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Warsaw, August 23rd 2022.

Review of the doctoral dissertation by

Robert Stryński MSc

entitled

„Proteome profiling of the parasitic nematode *Anisakis simplex* s. s.”

The basis for this review is the letter of prof. dr hab. Nina Smolińska, Chairwoman of the Scientific Council of the Discipline of Biological Sciences, University of Warmia and Mazury in Olsztyn, from the July 22nd, 2022.

The doctoral dissertation of Robert Stryński, MSc was prepared at the Department of Biochemistry, Faculty of Biology and Biotechnology, University of Warmia and Mazury in Olsztyn, Poland and at Department of Food Technology, Institute of Marine Research, Spanish National Research Council in Vigo, Spain under the supervision of Elżbieta Łopieńska-Biernat, PhD, DSc, prof. UWM in the role of the supervisor and Dr. Mónica Carrera Mouriño, PhD in the role of the auxiliary supervisor. Taking into account the subject of the dissertation and the fact that research was carried out in two scientific centers (in Poland and Spain), the participation of the auxiliary supervisor is justified and does not raise any doubts.

The doctoral dissertation of Robert Stryński consists of two published original papers, in which the PhD student is the first author. The research 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 PhD candidate was a participant in these projects or their author. However, it is worth emphasizing that Robert Stryński was the recipient of the scholarship

form the POWR program funded by the European Social Fund. It has proved effectiveness in obtaining external funds for his research.

It is worth mentioning here that Robert Stryński is the co-author of the publication "Genome-wide analysis of *Anisakis simplex* sensu lato: the role of carbohydrate metabolism genes in the parasite's development", which certainly contributed to a better identification of proteins and the interpretation of the research results of the PhD student and other researchers.

According to the dissertation sent to me for evaluation, the PhD candidate performed his analyzes in cooperation with researchers from Poland and abroad (PhD thesis p.11). This ability to undertake fruitful cooperation also deserves noting.

The results of Robert Stryński's dissertation results are presented in the following original scientific articles:

- Stryński R., Mateos J., Pascual S., González Á. F., Gallardo J. M., Łopieńska-Biernat E., Medina I., Carrera M. Proteome profiling of L3 and L4 *Anisakis simplex* development stages by TMT-based quantitative proteomics. *Journal of Proteomics*. 2019; 201: 1-11, <https://doi.org/10.1016/j.jprot.2019.04.006> (IF = 4.044; MEiN 100 points).
- Stryński R., Mateos J., Carrera M., Jastrzębski J. P., Bogacka I., Łopieńska-Biernat E. Tandem Mass Tagging (TMT) reveals tissue-specific proteome of L4 larvae of *Anisakis simplex* s. s.: enzymes of energy and/or carbohydrate metabolism as potential drug targets in anisakiasis. *International Journal of Molecular Sciences*. 2022; 23(8): 4336. <https://doi.org/10.3390/ijms23084336> (IF = 6.208; MEiN 140 points1).

The above titles belong to journals with a high impact factor (IF), as for the research field represented by Robert Stryński MSc. This proves that they belong to the group of leading periodicals in the discipline. In addition, the results of the doctoral dissertation were probably presented by the PhD student at scientific conferences.

On the basis of the dissertation, Robert Stryński MSc is applying for a doctoral degree in the discipline of biological sciences. In the review below, I focused on evaluation criteria tailored to the requirements of these doctoral dissertations.

Robert Stryński formulated two following research hypotheses:

1. L3 and L4 larvae of *A. simplex* nematodes differ in their proteomic profiles at the global level;

2. the tissues, the fully functional intestine of the L4 stage of *A. simplex*, in contrast to the L3 stage, and the cuticle are characterized by specific proteins responsible for growth, development and contact with the host.

The hypotheses are presented in the Objectives section. In my opinion, this chapter should be called *Hypotheses and Research Objectives*. Unfortunately, it does not include the goals that were to be achieved in order to verify the hypotheses set by the PhD student. There is a fundamental difference between research goals and hypotheses.

I consider the selected topic of the dissertation to be justified. Among food biological hazards for human and animal health, infective agents - including parasites - are particularly important. Globalization has increased changes in our eating habits, including the widespread consumption of raw, dried, marinated or smoked fish. Simultaneously there is alarming increase in the number of unidentified food allergies among the human population. Recent studies indicate that the cause may be due to sensitization to *Anisakis* spp. antigens, representing a public health issue, whose clinical manifestations are characterized by digestive disorders, asthma, dermatitis, and even anaphylaxis.

