



**MEDICAL
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DEPARTMENT OF GENERAL BIOLOGY AND PARASITOLOGY

Warsaw, March 16th 2023.

**Review of the doctoral dissertation by
Hynek Mazanec MSc
entitled
„Isolation and characterisation of extracellular vesicles of parasitic helminths.”**

The basis for this review is the letter of doc. RNDr. Eva Nováková, PhD., Program director of PhD studies in Parasitology at Faculty of Science, University of South Bohemia, from the March 8th, 2023.

The doctoral dissertation of Hynek Mazanec, MSc was prepared within School of Doctoral Studies in Biological Sciences of the University of South Bohemia in České Budějovice under the supervision of doc. RNDr. Roman Kuchta, Ph.D. from the Institute of Parasitology, Biology Centre, Czech Academy of Sciences in České Budějovice.

The doctoral dissertation of Hynek Mazanec consists of two published original papers, in which the PhD student is the first author, in addition one article is under preparation and one with his minor participation (5%), to which he provided figures and took part in developing the roadmap for studies of extracellular vesicles from parasitic helminths. Despite his minor participation in the preparation of the last publication, it shows his commitment to the work in the leading research teams working on the topic chosen by the doctoral student, and the ability to establish valuable cooperation.

It is worth mentioning here that Hynek Mazanec presented his research results during national and international conferences and was invited to give the lecture at 20th International Conference on Diseases of Fish and Shellfish in 2021. Moreover he is an active academic teacher and supervisor of bachelor thesis at the Faculty of Science, University of South Bohemia, Czech Republic.

The research of Hynek Mazanec PhD thesis was carried out as part of research projects listed in the PhD thesis and in the accompanying publications. It is not known, however, whether the Hynek Mazanec was a participant in these projects or their author.

Hynek Mazanec dissertation consists of 106 numbered pages, including copies of scientific articles presenting the results of the research, and the statements of the co-authors. Its layout is typical for doctoral dissertations.

The original results of Hynek Mazanec dissertation are presented in the following articles:

- **Mazanec, H., Koník, P., Gardian, Z., Kuchta, R. (2021).** Extracellular vesicles secreted by model tapeworm *Hymenolepis diminuta*: Biogenesis, ultrastructure and protein composition. *International Journal for Parasitology*, 51, 327–332. [IF 2021: 4.25]. In which he conceived the study, carried out the isolations, TEM observations, proteomic analyses and drafted the manuscript. (85%)
- **Mazanec, H., Bušková, N., Gardian, Z., Kuchta, R. (2023).** Secretion of extracellular vesicles during ontogeny of the tapeworm *Schistocephalus solidus*. *Folia Parasitologica*, 70:003. [IF 2021: 1.614]. Where he conceived the study, established the culture, performed TEM observations and drafted the manuscript. (70%)
- **Mazanec, H., Sotillo, J., Koník, P., Gardian, Z., Bílý, T., Jirků-Pomajbíková, K., Kuchta, R.** Biogenesis machineries and secretion dynamics behind different populations of extracellular vesicles of tapeworm *Hymenolepis diminuta*. (Manuscript in preparation). Here Hynek Mzazanec conceived the study, carried out the isolations, TEM observations, performed proteomic analyses and drafted the manuscript. (85%)

Additionally, Hynek Mazanec is the co-author of the review paper also included as the part of his PhD thesis. This paper represents guidelines for studies of extracellular vesicles from parasitic helminths (White, R., Sotillo, J., Ancarola, M. E., Borup, A., Boysen, A. T., Brindley, P. J., Buzás, E. I., Cavallero, S., Chaiyadet, S., Chalmers, I. W., Cucher, M. A., Dagenais, M., Davis, C. N., Devaney, E., Duque-Correa, M. A., Eichenberger, R. M., Fontenla S., Gasan, T. A., Hokke, C. H., Kosanovic, M., Kuipers, M. E., Laha, T., Loukas, A., Maizels, R. M., Marcilla, A., Mazanec, H., Morpew, R.M., Neophytou, K., Nguyen, L. T., Hoen, E. N., Povelones, M., Robinson, M. W., Rojas, A., Schabussova, I., Smits, H. H., Sungpradit, S., Tritten, L., Whitehead, B., Zakeri, A., Nejsun, P., Buck, A. H., Hoffmann K. F. (2023). Special considerations for studies of extracellular vesicles from parasitic helminths: A community-led roadmap to increase rigor and reproducibility. *Journal of Extracellular Vesicles*, 12, 12298. [IF 2021: 17.337]). In this article Hynek Mazanec contributed to the review assembly, provided data evidence and reviewed the final manuscript (5%).

