



Confidential

Review of USB FFPW PhD Thesis

First name(s), surname, titles of the PhD student: M.Sc. Pavlo Fedorov	First name(s), surname, titles of supervisor: Assoc. Prof. M.Sc. Boris Dzyuba, Ph.D.
Title of PhD thesis: Fish spermatozoa metabolites content in various physiological conditions	
REVIEWER:	
Surname: Kowalski	Institution: Institute of Animal Reproduction and Food Sciences, Polish Academy of Sciences, Olsztyn 10-747, Poland
Name: Radoslaw	
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Please describe your professional relationship to the PhD student: no relationship	Please describe your field of expertise: Fish sperm biology

QUESTIONNAIRE

Originality, scientific importance, perspectives and impacts of results presented in the PhD thesis for basic and/or applied research

Evaluate competitiveness of the PhD thesis in the international context and compare its level with the current state of the art in the field (**extent ¼ – ½ page**):

The doctoral thesis written by Pavlo Fedorov is composed from four already published articles in reputable journals and two articles already submitted for publication. This is a huge amount of work and shows extraordinary quality of the PhD thesis. The data presented in the publication described a not well-studied topic concerning fish sperm energy metabolism. The practical approach to this study was very challenging owing to the short duration of fish sperm motility. Therefore, conventional methods used for energetics of mammalian sperm could not be used. The applicant recognized the problems and participated in mini review-like publications such as "Energetics of fish spermatozoa: the proven and the possible" published in a highly cited journal-Aquaculture. To investigate chosen topics, he decided to use liquid chromatography combined with high-resolution mass spectrometry in product scan mode. This novel approach shows high originality in comparison to other techniques used for measuring sperm energetics path. The methodology was further used in a study on eel sperm maturation. Another manuscript published in the Journal of Animal Sciences (highly cited journal) was aimed to study the bioenergetics aspects of sturgeon sperm maturation. The data used in this manuscript were collected using a relatively novel approach that utilizes Liquid chromatography/mass/high-resolution mass spectrometric (LC/MS/HRMS) techniques. The novelty of this publication is



thesis is valid and fulfills the objectives of the work, as well as meet the standards for academic excellence.

OVERALL COMMENTARY ON THE PhD THESIS

Please write comments in extent of 1-2 pages:

Energetic approach of sperm physiology is a topic of great interest among spermatologists. However, due to methodological difficulties, the measurement of energetic molecules in sperm in a short period of time was not possible. Herein, the PhD applicant demonstrates methods and results, which enlarge our knowledge regarding energy utilization by spermatozoa during active motility period as well as the quiescent state. The detailed aims of the presented study were to:

1. develop analytical approach for description of the creatine- and adenylate phosphates content prior to and during the motility period of fish spermatozoa.
2. study the bioenergetic aspects of sturgeon sperm maturation;
3. study the creatine- and adenylate phosphates content during the motility period in European eel spermatozoa at different phases of hormonal treatment and during the short-term storage.
4. study the osmolality effect on whitefish sperm motility and creatine- and adenylate phosphates content.

All questions were adequately addressed with appropriate methodologies. Moreover, the thesis also contains a mini-review which is not described as a thesis goal, but understandable from the document's structure. Starting thesis with a mini-review-like form is little risky as it requires experience, which may be absent among young scientists. However, with the enormous support from experienced researchers (share of applicant was 15%), this was accomplished with success. The first task allows developing fast and reliable method of energetic compounds measurements within a short period of time with the use of LC/HRPS method. The adaptation of this technique to fish sperm allows the measurement of the concentration of several compounds of interest at the same time. Authors concentrated on macroenergetic compounds such as ATP, ADP, creatine phosphate and creatine. Also, secondary messenger, cAMP, which plays a crucial role in fish sperm motility, was investigated. The results of concentration latter included that compounds were combined with desired moment of movement (whitefish, eel, and sturgeon) or maturation state (sturgeon, eel). Despite well-structured discussion, I found some minor comments regarding manuscripts prepared for submission. The first question is related to the Fig. 1 from page 58 where we can see the results of macroenergetic molecules concentration in relation to time post-activation. However, there is lack of corresponding motility data, which makes it difficult to understand the data. Regarding Fig. 2 and 3, I would insist to uniform the x-axis description, either days or hours. Also, the result showing elevated concentration of creatine in samples stored at 20°C needs to be discussed



(reduced rate of metabolism, necrosis?). Moreover, the storage in such a high temperature might result in bacteria propagation. I did not find any indication if an antibiotic was used in preservation buffer; cited publications showed only the ionic composition of mentioned buffer. Please clarify these issues and discuss accordingly.

