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The Ph.D. thesis by **Muhammad Aslam** entitled '**Aposematism and toxicity of Coccinellidae**' deals with a very attractive topic, while at the same time brings interesting novel insights. I have some minor criticisms on the work regarding references (scientific literature), somewhat chaotic experimental design at some points, and data analysis not always clearly described. Despite this, I consider the work to be of high overall quality and **I unequivocally recommend it for a successful defence**. The introductory part 'INTRODUCTION' nicely introduces the reader into the investigated problematics and acquaints him/her with the current state of knowledge. The work has a clear structure and I appreciate the number of manuscripts that make up its core. In addition, the six manuscripts cover a greater number of well-related topics and are not just repeating a single idea/concept. Language quality (at least before page 96) is very good. I firmly believe that all manuscripts will be successfully published in scientific journals soon.

As a reviewer, I have a small complaint about the formatting of the electronic version of this thesis, which served as the source for my comments attached below. Numbering of lines across the thesis (at least for unpublished manuscripts) would greatly facilitate the reviewers' work (allow more detailed commenting on the text).

Detailed/minor comments:

INTRODUCTION:

Especially in this section literature sources are sometimes not as rich as I expected – e.g. just a single source in the chapter 1.2.

Chapter 3.3. (Polyamines) is a pure description of chemical composition of polyamines. Is there a known biological importance/function of these compounds? Otherwise, why are polyamines included in the introduction?

Several paragraphs looks like 'ctrl+C ctrl+V' from manuscripts. In my opinion, there is no ethical problem to use your own text twice, but reader can be bored later (when reading your manuscripts).

AIMS AND OBJECTIVES OF THESIS:

III. Why did you expected more deterrence in large-sized ladybirds? Is there a biological explanation/theory-driven hypothesis?

ARTICLE 1:

In general, a very nice paper – congratulations!

Do I understand well that no regular post-hoc tests (e.g. Tukey) were applied?

Why no regular control treatments were applied (used for statistical analyses)? Why attack/eating rates were not measured/presented for mealworms – were all mealworms attacked and consumed immediately (in a few seconds)? It would be nice to show this information also in Figures (2 and 3).

Do attack/consumption rates decrease also for mealworms in the case of *Gallus*?

It is a pity that adult ladybirds were not included in the experiment and thus only a comparison to previously published data is discussed (Veselý et al. 2016). Is it possible that larvae and pupae are more strongly chemically protected than adult ladybirds?

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ARTICLE 2:

Better to move the 'experimental area' section into '*Experiment 1*', as it is not related to the experiment 2.

My experience is that the plasticine is quite tasty for some animals (see consumption by gastropods recorded in your study), but on the other hand acrylic paints are very stable and inert material (probably nobody consumes it). Is it possible that the difference between aposematic models and these green and black are caused by acrylic coating? Why was the same coating (green and black) not used for non-aposematic models?

Did you consider treatments including also chemical defence (i.e. models coated with extracts from ladybirds)? This can provide very elegant comparison of the effects of visual and chemical stimuli. I can imagine that in the ARTICLE 1 there was a limited number of birds available – but it is no problem to made thousands of artificial larvae.

I was also thinking about the possibility to include real ladybirds (larvae) as one of treatments – but how to make them immobile and preclude their deterioration after a few days … But real *H. axyridis* pupae can be easily used for this experiment than . Can you estimate whether predation rates for real larvae would be much lower than these observed for aposematic plasticine models?

How it is possible that the interaction between the type of larva and predator ($F_{2,145} = 1.29$; $P = 0.28$) was not significant but birds are deterred by aposematic colouration and arthropod predators not (see your own text)?

In Figure 2 there is a visible tendency to attack green models more frequently also in the case of arthropod predators – is there any explanation for this pattern?

ARTICLE 3:

This is a very interesting work. A tool that allows to measure/estimate age for ladybirds collected in nature can be useful for a wide range of future studies.

Are you considering to publish existing data (or extend it with additional measurements) in a regular scientific paper?

I can't understand this statement: 'There was no decrease of carotenoids in individuals with large melanic patterns. This means that carotenoids are not deposited under black spots.' No decrease in melanics indicates that carotenoids are stored under black spots rather than their absence.

Do you expect that other conditions besides temperature can affect the speed of carotenoids accumulation (e.g., starvation, pathogen load, mating frequency, etc.)? Are you considering to perform some additional experiments investigating these effects?

