



19 September 2022

To Whom It May Concern,

I am writing this letter for evaluating the scientific significance and novelty of the Ph.D. thesis entitled "Reproductive diapause in males of the linden bug, *Pyrrhocoris apterus*" written by Markéta Hejníková.

Diapause is genetically-determined, hormonally-regulated developmental arrest and is crucial for seasonal adaptation. Diapause at embryonic, larval, nymphal, and pupal stages are characterized by developmental arrest or suspension, whereas that at the adult stage manifests as a cessation of reproduction, in which the development of the reproductive organs is arrested. Both sexes enter diapause at the adult stage in many species, but sexual differences are also known. The ovaries of diapausing females are small and the oocytes contain little or no yolk. However, the testes of males remain small during diapause in some species but are well-developed and contain sperm in others. By contrast, developmental suppression of the accessory gland (the organ that produces spermatophores and factors responsible for sperm activation) is a good characteristic of male diapause. Physiological mechanisms underlying adult diapause have been investigated mainly in females possibly because of its distinctive phenotypic characteristic, whereas those underlying male diapause has been paid little attention to. This thesis has approached this issue and clarified the sexual difference in hormonal diapause regulation, which is very novel and opens a new door in this field.

This thesis consists of the following section: Overview, Chapters 1-3, Discussion and Summary, and Bibliography. I enjoyed reading it and found the significance of scientific achievements of this study.

The Overview describes the background and some questions in this field. The Overview nicely covers entire topics related to male diapause. However, it seems rather shallow when compared with Chapters 1-3. Because the main topic of this thesis is sexual differences and male diapause, a more detailed explanation of them is required. The scientific motivation of this author is still unclear to me from this Overview section. The readers of this thesis understand that information on male diapause is quite limited, but they do not understand why it is worthy to investigate and what is the author's expectations and hypothesis.

Chapter 1 clarified the significance of Met and Tai, components of the juvenile hormone (JH) signaling cascade, in the amounts of accessory gland proteins (ACPs), hexamerin expression, and longevity in male *P. apterus*. The experiments were well-performed, and the results are adequately interpreted. So, I just have a few questions.

- (1) Interestingly, silencing of Met and Tai as well as allatectomy had not affected fecundity and fertility in males, like the mating behavior reported in previous studies. Thus, this study stimulates discussion on the role of JH in male reproduction. Although the author describes some possibilities in the discussion section, I would like to ask the author more specifically what the author's hypothesis is and how the author can approach it. The author mentioned the possibility that the development of nymphs or the next generation may be affected. But is it indeed possible? Do you know any literature indicating such results? I also would like to ask the author whether the finding can apply to other insect or arthropod species.
- (2) Diapause, the natural absence of juvenile hormone (JH), had a stronger effect on the parameters of ACP accumulation, hexamerin accumulation in the hemolymph and its expression in the fat body, and longevity, than the ablation of the corpora allata (CA), the JH synthetic organ, and the silencing of Met and Tai. These results suggest an additional regulating agent in these parameters. However, this point was not discussed in detail, not only in this thesis but also in other literature. I would like to ask the author's thought on this agent.

Chapter 2 is the main chapter in this thesis. This clarified the sexual dimorphism of diapause regulation in *P. apterus*. Again, the experiments were well-performed, and the results are adequately interpreted. So, I have only some questions.

- (1) Met is dispensable for diapause termination in males that were chilled for longer periods but is indispensable in males that were chilled for 1 week. This discrepancy may indicate some relationships between diapause intensity and JH signaling, but this was not discussed. I would like to ask the author what causes this discrepancy and what is the underlying physiological mechanisms.
- (2) Fig. 2B & C indicate that the JH signaling pathway, Met and Tai, is necessary for diapause termination in response to JH analog application under short days, whereas Fig. 2D-F reveals that Met and CA are unnecessary for diapause termination under long days. The discussion of this chapter did not explain the reason for the discrepancy adequately. What is the underlying mechanism? The last sentences of Fig. 4 legend would be related to this point. Further discussion is welcomed.
- (3) The author's hypothesis that the accessory gland may produce JH or act as its storage site is fascinating. So I would like to ask the author how you can verify the hypothesis and how you can determine the relative significance of the MAG and the CA in diapause regulation.
- (4) If the male accessory gland produces JH or stores it, is the female's counter organ, i.e., the female's accessory organ or the ovary, able to function similarly? Is there any literature on this topic?

In Chapter 3, the author tried to expand our knowledge of the neuropeptides regulating diapause and studied the role of ELFa. Unfortunately, this neuropeptide appears NOT to be related to diapause, but I like this challenge. Such attempts move science forward. However, I am concerned about whether this chapter is adequate for this thesis. Because this chapter has focused on female diapause, not a male one. Although I highly evaluate this paper, I cannot evaluate this chapter as a part of the thesis.

Discussion and summary adequately described the results obtained in a series of experiments.

- (1) I do not understand why the author can say that ELFa is a very promising neuropeptide in male diapause in the second paragraph.

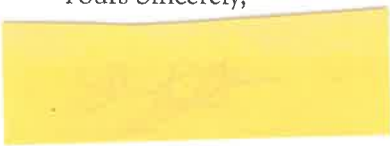
I was disappointed with the poorly-formatted bibliography section.

Importantly, I am largely concerned about the authorship of chapter 2. The author's contribution which is shown in Insect Biochemistry and Molecular Biology said that this paper was not written by the Ph.D. candidate, M. Hejnikova. Is this acceptable as a part of the Ph.D. thesis? Is it unnecessary for the Ph.D. candidate to write chapters or thesis?

Additional minor comments are written in a pdf file.

In summary, although I am still concerned about the authorship of chapter 2 and the adequacy of chapter 3 as a part of thesis, I am thoroughly satisfied with this thesis, which approached very important questions in insect physiology. Here I conclude that this thesis is adequate and sufficient for the Ph.D. degree.

Yours Sincerely,



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