Nematodes of the Anisakidae family are worldwide distributed marine fish and mammals parasites. The larvae live in the gut, visceral peritoneum, and flesh of many marine fish and cephalopod species. Humans becoming accidental hosts of the *Anisakis* parasite when swallow raw or undercooked fish containing L3 larvae. Within a few hours of being ingested, *Anisakis* larvae, when alive, penetrate the mucosal layers of the gastrointestinal tract, causing direct tissue damage resulting in anisakiasis. This acute gastrointestinal is manifested by clinical symptoms ranging from nausea to vomiting, diarrhea, mild to severe abdominal pain, and intestinal obstruction, mimicking other common gastrointestinal disturbances, such as acute appendicitis, gastric ulcer, or tumors, thus making the diagnosis of anisakiasis difficult. Diagnosis is generally obtained through endoscopy, serum-specific anti-*Anisakis* IgE determination, or surgery if the worm has embedded. Visualization and morphological identification of the larva(e), removed during endoscopy or surgery or expelled by patients' cough is the conclusive assessment. Because of numerous technical difficulties and lack of the unambiguous diagnostic criteria and laboratory algorithms, anisakiasis is most probably under-diagnosed.

Nowadays, mass spectrometry biotyping is a rapid, easy and validated method for accurate microbial phenotypic identification approved for routine use and identification of pathogenic microorganisms, such as bacteria, yeasts, and filamentous fungi. Unfortunately, this method is not so widely included in the diagnosis of parasitic diseases. A recent application of MALDI-TOF MS for *Dirofilaria* and *Ascaris* protein-based profiling showed a promising scenario towards clinical applications involving biotyping of helminths, and probably protozoans in the near future. Identification of proteins of different developmental stages can provide not only new diagnostic markers but also indicate specific and key proteins for a given stage. Particularly interesting are those involved in the invasion mechanism, immunomodulation and metabolic processes. These proteins can be of great importance in the search for new diagnostic and therapeutic methods, and in our understanding of the key mechanisms of the parasite's developmental biology. Therefore, I believe that apart from the cognitive aspects, results presented in the Robert Stryński's PhD Thesis may also have a practical aspect.

Robert Stryński's doctoral thesis consists of 99 numbered pages, including copies of scientific articles containing the results of the research and the statements of the co-authors. Its layout is rather typical for doctoral dissertations.

The thesis begins with a summary, a table of contents and a list of abbreviations used. In a summary, prepared in Polish and English, the PhD student synthetically presents the main assumptions and research results. Then the author lists the articles that constitute the essence of the doctoral dissertation. On the following pages, the author briefly presents the original value of the research, indicating the most important achievements, such as:

- the largest dataset of *A. simplex* proteins for two developmental stages (L3 and L4) identified to date;
- usefulness of the identified tissue-specific proteins to initiate further molecular studies related to this parasite, and targets for new drugs against anisakiasis.

At this point, the question arises what criteria for the inclusion and exclusion of the identified proteins were used by the author to conclude that his results represents the largest dataset of *A. simplex* proteins for two developmental stages. Are they comparable to those used by other authors? Maybe they are more restrictive or milder? If they differ from previous studies, why criteria used by author were included?

Next, the authors' contribution in the preparation of the publication, research funding and scientific cooperation are provided. In the following chapters, the author presents in a synthetic way the most important informations and a review of the literature referring to the research topic. This part is complemented by carefully selected figures. After this synthetic introduction, he discusses the hypotheses, not fully presenting the objectives of the research - as I mentioned above. Then the author smoothly proceeds to the presentation of the results of the published research. In the presented articles, the PhD student verifies the hypotheses presented in the dissertation in a logical and coherent manner. It is worth emphasizing that the results were additionally presented in very well-designed and thought-out figures prepared with the use of modern bioinformatics tools. The whole dissertation ended with the chapter "Conclusions", which in my opinion contains a summary. At this point, I would ask the PhD candidate for a synthetic indication of the most important conclusions. The cited literature has 185 items (including original papers of the PhD student) to a large extent these are papers published in recent years, which also proves the topicality of the issue raised.

