formula for its calculation given in the first paper on page 29 (1138) is wrong. **(3)** Will you show us a correct one? And will you find, please, original measured data (volume of hemolymph, osmolality reading on Vapro) for the winter samples in Kubova Huť?

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The tight statistical correlation between hemolymph osmolality and SCP (page 32 below, Fig. 6) is apparent only if you look at the entire year, but within the period December to March, there is no or opposite relationship worth to be explained. It was partially tried in the discussion. In figure 4, the blue line could not be the result of given negative exponential with "top" set to 100. **(4)** What was the correct formula?

I do not have any comments to the second paper except for the inset in figure 2, which does not show Pearson's correlation. I do not have any comments to the third paper except for the equation on page 53, which is not exponential.

Missing of chapter explaining the methods used in the fourth paper, where our candidate is the first author, is very confusing. There are some other inaccuracies that hopefully will not appear in the final published article. The four digits precision of values of temperature given in table 2 is non-sense. The sentence "No larva, either supercooled or frozen, was able to survive at temperatures below SCP" (page 70 right column, in the middle) should be reworded. In the continued sentence, I would not call those temperatures used as being "just above the SCP" – there was a difference by several degrees.

I much appreciate the discovery of inoculated freezing tolerance in the winter caterpillars. I also like the finding of high survival following alternating temperatures. I was surprised (like the authors were) by the caterpillars retaining low supercooling point in April when the environmental conditions do not require such high cold hardiness capacity, and with low concentration of cryoprotectants. **(5)** Does this phenomenon occur in more insect species?

Generally, the submitted thesis and the contribution of the candidate to the four studies are fairly sufficient for awarding him the grade of PhD.

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Review of "Insect overwintering: physiological and biochemical adaptations to low temperatures" by Jan Rozsypal

General Thoughts:

The thesis consists of a general introduction on insect cold physiology and 4 accepted/published original manuscripts that are all published in well-respected international journals. Jan is lead author one paper and co-author on the other three. The focus and methods used in the papers represent a broad range of experimental techniques and the species studied are also somewhat different representatives of insect cold resistance, such that Jan's thesis cover a versatile range of physiological topics within insect cold physiology. The introduction as well as the paper are all of high quality and demonstrates a broad and detailed understaning of insect cold biology. The suggestions for questions should therefore more be seen as topics for discussion rather than critism of this interesting PhD.

Introduction:

Jans thesis starts with a general introduction to the cold biology of insects covering the different cold tolerance strategies and their relation to temperature and water/ice relations. There is also a section describing the different model animals used and how their cold biology fits into this general scheme.

Question to introduction:

Several of the studied species are commercially important. Try to give concrete examples of how increased understanding of these species winter biology can be used for controlling/conserving important insect species.

Comments to paper 1:

The paper describes different aspects of the cold physiology of *I. typographys* with a focus on regional and seasonal differences. The findings support that *I. typographys* are freeze avoiding species that seasonally alter their supercooling point (presumably in a manner linked to accumulation of sugars and polyols). They also show a considerable high mortality at sub-zero temperatures indicating a cost-benefit, where animals overwintering in the mild litter layer may become energetically challenged, while animals overwintering under the bark may be exposed to lower temperatures, but retain the ability to feed during mild periods.

Question to paper 1: The use of different populations to highlight specific adaptations is a strong approach to infer physiological adaptations. Comment of the applicability of the two populations used in the present study and discuss how genetic/phenotypic differences can be resolved using this approach?

Comments to paper 2: This paper adds to the growing knowledge of the cold biology of the firebug *P. apterus* in which many aspects of cold tolerance has previously been examined by Kostal and co-workers. Specifically this paper addresses the putative role of thermal hysteresis proteins and accumulation of amino acids during winter diapause and demonstrates small, but significant induction of thermal hysteresis and some accumulation of amino-acids that contribute to the increased osmolality of winter acclimated animals.