The second manuscript prepared for publication and describing the subpopulation structure dynamics in different osmolality of activation buffer also raise some questions. The very first one is that the introduction section lacks information as to what theory supports the study hypothesis. Is it osmotic shock value (rate of membrane depolarization)? It should be more precisely expressed to make it easier for the readers to understand author's intentions.

Another comment is related to the statement of the author presented in the page 76 i.e. „(...) four sperm subpopulations with distinct characteristics exist in whitefish sperm“. According the presented data, they do not appear instantly, so the word "exist" is rather too strong. As we can see from the data (clearly and nicely shown), these subpopulations are appearing during the course of motility as an effect of decreasing energy in the sperm. According my understudying of the data, sperm, while swimming change their pattern of movement (which is also stated in the discussion). Interesting points are: which sperm change into slow and linearly moving ones, and which into slow and circular ones? Despite the absence of exact data to explain this phenomenon, I encourage to discuss basis on the available bibliography. One indication I can give is that, the circular sperm must have flagellum shaped asymmetrically (hyperactive-like pattern). This is known to be triggered by high calcium influx into the sperm flagellum. Please try to discuss this briefly in manuscript. My last comment is related to the objective of this study. Of course, osmolality seems necessary to be tested; however, I would like to encourage the author to further investigate pH effects on energy utilization by the sperm. As the energy production in the cells utilizes protons and depends on proton gradients (intra/extracellular), I would expect greater differences in energetic pathways when media with different pH will be used for sperm activation. This study could help to better understand the influence of pH on salmonid sperm motility and in addition, may further explain why the samples contaminated by urine losses ability to be activated in low pH (Nynca, J., Dietrich, G.J., Kuzminski, H., Dobosz, S., Ciereszko, A. Motility activation of rainbow trout spermatozoa at pH 6.5 is directly related to contamination of milt with urine. *Aquaculture*. 2012; 300-333:185–188). Please take this into account when designing further experiments.

The last but not least to evaluate is acknowledgment. This section, although did not represent scientific value, is a great evidence of the applicant maturity. I can just say that this part is really well done!

Despite all of my criticisms towards reviewed manuscripts, the overall scientific value of presented results is high. It is my pleasure to recommend the presented thesis as a PhD defense document fulfilling all rigorous criteria anticipated from the doctoral candidate.

FINAL RECOMMENDATION




Fakulta rybnářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice
Czech Republic

- PhD Thesis can be recommended for defence**
 PhD Thesis can be recommended with reservations for defence
 PhD Thesis can not be recommended for defence

13.06.2017, Sesoko, Okinawa, Japan
Date and place


Radoslaw Kowalski
Name and signature



Fakulta rybnářství
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Review of USB FFPW PhD Thesis

First name(s), surname, titles of the PhD student: M.Sc. Pavlo Fedorov	First name(s), surname, titles of supervisor: Assoc. Prof. M.Sc. Boris Dzyuba, Ph.D.
Title of PhD thesis: Fish spermatozoa metabolites content in various physiological conditions	

REVIEWER:

Surname: Gorbenko	Institution: V.N. Karazin Kharkiv National University, Department of Nuclear and Medical Physics
Name: Galyna P.	
Titles: Prof.	E-mail: galyna.p.gorbenko@karazin.ua
Please describe your professional relationship to the PhD student: I have known Pavlo Fedorov in my capacity as a Professor at the Department of Molecular and Medical Biophysics where I was lecturing the courses of Physics of Biomolecules, Molecular Biology and Genetics, Sensorics and Luminescence Technologies in Medicine and Biology. Likewise, I was a supervisor of Pavlo's diploma work devoted to the testing of novel fluorescent probes for biomedical applications.	Please describe your field of expertise: Membrane biophysics, protein-lipid interactions, protein misfolding, fluorescence spectroscopy, computer modelling

QUESTIONNAIRE

Originality, scientific importance, perspectives and impacts of results presented in the PhD thesis for basic and/or applied research