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

1. The largest dataset of *A. simplex* proteins for two developmental stages (L3 and L4) identified to date.
2. Selection of L3 and L4 stage-specific proteins, which could be used as targets for control/elimination of this parasite.
3. Indication of potential novel allergens of *A. simplex* (pseudocoelomic globin, which was assigned to L3 larvae, prolylcarboxy peptidase like protein 5, and L-threonine-3-dehydrogenase, which was selected as one of the L4-specific proteins).
4. Comparative analysis of the individual tissues, which revealed a total of 272 differentially regulated proteins, with 133 proteins more abundant in the cuticle and 139 proteins in the intestine, with a particular note to the enzymes involved in glycolysis and the tricarboxylic acid cycle, as these metabolic pathways were most enriched by cuticle and intestinal proteins, respectively, in the L4 stage of *A. simplex*.
5. Analysis of oxoglutarate dehydrogenase (OGDH) and folliculin (FLCN) presence and structure in L3 and L4 larval homogenates, as potential candidates for controlling the survival of *A. simplex*.

6. Tissue-specific host-parasite interactions studies, indicating that OGDH from the parasite intestine was particularly interactive, and polyubiquitin-C was the major identified host target interacting with *A. simplex* proteins. Cuticular proteins of *A. simplex* interacting with the human or dolphin host included serine/threonine protein phosphatase, 2 N-domain-containing peptidase M1 proteins, and cofactor-independent phosphoglycerate mutase.
7. Suggestion that the search for novel molecules that can inhibit the iPGM of *A. simplex* may be the basis for further exploration of anisakiasis control methods.
8. Presented results discussed new possibilities in the study of enzymes of energy and carbohydrate metabolism as potential drug targets in anisakiasis.

The results of the analyzes carried out by Robert Stryński have already been published and thus assessed by at least several reviewers and modified in accordance with their suggestions. However, I have a few questions that I would like to ask the PhD student for clarification. In addition to my previous comments, I present below a few minor remarks to the substantive part of the dissertation.

- The title of the dissertation should be more precise and better reflect the research results. At present, one might suppose that all developmental stages have been profiled, and this is not the case.
- Many times in the text, the PhD student mentions fish as hosts of *Anisakis*. It is worth clarifying that it is about marine fish. Additionally, for example, in the abstract, the doctoral student mentions "*A. simplex* is a parasitic nematode that causes anisakiasis in humans". In fact, anisakiasis is a disease that mainly affects marine mammals, and humans become infected accidentally.
- The taxonomic classification and species names provided in the dissertation should comply with the current guidelines contained in the "International Code of Zoological Nomenclature". For example, on page 20 there is "(genus) *Anisakis*" and it should be "Genus: *Anisakis*, Dujardin, 1845". Is the PhD candidate sure that the entry *Anisakis simplex* Rudolphi, 1920 (p. 20) is full and correct?
- In my opinion, chapter 2.3 "Proteomics" lacked a little more attention to the application of particular methods in parasitological research. Especially in the

context of parasites biology, diagnostics, searching for new diagnostic methods and therapeutics.

- Was a protease inhibitor used in the isolation of *Anisakis* proteins?
- The doctoral student reports that all analyzes were made in triplicate. Were they technical or biological replicates?
- Did *Network analysis* allow the identification of particularly interesting proteins/protein-protein interactions? What is the biological significance of these observations?
- Has the analysis of the presence of host proteins in the isolated intestines of the larvae been carried out? How do you know there were no host contaminants in the samples analyzed?
- Did sequencing of the *Anisakis* genome improve protein identification? I am referring especially to the first publication in the dissertation cycle.
- This thesis begs the question of the availability of positive patient sera. Were immunoblots performed / planned with the use of sera from infected patients and identification of immunoreactive proteins? This could provide information on potential diagnostic antigens.

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 minor comments contained in my review, I highly appreciate the doctoral thesis of Robert Stryński, MSc. The obtained results are undoubtedly valuable and significantly expand our knowledge of the biology of *Anisakis simplex*. In my opinion, many of them can also find practical application. The use of a properly selected methodology and the variety of analyzes carried out 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 maturity and independence of the PhD candidate as well as the scientific value of the results obtained.

Final conclusion

I would like to emphasize that I highly appreciate the PhD dissertation by Robert Stryński MSc and state that it undoubtedly meets the requirements for doctoral dissertations required by law. Therefore, I am asking the Scientific Council of the Biological Sciences Discipline of the University of Warmia and Mazury in Olsztyn to admit Mr. Robert Stryński 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 and exceptional value of the research results published in high quality scientific journals, whose first author is Robert Stryński, **I am pleased to ask the Scientific Council of the Biological Sciences Discipline of the University of Warmia and Mazury in Olsztyn to award the doctoral dissertation of Robert Stryński, MSc.**

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