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Reviewer's Report on PhD Dissertation Thesis of RNDr. Ing. Emad Ibrahim entitled: Study of adipokinetic hormone role in insect stressed by entomopathogenic nematodes

The PhD thesis of Emad Ibrahim is focused on the role of adipokinetic hormone, adenosine and vitellogenin in the insects infected by entomopathogenic nematodes and fungus. As an expert in EPNs I find the general topic very interesting both from the theoretical and also practical point of view. In these days, many chemical pesticides are no longer available on market, and the need for effective biocontrol agents is higher than ever. The possible way of boosting nematode performance against insects, that is suggested in the study sounds fascinating, and if it proves to be possible, that would definitely have a great practical impact for biocontrol.

The present thesis is formed by introduction, two published papers and a submitted manuscript. Emad Ibrahim is the first author in the two published studies, and both studies were published in a respected journal.

The introduction part comprises 16 pages of text, and gives a nice review on insect immunity and the role of hormones. My only remarks go to the part on entomopathogenic nematodes that, in my opinion, could have been prepared more carefully. In the text, it is stated that "EPNs have been described from 23 nematode families". This is a misinterpretation, as in 23 nematode families, we can find nematodes with association with insects. That includes phoresis, facultative parasitism, obligate parasitism and pathogenesis. In fact, EPNs are formed by members of two families and the "entomopathogenic" way of life among nematodes is quite unique. Later on we can read that "the relationship is not obligated, as nematodes can kill the host in the absence of their mutualistic bacteria". Indeed, several species can kill the insect without bacteria in experimental conditions, but the reproduction is strongly decreased. Under natural conditions, it would be impossible for EPNs to survive without their symbionts so the symbiosis really is obligate, at least for the nematode.

The objectives of the thesis that are formulated on page 30 are quite brief and in my opinion, this part would benefit from more details.

The rest of the thesis is formed either by papers that have already passed the review process or are presently under review and therefore I will limit my remarks only to several comments and questions for candidate.

In the first and second paper, the authors observed that the co-application of EPNs and AKH or adenosine increased the mortality in comparison to EPN only treatment, both in 24 hours and in 48 hours after the inoculation. My question is, what happened next? Did the mortality in EPN only treatments remain at ca 50%, or had it later grown to 100% as in hormone treatments? In studies of EPN mortality, the observation is usually done for at least 3-4 days. If the experiment was finished after 48 hours, we would not know, if there was indeed increase in mortality, or rather a faster onset of mortality (which would be practical as well).

In the discussion of the first paper, the authors hypothesise that increased metabolism induced by AKH might lead to faster degradation of the toxins, but it occurred too late, after the toxic effects have been produced. Did the authors consider pre-inoculation of AKH several hours before the nematodes? Eventually performing the observation with a lower (possibly sub-lethal) dose of nematodes? These approaches could possibly show the positive effect of AKH in handling the infection.

The submitted manuscript nicely connects to the published papers. The antibiotic activity of vitellogenin is interesting. I have just a small remark on the naming of bacterium. The symbiont of *S. carpocapsae* is referred to as *Xenorhabdus* spp. Shortcut "spp" means plural form of "species", so this would suggest that there are more *Xenorhabdus* species associated with *S. caprocapsae*, which is not true. There is only one and its name is *X. nematophila*.

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In conclusion, I can state that the candidate has performed a valuable research and obtained new original results that can have a high practical impact. The scientific value of the results is very high, and the topic would definitely deserve further research with more insects and more doses of nematodes. The candidate's publication activity is sufficient. In my opinion, the present dissertation thesis fulfills all requirements posed on theses aimed for obtaining PhD degree.

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Referee's report on PhD Dissertation Thesis

Study of adipokinetic hormone role in insects stressed by entomopathogenic nematodes

Author: RNDr. Ing. Emad Ibrahim

School of Doctoral Studies in Biological Sciences, University of South Bohemia in České Budějovice, Faculty of Science

Referee: Mgr. Martina Gáliková, PhD.

The article-based doctoral thesis by RNDr. Ing. Emad Ibrahim aimed to investigate the processes associated with the defense against infection by entomopathogenic nematodes. The thesis focused on two factors likely involved in the host response: Adipokinetic hormone (AKH) and vitellogenins. The thesis comprises three manuscripts; RNDr. Ing. Emad Ibrahim is the first author on two of them. These two publications deal with the role of Adipokinetic hormone (AKH) signaling in the defense against *Steinernema carpocapsae* in two model insects, firebug *Pyrrhocoris apterus* and fruit fly *Drosophila melanogaster*. The papers show that infection by *S. carpocapsae* increases AKH signaling in both fruit fly and firebug. Interestingly, this increase in AKH signaling is a maladaptive response of the host to the infections. In the firebug, deficiency for the AKH receptor improves survival upon nematode infection, while co-application of AKH during the infection increases mortality rate. Similarly, deficiency for the AKH increases resistance to the entomopathogenic nematodes, while co-application of the hormone during the infection increases mortality rate also in *Drosophila*. As shown in both studied insects, the co-application of AKH and parasitic nematodes increases metabolic rate of the host, while application of the hormone itself

