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A review of the doctoral thesis of Anna Żmudzińska
'Age related changes in proteome and quality parameters of sonicated dog epididymal spermatozoa'
completed at the Department of Animal Biochemistry and Biotechnology
University of Warmia and Mazury in Olsztyn
under
the supervision of Marzena Mogielnicka-Brzozowska, post-doctoral degree, professor of UWM
and second supervisor Magdalena Zielińska, post-doctoral degree, professor of UWM

This doctoral thesis review was undertaken at the written request of Professor Tomasz Daszkiewicz, Chairman of the Scientific Council of the Discipline of Animal Science and Fisheries, University of Warmia and Mazury in Olsztyn, of 8 November 2022 (no. WBZ-DZ.6350.3.1.2021).

Scientific problem and importance of the research

Due to continually growing interest in dog breeding, there is a need to explore the specifics of reproduction of this animal species, particularly in terms of improving assisted reproduction techniques. Most studies on dog semen involve the use of sperm collected from ejaculates. In cases when it is not possible to evaluate ejaculate sperm, an alternative is to acquire them from the epididymis. This possibility may prove to be highly significant for the further development of assisted reproduction techniques and conservation of genetically valuable animals. Analyses of the properties of semen obtained from the epididymis may prove to be crucial in the conservation of endangered canid species, particularly in the case of the sudden death of an individual with valuable genetic resources. Another important aspect of research on canine semen is the increasing incidence of various diseases which can exclude genetically valuable individuals from reproduction. Therefore there is a need to assess the degree of development of sperm collected from the epididymis. During the passage of semen through the epididymal duct, changes in sperm structures take place. Protein components originating in the epididymal ducts play an important role in this process. Both the interaction

of proteins with the plasma membrane of sperm and the recomposition of internal proteins play a fundamental role in the maturation of sperm in the epididymides. Epididymal sperm differ from ejaculate sperm in that they are coated only with proteins of the epididymal fluid and have no contact with proteins secreted by the accessory glands. Protein functions, which are closely linked to the fertilization capacity of sperm, can be influenced by a variety of factors. Little is known of the molecular mechanisms associated with changes in the composition of proteins and their effect on sperm quality during the maturation of sperm in the epididymides. The doctoral thesis submitted for review, written by Anna Żmudzińska, is clearly in line with these issues, and the research undertaken by the candidate is fully justified.

Formal and substantive evaluation

The doctoral thesis submitted for review, entitled 'Age related changes in proteome and quality parameters of sonicated dog epididymal spermatozoa', is based on a thematically coherent set of two original scientific papers published in journals listed in the Journal Citation Reports database:

1. Żmudzińska, A.; Bromke, M.A.; Strzeżek, R.; Zielińska, M.; Olejnik, B.; Mogielnicka-Brzozowska, M. Proteomic Analysis of Intracellular and Membrane-Associated Fractions of Canine (Canis lupus familiaris) Epididymal Spermatozoa and Sperm Structure Separation. Animals 2022, 12, 772. DOI: 10.3390/ani12060772 (IF = 3.231; pts = 100).
2. Żmudzińska, A.; Wiśniewski, J.; Młynarz, P.; Olejnik, B.; Mogielnicka-Brzozowska, M. Age-Dependent Variations in Functional Quality and Proteomic Characteristics of Canine (Canis lupus familiaris) Epididymal Spermatozoa. International Journal of Molecular Sciences 2022, 23, 9143. DOI: 10.3390/ijms23169143 (IF = 6.208; pts = 140).

The candidate is the first author of both papers, and her declared participation in the preparation and development of the manuscripts is high, at 65%, which was confirmed by an appropriate statement. The candidate's contribution to the papers consisted in planning and developing the research methodology, acquiring and describing the research material, adapting the software for the analyses, carrying out the research, analysing and interpreting the results, writing the manuscript, and carrying out the editorial process. Both papers were published in journals with international scope, with an impact factor (total points 240 and IF 9.439). It should be noted that these papers received positive reviews from international experts associated with the subject matter and are of high scientific value.

The doctoral thesis submitted for review is written in English and contains 85 pages. The chapters are as follows: Table of Contents (p. 3), Acknowledgements (p. 4), List of

Papers (p. 5), List of Abbreviations (p. 6), Abstracts (p. 7-10), Introduction (p. 11–12), Aims (p. 13), Material and Methods (p. 14–18), Results and Summary of Papers (p. 19–21), Conclusions (p. 24) and Bibliography (p. 25–30), containing 50 literature references.

The doctoral thesis takes the form of a typescript succinctly describing the research results contained in the series of publications. It forms a coherent and logical whole and contains all the essential elements characterizing this type of work.

The title is correctly formulated, although mainly reflective of publication no. 2, while expressing little of the scope of the research in publication no. 1. In the introduction to the thesis, the candidate has concisely introduced the subject of the research and presented information demonstrating the importance of the issues undertaken. The author has used well-selected literature sources to present the most important questions concerning the role of proteomic analyses in explaining the functions of proteins at various stages of sperm maturation in the epididymal duct and the process of sonication in isolation of sperm proteins and cell structures. The arrangement of content in this chapter is well thought-out and reveals a good knowledge of the subject matter taken up by the candidate in her research.