The above journals belong to the group of leading periodicals in the field, this proves the quality of the conducted research, which has already been critically assessed by independent experts. The databases show that these publications are cited by other researchers, so they aroused the interest of the scientific community.

In his thesis Hynek Mazanec formulated several aims and objectives (p. 41). The ultimate goal was to evaluate the presence of extracellular vesicles (EVs) in two species of tapeworms (*Schistocephalus solidus* and *Hymenolepis diminuta*) and explain the mechanism of their secretion in this group of parasites. Detailed goals are interesting and may provide important information related to parasite biology and survival strategies.

I consider the selected topic of the dissertation to be fully justified and interesting. Research in recent years has indicated a number of mechanisms used by parasites to survive in the hostile environment of the host. Some of them lead to immunomanipulation, and others allow to change the behavior of the infected host to ensure the parasite's biological success. It has been known for some time that the carriers of these molecular modulators can be extracellular vesicles (EVs). EVs are small membrane-bound vesicles that are generally classified based on their size, biogenesis and composition. The current perception about EVs has changed drastically due to their newfound role as mediators in the transmission of complex biological signals and immune responses. Nowadays EVs are considered as key players implicated in intercellular crosstalk between parasite and its host, without direct cellular

contact. These vesicles of various origins can carry as their cargo a wealth of bioactive molecules such as proteins, lipids, DNA, mRNAs and miRNAs, through which they can regulate their targeted cells. Moreover parasite-derived vesicles most probably mediate diverse environmental adaptation, especially in species with complex lifecycle and host change. Apart from the seemingly obvious role of EVs as carriers of intercellular mediators, they are also considered as a promising target for immunotherapy of autoimmune diseases.

Although important discoveries concerning the role of EVs in parasitic diseases have been made, there is still much left that is poorly understood. For example, the nature of the cell machinery responsible for EV release and uptake and the mode of transport to their final destinations are unclear. Moreover, although some cargo components seem to be selectively loaded into EVs, how the cargo is loaded and released mostly remains unknown, it means that EV biogenesis mechanisms remain elusive. Therefore, research conducted by a doctoral student broaden our knowledge in this area, in particular with regard to parasites such as tapeworms. His work focused on studying the patterns of EVs secretion in tapeworms with different life cycles and host species.

Identification of molecules from different developmental stages and species may indicate specific and crucial mechanisms related to developmental and invasion stage of the parasite. Particularly interesting are those involved in the invasion mechanism, host modulation/manipulation (immune and neuro system) and metabolic processes. These proteins can be of great importance in our understanding of the key points of the parasite's developmental biology, but also in the search for new diagnostic and therapeutic methods.

The thesis begins with an introduction (40 pp.), in which the author presents in a synthetic way the most important information related to his research and a review the most important literature. This part is complemented by carefully selected figures and tables. After this synthetic introduction, he discusses the aims and objectives, however, the research hypotheses are not clearly presented. Then the author smoothly proceeds to the presentation of the results of the published research. In the presented articles, the PhD student verifies his goals in a logical and coherent manner. It is worth emphasizing that the results are additionally presented in very well-designed and thought-out figures, also prepared with the use bioinformatic tools (paper in preparation). The whole dissertation ended with the chapter "Summary", in which the PhD student synthetically presents the main assumptions and research results. On the following pages, the author briefly presents the original value of the research,

indicating the most important achievements. In my opinion, apart from the summary, the doctoral thesis should contain clearly defined conclusions that do not repeat the results but are based on them. Therefore I would ask the PhD candidate for a synthetic indication of the most important conclusions.

In my opinion, the most important results presented in Hynek Mazanec dissertation are:

1. Development of a method allowing to obtain reproducible results of research on extracellular vesicles in tapeworms.
2. Description of the biogenesis of EVs in adult stages of the model tapeworm *Hymenolepis diminuta* maintained in a controlled life cycle in the laboratory conditions.
3. Indication of novel mechanisms in EVs biogenesis, such as the involvement of dense bodies and the formation of beaded protrusions.
4. Indication the influence of host proximity on EVs biogenesis by observation of a subpopulation of large, direct-budding vesicles presented only after immediate fixation upon removal from the host intestines.
5. Drawing attention to the differential secretion of vesicles in freshly collected tapeworms in comparison with *in vitro* culture. Overall, the results provided evidence for the negative effects of prolonged cultivation on the composition and structure of EVs.
6. Partial elucidation of mechanism behind the formation of beaded protrusion and their subsequent dissociation into single or elongated forms of EVs.
7. Detailed study of the secretion of EVs along the life cycle of a tapeworm *Schistocephalus solidus*
8. Detection of tegumental structures e.g., beaded protrusions, distorted regions of the tegument, which appear to be unique for tapeworms.
9. Mass spectrometry identification of proteomic cargo in EVs of tapeworms.
10. Participation in the discussion on the common obstacles in the study of helminth-derived EVs resulting in guidelines specific to the studies of helminth-derived EVs. This should result in the reproducibility and comparability of studies across different organisms.