increases neither metabolic rate nor mortality (at least not in the firebug). Thus, the interference with the host's metabolic rate seems to be the most plausible mechanism whereby AKH increases mortality of insects infected by entomopathogenic nematodes. This finding of the thesis is very interesting and important for the general understanding of the immunity in insects. Moreover, since entomopathogenic nematodes are currently used as means of pest control, results of the thesis may turn useful for the development of better strategies in the insect pest management.

The third manuscript of the thesis investigates the role of vitellogenin the immune response of *Pyrrhocoris*. Authors show that in males, both nematode and fungal infection increases expression of vitellogenin gene. Moreover, infection by entomopathogenic nematode increased also the protein levels of vitellogenin. The purified protein reduced growth of the bacterial symbionts of the nematode, which contributes to the pathogenicity of the nematode. Thus, the increase in vitellogenins following the infection is likely an adaptive immune mechanism of the host.

Altogether, the manuscripts by RNDr. Ing. Emad Ibrahim are novel, interesting, and have significantly enhanced the knowledge on insect immune response. Two of the papers were published in the Journal of Insect Physiology; the last manuscript was submitted to the Journal of Experimental Biology. Taken together, the publication achievements of RNDr. Ing. Emad Ibrahim are clearly impressive.

However, a PhD thesis is also an opportunity to show analytical thinking, which can be manifested by putting the published results into a broader perspective, for example, in a general discussion & conclusion section. In my opinion, the thesis is missing this aspect. Instead of a broader perspective, author just reiterates the findings of each paper separately. The introduction part is sufficiently long, but the quality is somewhat heterogeneous. Author starts with a chapter on AKH. I have several general comments to this part. Firstly, AKH does not have the same functions in all insects, thus, it would be useful to specify in the introduction when author writes about *Drosophila*

and when he just refers to the knowledge based on gain-of-function experiments in other insects. This is important, for example, when talking about the signaling pathway downstream of AKHR in the fruit fly fat body – it is far from being well investigated. Several studies addressed this issue, showing that the fly is likely different than assumed by the classical models by Gerd Gäde almost 20 years ago. Actually, quite a lot has been discovered since then. It is a pity that many key works are not mentioned, and the author focuses mainly on works of his PhD supervisor. Secondly, it would be very useful to discuss into more detail the potential molecular mechanisms whereby AKH regulates immunity. That said, the quality of this part is still good, it just contrasts with the following, much better chapters. For example, the subsequent chapters on the entomopathogenic nematodes and on the insect immune system are very well written, and explain the topic in a really captivating manner. This part clearly documents the writing and didactic skills of the author.

Altogether, RNDr. Ing. Emad Ibrahim presented in his thesis novel and significant findings. The publication achievements of the author are indeed impressive. The thesis clearly documents author's research skills, didactic and writing skills, as well as scientific independence. Thus, the thesis fulfills all requirements for obtaining a PhD degree.

Bratislava, 15th of April 2019

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CAR HAS
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Topic: The review of **Emad Ibrahim's PhD thesis** entitled: **Study of adipokinetic hormone role in insects stressed by entomopathogenic nematodes**

To whom it may concern, Budapest, 15th April, 2019.

I'm honoured for the respect to perform the review of the above entitled Ph.D Thesis submitted to University of South Bohemia, Faculty of Sciences, School of Doctoral Studies in Biological Sciences (České Budějovice, Czech Republic) 91 pp.

According to the information provided by the Head of the Committee for PhD studies in Physiology and Developmental Biology study program before I received this work a formal checking has been performed, and it was found that the thesis has met all necessary formalities of the Faculty of Sciences study rules. So, accordingly, I fully accept this and from now onwards only the contents will considered.

I have carefully read and evaluated the thesis and I place in advance **that overall I have no any hesitation to recommend awarding the Ph.D. degree to the Candidate Emad Ibrahim after a successful defence.** Below I will put forward my comments and questions.

The **(1) Introduction** has provided a very extensive and clear overview of different stressors and stress in insects. This is a hot topic in scientific research describing the environment as the most serious stress factor, while internal stress factors exist as well. The environment stress factors are in the centre and the focus is currently on the entomopathogenic nematodes (EPNs). It is well described that in insects, the major defence biochemical and physiological reactions under unfavourable conditions are controlled predominantly by members of the adipokinetic hormone/red pigment-concentrating hormone family (AKH/RPCH family).