Following the concise and informative introduction to the most important issues of the paper, the candidate presented the objectives and assumptions of the research. The main research objective was to investigate the relationship between the functional quality and proteome of epididymal spermatozoa of dogs differing in age. This aim was achieved by meeting a set of specific aims (points I–VII). The aims presented in points I–III were achieved in publication no. 1, while aims IV to VII were met in publication no. 2. They concerned analysis of the effects of various sonication parameters on the type and percentage of morphological changes in sperm collected from the dog epididymis and on the total content of intracellular proteins and proteins associated with the sperm membrane, as well as identification of differences in protein composition and identification of selected proteins by mass spectrometry (publication no. 1); and evaluation of the functional quality of sperm and proteome analysis of epididymal sperm depending on the age of the dog (publication no. 2). In my opinion the objectives of the research – both the overall aim and specific aims – were correctly formulated and raise no objectives. However, I believe that in this part of the dissertation, research hypotheses should be formulated before the aim of the research is presented. While such a hypothesis was put forth in one of the papers making up the scientific achievement, there was no such hypothesis in the other paper.

The ‘Material and Methods’ chapter is developed correctly and in detail. The author describes the procedures and experimental material correctly and in a manner that is easily

understood. The material is described as semen collected from the epididymides of ‘from 15 to 30 dogs’. In my opinion it should be precisely stated which analyses were conducted using 15 dogs and which using 30 dogs; is it possible that some individuals were used in the studies described in both papers? The choice of research material and the methods used should be considered adequate to the assumptions made by the candidate when she undertook the research. The material was of sufficient sample size and was analysed using advanced diagnostic techniques. The statistical analysis of the results raises no objections. Given the specifics of this type of research and the difficulties involved in conducting it, including collection of suitable research material, the candidate can be said to have organized and carried out the analyses in a consistent and very well-thought out manner.

In the next chapter, ‘Results and Summary of Papers’, the candidate describes the results presented in the attached publications in a concise manner. The research presented in publication no. 1 showed the presence of two protein fractions in sperm, intracellular (SIPs) and membrane-associated (SMAPs). These protein fractions were shown to take part in important metabolic pathways in epididymal sperm, which may suggest their potential as biomarkers of semen quality. In this study the candidate also determined the optimal sonication parameters for separating individual morphological structures of sperm and isolating proteins. She found that sonication using an Omni Sonic Ruptor 250 ultrasonic homogenizer with power of 150 W, carried out for 10 minutes, results in successful isolation of sperm structures. It should be noted here that these analyses were carried out using semen collected from the epididymides of dogs in an age range of 1 to 6 years. This raises the question of whether the sonication process may be influenced by the age of the dog. In the second publication included in the series upon which the thesis is based, the candidate did assess the impact of the dogs’ age on semen quality and proteomic analysis, but using only one sonication parameter.

In publication no. 2 the candidate attempted to determine the relationship between the functional quality and proteome of epididymal sperm of dogs of various ages. She showed that semen quality parameters were significantly lower in older dogs (78–132 months – group III) than in dogs at the age of 12–41 months (group I). The candidate identified 865 proteins in the first group, 472 in the second, and 435 in the third. Many age-dependent differences were detected in the proteome composition of the sperm, and these were associated with important metabolite pathways, which may suggest that several proteins are involved in sperm maturation and may be potential biomarkers of ageing.

The candidate formulated six conclusions on the basis of the research results. In my opinion, this chapter should be titled ‘Findings and Conclusions’, because most of the points represent findings rather than conclusions. Reading this part of the thesis alone does not provide a full picture of the research presented. I suggest revising points 1 and 6, and I would also consider combining points 4 and 5. Conclusion 1 is too general. In point 6 it would be good to specify by the type of analysis, so that the reader could have a full picture of the usefulness of these analyses without knowing the entire contents of the paper.

To conclude, the comments and suggestions expressed above are editorial comments which do not in any way diminish the substantive value of the doctoral thesis. I have a positive opinion of the entire thesis, which is a valuable scientific paper. It is indicative of the author’s good practical and scientific preparation and her mastery of the skills of developing and editing a scientific work. I am impressed by the wide range of laboratory analyses performed by the candidate, requiring dedication and precision, and her skill in using modern analytical techniques.

Taking into account the usefulness of the research, the means and scope of its realization, and the innovative nature of Ms Anna Żmudzińska’s doctoral thesis, I conclude that the thesis submitted for evaluation, ‘Age related changes in proteome and quality parameters of sonicated dog epididymal spermatozoa’, fully meets the requirements for doctoral theses specified in art. 187 of the Act of 20 July 2018, Law on Higher Education and Science (consolidated text: Journal of Laws of 2022, item 574, as amended).

In view of the above, I present to the Scientific Council of the Discipline of Animal Science and Fisheries, University of Warmia and Mazury in Olsztyn a motion to admit Anna Żmudzińska to the subsequent stages of the doctoral process.

Anna Wysocińska