Question to paper 2: It is suggested that THF may play a role in protecting inoculative freezing, what are the data to support this and how would you design an experiment to test this hypothesis

Comments to paper 3: This paper describes the metabolomics and lipidomic changes associated with cold acclimation in *D. melanogaster.* The results indicate a putative role of particular metabolites (trehalose and proline) as well as minor restructuring of membrane composition which are qualitatively (but not necessarily quantitatively) similar to observations from previous studies and highlights how these changes may be particularly important in relations to increased tolerance for indirect chill injury.

Question to paper 3: The present study represents one of the most comprehensive investigations of metabolomic and lipidomic changes during cold acclimation in D. melanogaster. The findings partially confirm several of the correlations between specific metabolites and cold tolerance found also in earlier studies. Discuss how one could possible devise experiments to bridge the gab from correlative observations to more mechanistic understanding of for example specific metabolites or membrane lipid composition.

Comments to paper 4: This paper describes some important features of the cold biology of the codling moth including field observations of winter survival. Amongst others, the study shows a marked accumulation of amino-acids, sugars and polyols. Interestingly, the cold tolerance did not correlate tightly with this accumulation although there was a (almost significant) correlation with the supercooling point. These results suggest that accumulation of diverse cryoprotectants are more important for freeze avoidance, and that other mechanisms may be more central to prevent the indirect chill injury that was assessed in the present study.

Question to paper 4: In this study (and several of the other studies) the supercooling point is used as a metric for insect cold tolerance. This may or may not be an appropriate measure to assess the cold tolerance of insects. Discuss when it is appropriate and when not and in an open discussion try to relate this to the pros and cons of other measures of cold tolerance (mortality, chill coma recovery, CTmin etc.)

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General Thoughts:

This thesis represents a substantial body of work on the basic cold tolerance parameters and underpinning mechanisms for four different species of insects. The four papers within reflect an excellent understanding of insect cold tolerance physiology and are all published in reputable journals.

Strengths:

Jan (and co-authors) does an excellent job of thoroughly characterizing basic cold tolerance parameters (i.e. limits of tolerance, supercooling points, freeze-tolerance vs. freeze-avoidance, etc.), using experimental designs that are ecologically relevant. Also, whereas metabolomics is typically reserved for laboratory studies, Papers I, III, and IV describe the metabolome of field-collected overwintering insects, something that is rarely done. Thus, there is no debating that the observed biochemical shifts are field-relevant. Finally, in these papers (and the Introduction), Jan and co-authors do a good job of critically examining previous literature and identifying gaps that need to be filled by their experiments.

Weaknesses: Overall, there isn't much I can say in this section, especially seeing that the papers have already been peer-reviewed and published in good journals. However, just a couple of minor suggestions for Jan moving forward: First, while the metabolomics experiments are excellent, the results are primarily descriptive, rather than testing specific hypotheses. Jan's expertise in metabolomics provides a powerful means to test specific hypotheses on the nature of low temperature metabolism, rather than simply identifying biochemical correlates of cold-hardening. This suggestion is beyond the scope of the thesis but something to consider in the future. Second, this is a very minor comment, but I think the stacked graphs in Figs. 3 and 5 of Paper IV are difficult to interpret, because you can't see precise levels of individual metabolites. I prefer the format of Fig. 3 in Paper II, although I appreciate this can get messy with a large number of metabolites.

Discussion Questions

1. What do your results suggest about the ability of insects to overwinter in a warming climate? Can we make any generalizations about the impacts of global climate change on the success of overwintering insects?
2. In your estimation, why do the winter polyols vary so significantly between species? For example, *Ips typographus* accumulates very little glycerol, whereas other overwintering species (e.g. *Eurosta solidaginis*) synthesize high quantities of glycerol in the winter. Feel free to speculate.
3. What are some factors that likely govern whether an insect is freeze-tolerant of freeze-avoiding? For example, *P. apterus* and *C. pomonella* both overwinter in similar microhabitats, yet only *C. pomonella* is capable of surviving internal freezing.

Signed: ____________________

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