The selection of EPNs for the studies is clearly stated as they are mass-produced and marketed worldwide as biocontrol agents against a wide range of insect species of agricultural or medical importance. Their mode of action and the various mechanisms induced after infestation is of critical interest therefore the approach to study their effect (by stress markers) under laboratory conditions is a very good approach. The various immune response possibilities are also described. The **Candidate** demonstrates and in depth knowledge of this area.

There is insufficient balance between some sub-titles in **Introduction** especially in the light of the topic of **Paper 2** and **3**. In **Paper 2** how AKH and adenosine interferes with EPN infection in *Drosophila melanogaster*, while in **Paper 3** "Changes in vitellogenin expression caused by nematodal and fungal infections in insects" is discussed (suggestion: instead of "in insects" I propose *Pyrrhocoris apterus*). Minimal attention is devoted to adenosine and vitellogenins in respect to their importance in defence mechanisms and to support the studies performed. It should be noted, however, that in the "Summary of PhD Thesis" Introduction the required equilibrium has been well achieved and the text is concise. In my view the title of the thesis itself does not fully cover the content and is a somewhat misleading.

A number of inconsistencies of **(1) Introduction** regarding references in text and list of references are dealt in a separate pdf file including sticky notes. The errors are due to typically non-careful copy-paste actions, since I cross checked these facts, with the two published papers and elsewhere.

In sum, the timeliness and relevance of the chosen topic is unquestionable which is well argued in the **Conclusions** on page 32, and in the two papers and submitted manuscript.

The **(2) Objectives** are very shortly addressed and it should have been better itemized to provide a clear guideline for the overall Ph.D. work and concept. This small paragraph is disproportional in my eyes to the work planned and performed. This is in accordance with my above statement that based on the **(1) Introduction** it is unclear what are the major hypotheses and questions formulated.

The **(3) Results (and Conclusions)** with similar wording from the abstracts summarize the main findings which I wish not to repeat. A bit longer, more detailed and rephrased **(3) Results** would have helped the reviewer to increase the confidence that the **Candidate** is fully competent in the field. One single introductory statement sentence for each paper is very limited in contrast to the content of the papers which are of high quality. Overall, I miss information that which experiments were worked out and performed by the **Candidate** (except in **Paper 3;** while it is also a bit vague what is meant under "Methodology" since no other role of the **Candidate** is listed among the tasks. Being the second author of this paper is insufficient proof of evidence). Nowadays, it's normal and general –especially in case of PhD students– that they work in a team with the help of the supervisor. Up till the defence, -if possible- I would like to ask for a detailed list from the **Candidate** with specific points and level of contribution for each paper to help me judge his competence. I wish to compose a full and correct view of his acquired expertise.

General remark: As for the nematode, fungus and hormone treatments in all three cases the methods and conditions are clearly described and/or referenced. However, this is not the case at numerous places in the three papers that how many individuals were tested (and/or pooled) in different biological replicates. Below are some examples:

Paper 1: 2.4 Metabolic rate measuring; Fig 4. what n= 6-7 means?

2.5 AKHR sequence and phylogenic analysis. This section is not specific enough and the statement "The sequence of AKH open reading frame was confirmed by PCR and Sanger sequencing" is not too convincing

2.6 Akhr RNAi; Fig 2. what n=4-5 stand for?

2.7 Quantification of Pyrap-Akh gene expression and Akhr RNAi efficiency. The quantity of starting material is not defined. n= 10-16?

Similar unclarities of sample preparation are discovered for section 2.9 and 2.10 as well.

Paper 2: 2.4 Metabolic rate measuring; Fig 3. what n= 4-8 represents?

2.5 AKH extraction and level determination by ELISA; Fig 2. what n= 5-8 stand for exactly?

2.7 Total anti-oxidative activity; number of individuals are not provided. n=5 means a pooled sample of 5 replicates?

Paper 3: Quantification of Vg gene expression (in fat bodies); Fig 3. what n=3 represents?

Gel electrophoresis and vitellogenin quantification (in haemolymph). The volume of the haemolymph is not defined, neither the replicates. Fig 4. what n=4 is?

As this manuscript has been recently submitted, later in the revision phase, I advise to better clarify the sampling conditions and make crystal clear their size, number, volume and the biological replicates.

In overall I'm positive that these promising studies will be continued. The investigation of interactions between infections elicited by nematodal and fungal stressors in combination with adipokinetic hormones and adenosine are very interesting. A wide range of stress markers were monitored and very well combined to extract as much results as possible. I accept the presented results and I wish the **Candidate** and the **Supervisor** success and fruitful accomplishments in this very interesting field.

Sincerely yours,

Adrien Fónagy (Ph.D;D.Sc)

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