ARTICLE 4:

This manuscript is more problematic (mainly methodologically) for me than the previous.

It is under review in *Insectes Sociaux* or *Insects* (just for my interest)?

Abstract/results: 'However, the differences were too small and variable to have biological meaning.' Can you explain based on which facts/existing evidence (published studies?) you decided that the differences observed are biologically irrelevant?

In chapter 1.5.2. you are writing about spiders but the title is 'Chemical defences against ants'.

Based on my experience there are large differences among ladybird species in their speed of haemolymph coagulation. At the same time, haemolymph contains a high concentration of

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alkaloids etc. Your solution (water + honey) probably can't preclude the coagulation. Were the solutions (extracts) that you used really homogeneous?

It seems that a linear decrease is not the best function to fit the relationship between extract concentration and acceptance by ants in all cases (see for example Figure 3). Did you considered/tried any non-linear functions?

Based on the description in methods, I expected that five concentrations were used for all ladybird species. Why there are just four for some species (e.g. Figure 4) and six for others (e.g. Figure 6)?

Do you have an explanation why small species (*Adalia*, *Tytthaspis*) seem to be more repellent than large ladybird species? Can it be related to allometry of body parts (e.g. exoskeleton or body fluids)? I noted that you mention in the discussion that there is no relationship between body size and effects on ants – however, did you really performed an analysis testing this? Maybe I missed it.

ARTICLE 5:

A very interesting study for me!

In the introduction, you refer almost exclusively to 'very old' literature (from the previous century:-) and mention that 'very little is known about scavenging on arthropod carrions'. I checked WoS briefly and there are several new studies published to this topic.

I miss the logic of the 3rd set of species – the 1st served as test/selection of suitable (palatable) species; the 2nd served for comparison of various ladybird species … but why did you selected the species combination used in the 3rd set?

I agree that data for 12 and 24 hours are interesting (and even longer exposition can be interesting = how long can dead ladybirds persist in the environment?) … but this can complicate the division of datasets to night and day time. Can be the absence of significant differences between day and night partly caused by this long exposition of carcasses?

Regarding statistical analyses, my first choice would be Cox proportional-hazards model – but it is just a matter of taste and results will probably be the same.

It is a pity that you didn't record the species scavenging particular carcasses – is it possible that different species were removed by different scavenger taxa? For example, just carabids were removing ladybird carcasses?

ARTICLE 6:

Why was the ladybird material used for the experiment so heterogeneous and the experimental setting so chaotic? There are different ages, different phenologies for different species – no clear systematic approach (e.g. last larval instar, young adult and old adult replicated for each investigated species).

Haxy 1m = 3 months old (or 1 month)? See also the legend to the Figure 1 – one month old *H. axyridis* are mentioned there.

Your computation of 'x' probably give advantage to larvae against adults as adult fresh mass include quite heavy exoskeleton. Can this 'bias' somehow affect your results?

Why GLM was used when data had normal distribution of errors? Did you used a regular post-hoc tests to analyse pair-wise comparisons?

Is it possible that relatively low toxicity values recorded for *Calvia* and *Coccinella* do not represent species-specific traits but rather the consequence of phenological stage (adults of these species were collected during their overwintering)? I can imagine that during winter

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season ladybirds have to devote their energy into more important functions than is being as toxic as possible (e.g. to limit chill injuries, to preserve energy reserves etc.).

In Prague, 24th February 2020

Ing. Michal Knapp, Ph.D.

Review of Ph.D. Thesis

Author: Muhammad Aslam, MSc

Title: Aposematism and toxicity of Coccinellidae. Ph.D. Thesis Series, 2020, No. 5. School of Doctoral Studies in Biological Sciences, University of South Bohemia in České Budějovice, Faculty of Science, 137 pp.

The presented Ph.D. Thesis was completed at the School of Doctoral Studies in Biological Sciences, University of South Bohemia in České Budějovice, Faculty of Science under the scientific supervision of doc. RNDr. Oldrich Nedved, CSc. The Ph.D. thesis, which is 137 pages long, contains a collection of six works artificially divided into four chapters. Two works are already published and four works have either been accepted or sent to the press or are still in the manuscript stage. In five works, the dissertant is the first and probably the corresponding author. One of the published works is published in the prestigious journal Ecological Entomology. (IF = 2.073), the second accepted by Entomologia Experimentalis et Applicata (IF=1.623). In all papers and MS the dissertant carried out experimental work and contributed to manuscript writing.