Evaluate competitiveness of the PhD thesis in the international context and compare its level with the current state of the art in the field (**extent ¼ – ½ page**):

The doctoral dissertation of Pavlo Fedorov is of greatest scientific interest and very high practical importance because the bioenergetic mechanisms underlying spermatozoa activation in fish so far remain largely unknown. This is not a trivial task, requiring the adjustment of existing techniques and elaboration of new methodologies for quantitative characterization of the balance between a variety of macroergic substrates. Analyzing the current state of the art in the fish spermatology shows that only a few attempts have thus far been made to evaluate the level of adenine nucleotides, creatine phosphate and other compounds in fish spermatozoa and no systematic



studies have been performed in this direction. Therefore, there is no doubt in originality, timeliness and urgency of the research program undertaken in the thesis.

Elaboration of the PhD thesis, objectives of the work and deliverables

Evaluate the overall level of elaboration of the PhD thesis (structuring of the main text, comprehensibility, logicity of the chapters and their ordering) and the originality of the selected approaches to solve the objectives; evaluate publications and whether the results described correspond to objectives of the PhD thesis (extent ¼ – ½ page):

The reviewed PhD thesis is clearly written and well structured. It is divided into 6 logically connected chapters. The first chapter represents a detailed review of both fundamental and applied problems associated with the energetics of fish spermatozoa. The importance of correct determination of a multitude of macroergic substrates is emphasized and the main objectives of the study are highlighted. The second chapter is focused on the methodological approach developed by the author for evaluation the content of creatine- and adenylate phosphates in the fish spermatozoa. In the remaining chapters the validity of this approach has been demonstrated while investigating the bioenergetics of sturgeon, European eel and whitefish spermatozoa. The thesis is technically sound and met the objectives set forth. The accomplishment of the planned research is confirmed by the papers published in the highly ranked scientific journals.

OVERALL COMMENTARY ON THE PhD THESIS

Please write comments in extent of 1-2 pages:

The PhD thesis by Pavlo Fedorov significantly contributes towards solving the really challenging problem concerning the role of bioenergetic processes in the metabolism and motility of fish sperm cells. There are solid fundamental and practical reasons for undertaking the proposed work because bioenergetics of fish spermatozoa so far remains poorly understood. To the best of my knowledge, this work is the first attempt to undertake a systematic extended study of fish sperm bioenergetics. The dissertation possesses a number of undoubted merits, among which are the following:

- A very comprehensive and versatile critical analysis of the studied problem performed in the Chapter 1, describing in detail the sources of energy for fish sperm motility, the relationships between the physical and chemical parameters controlling the flagellum behavior, the practical aspects of maintaining the sperm quality, etc.
- A worthwhile research programme providing a framework for coherent understanding of the involvement of macroergic metabolites in the physiological processes of spermatozoa and elaboration of efficient ways of preserving the fish sperm;
- The development of novel LC/HRPS method for simultaneous quantification of the creatine, creatine phosphate and adenine nucleotides, offering the advantages over NMR technique. Importantly, the performance of this method have been thoroughly evaluated and the correction factors allowing for possible loss of the target compound during the fixation of sperm cells have been introduced;
- The confirmation of the hypothesis that the maturation of testicular sperm in sturgeon is



coupled with the mitochondrial respiration and ATP regeneration via creatine kinase reaction;

- The quantification of creatine- and adenylate phosphates during the motility period in European eel spermatozoa at different stages of hormonal treatment and in the course of in vitro storage;
- Gaining insights into the effect of environmental factors such as osmolality and ionic composition on the content of macroergic substrates and motility of whitefish spermatozoa.

I find such studies really interesting and very important for many potential applications. All presented data are statistically significant and interpretation of the obtained results is well argued.

As for the critical remarks, since the cluster analysis was made only in the case of whitefish sperm, it seems reasonable to explain whether there exist different cell subpopulations in European eel and sturgeon sperm and how this fact may affect the conclusions drawn.

In my opinion, the thesis by Pavlo Fedorov meets all requirements posed on theses aimed at obtaining a PhD degree and is ready to be defended in front of the Scientific committee.

FINAL RECOMMENDATION

- PhD Thesis can be recommended for defence
 PhD Thesis can be recommended with reservations for defence
 PhD Thesis can not be recommended for defence

...May 30, 2017, Kharkiv.....
Date and place

Gorbenko Galyna.....
Name and signature