As mentioned above, the results of the Hynek Mazanec research have already been largely published, so they have been evaluated by other reviewers and modified according to their suggestions. However, I have following comments and questions that I would like to ask the PhD student for clarification and to stimulate the discussion.

- I have the impression that the title of the dissertation does not fully reflect the obtained results? The results of the research concern two relatively different species of tapeworms, not the representatives of all helminth groups. In my opinion this should be specified in the title of the dissertation either by listing the species of parasites or changing it to, for example "Isolation and characterization of extracellular vesicles in selected tapeworm species". Otherwise, one can get the impression that the doctorate presents research results from a number of species belonging to different taxa.
- One of the objectives was to establish a stable and sustainable life cycle of *S. solidus* under laboratory conditions. The adults have been obtained through cultivations in an artificial medium. Wasn't the maintenance of *Schistocephalus* in laboratory conditions previously developed?
- As mentioned by the author, existing studies suggest intermediate host manipulation (first and second). He states that this makes *S. solidus* a suitable model for studying of EVs with immunomodulatory potential. Don't you think that in addition to their immunomodulatory functions, the molecules released by the parasite can affect the host's nervous system and thus lead to changes in its behavior. Do you know studies related to this topic?
- What is the location of the larvae in the intermediate hosts of *S. solidus*, and is it possible that they interact mechanically with the host nervous system, which may cause behavioral changes - especially in the copepods. If not, is there evidence that the mechanical impact is of minor importance?
- Author decided to establish an isolation protocol for the separation of EVs from tapeworms? Wasn't the protocol for isolating exosomes from tapeworms previously known? What is new in the protocol proposed by PhD student?
- I understand the aims and objectives and I'm sure that received results are novel and valuable, however, I would like to know what was the research hypothesis that was

verified by the implementation of the assumed goals? What was the starting scientific question?

- Was a protease inhibitor used during preparation of proteins for MS analysis?
- How many technical and biological replicates were used for MS study? What were the exclusion criteria for MS results?
- How the identification of potential immunomodulators in the EVs cargo was evaluated? Were any immunoproteomic techniques or functional studies used, or were immunomodulators selected based on literature data?
- Were attempts made to analyze the complete content of EVs (DNA, mRNA, miRNA, lipids)? If not, were there any technical obstacles?
- Did *String interaction network analysis* allow the identification of particularly interesting proteins/protein-protein interactions? What is the biological significance of these data?
- It is worth noting that before the use of terms such as extracellular vesicles, exosomes, etc., the authors used a different nomenclature? Is the doctoral student familiar with previous ultrastructural studies indicating the presence, release mechanism and importance of extracellular vesicles in other tapeworm species?
- Finally, I would like to ask the PhD candidate to indicate, which of the results he would like to consider as the most important and why?

Regardless of the aforementioned minor comments, I highly appreciate the doctoral thesis of Hynek Mazanec, MSc. The obtained results are undoubtedly valuable and expand our knowledge on the role of EVs in the biology of selected tapeworm species, with particular emphasis on their biogenesis. In my opinion, some of these results may help in the future evaluation of the practical application of parasite derived molecules eg. in studies on their use in autoimmune diseases. The use of a properly selected methodology and the variety of presented analyzes prove a comprehensive approach to solve the research problem. Moreover, the publication of the results as the first author in well-established journals proves the scientific maturity and independence of the PhD candidate as well as the confirms the scientific value of the results.

Final conclusion

I would like to emphasize that I highly appreciate the PhD dissertation by Hynek Mazanec MSc and state that it undoubtedly meets the requirements for doctoral dissertations. Therefore, I am asking the Scientific Council of University of South Bohemia in České Budejovice to admit Hynek Mazanec MSc to further stages of the defense of his doctoral thesis.

The presented dissertation is characterized by high-quality research carried out with the use of modern methods and has significant cognitive values. Appreciating the high value of the research results published in well-known journals, in which Hynek Mazanec is the first author, I am pleased to ask to award the doctoral dissertation of Hynek Mazanec, MSc.