This Ph. D. thesis deals with a wide variety of problems with the main focus on various aspects of the warning coloration and toxicity of coccinellids and the effect of these factors on vertebrate and invertebrate behavior. Attention was paid to the predatory behavior of birds, the influence of toxic substances on the ants of the genus Lasius and the crustaceans of the genus Daphnia, the deposition of carotenoids in the elytrae of two species of coccinellids and the disappearance of palatable and toxic carcasses of arthropods exposed free in the natural environment. The research topics that the dissertant investigated have not been sufficiently studied so far and the results are thus of interest to a wider audience.

First work, Aslam, M., Veselý, P., & Nedvěd, O. (2019). Response of passerine birds and chicks to larvae and pupae of ladybirds. Ecological Entomology. 44 (6): 792-799, studies response of three bird species provided with the coccinellid larvae and pupae as food. I have two questions. First, as predators were two species of passerine birds caught from the open and two-day old chickens borrowed from an industrial farm: As far as I know, chicks in industrial farms are sexed after hatching and only females are left for further breeding. I wonder if the experimental chicks were sexed and, if so, whether wild birds were also sexed and only females were used for experiments? Could predator sex influence acceptance and consumption of prey? I also would like to know whether mealworms, an unnatural food provided to chickens, could influence them in the choice of insect prey.

In a closely related second work Aslam, M., Nedvěd, O. & Sam. K. (2020). Attacks by predators on artificial cryptic and aposematic insect larvae. Entomologia Experimentalis et Applicata. (accepted) the authors made artificial, differently colored models of larvae from plasticine and found that models imitating aposematic prey repel birds. I wonder whether the differences in the basic color of the artificial larvae, the blue color called "black" in the text of the figure on page 36 and green color, were created using differently colored plasticine or by surface coloring of the originally monochromatic plasticine. If plasticine of different colors was used, could this difference affect the predatory behavior of birds? I also remind that mice are eating plasticine intensively - could mice participate in predation of models in nature? The authors discriminated the categories of predators based on the damage to plasticine models of larvae. On page 33, the PhD thesis states: „This method is successfully used and described in detail in previous studies..." Since this distinction is essential, more details concerning the method should be given in the PhD defense!

Further already published paper Nedvěd O., Aslam M., Abdolahi R., Sakaki S., Soares A.O. (2019). Age and temperature effects on accumulation of carotenoids in ladybirds. IOBC WPRS Bulletin, 145: 33-36. (Summary) deals with the influence of temperature and age of coccinellids adults (Coccinella undecimpunctata and Harmonia axyridis) on the deposition of carotenoids on ground colour of the elytrae. The work is an important contribution to determining the adult age in natural populations. One small question: where did the authors catch Coccinella undecimpunctata in the Czech Republic?

The manuscript Aslam, M., & Nedvěd, O.: Response of the ant Lasius niger (Hymenoptera: Formicidae) to extracts from ladybirds (Coleoptera: Coccinellidae) has already been sent to „Insects", a journal where the review process begins with the creation of a kind of provisional „reprint". What is the present situation of review proces? The authors quantify the effect of the concentration of coccinellid repellents dissolved in honey on Lasius ants' behavior. The work brings clear and expected results. The introductory part is in the form of a small review, which does not, however, completely relate to the further examined issue and in some parts (e.g. paragraph 1.1) it is difficult to understand. Part (2.2) is unnecessarily long.

The work Aslam, M., & Nedvěd, O. Scavenging rate of palatable and toxic arthropod carrions during day and night (manuscript) deals with a little investigated problem of scavenging invertebrate carcasses. The author(s) conclude that the repulsive effect of coccinellids persists even after death. A very interesting topic would deserve still further elaboration. Also the last manuscript Aslam, M., & Nedvěd, O. Toxicity of extracts from ladybirds (Coleoptera: Coccinellidae) for water flea Daphnia magna (Cladocera) provides very interesting results concerning the toxicity of different coccinellid species. Important is establishing the absence of a difference in the toxicity of melanic and non-melanic forms of Harmonia axyridis.

In conclusion, the thesis is of high quality and fully meets the conditions required for the defense of Ph.D. thesis. This work clearly demonstrates that Muhammad Aslam is a promising and competent young researcher. I propose that the thesis be admitted to the approval procedure, classified to the best grade and, after a successful defense, that Muhammad Aslam receive the appropriate scientific degree in accordance with the directives.

V Praze

29. 2. 2020

RNDr Alois Honěk, CSc.