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

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

Confidential

Review of USB FFPW PhD Thesis

First name(s), surname, titles of the PhD student: Yevhen Horokhovatskyi, M.Sc.	**First name(s), surname, titles of supervisor:** Assoc. Prof. M.Sc. Borys Dzyuba Ph.D.
Title of PhD thesis: Applied aspects of fish sperm cryopreservation	

REVIEWER:

Surname: Butts	**Institution:** Auburn University School of Fisheries, Aquaculture and Aquatic Sciences 211 Swingle Hall, Auburn, Alabama, 36849 United States of America
Name: Ian Anthony Ernest	
Titles: Dr.	**E-mail:** iana.e.butts@gmail.com
Please describe your professional relationship to the PhD student:	**Please describe your field of expertise:** Reproductive Physiology in Aquatic Species

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)**:

Aquaculture is the fastest growing food producing sector in the world. As aquaculture continues to expand it will rely on selective bred and genetically improved stocks. As such, gametes from these selectively bred and genetically improved fishes should be frozen in perpetuity for future spawning and conservation efforts. Unfortunately, cryopreservation still induces cellular injuries which can jeopardize these freezing/thawing efforts. The PhD thesis by Yevhen Horokhovatskyi, as titled, examines applied aspects of fish sperm cryopreservation with the goal of improving freezing techniques, to assist the sustainability of aquaculture. More specifically, the student conducted three research chapters. In research **Chapter 2** lipid composition was linked to sperm cryo-resistance in common carp; **Chapter 3** worked on decreasing variability and improved post-thaw motility and freezing ability for sterlet sturgeon sperm, while **Chapter 4** was extremely novel as it examined sperm subpopulations and how they are impacted, specifically at the proteomic level in sterlet sturgeon. For dependant variables, Yevhen measured sperm performance using computer assisted sperm analyses system, live/dead viability using florescent probes, embryonic development, major lipid classes, and conducted extensive proteomic analyses. As a combined unit, these are solid approaches that represent state of the art in the field of fish spermatology, especially the protein work which was conducted in Poland. Together, the measurement of these above traits with the applied/basic science hypothesis he applied are adding new results which shall improve of ability to freeze/thaw cells, for not only fishes but other organisms.

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, logicality 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 PhD thesis by Yevhen Horokhovatskyi consisted of five chapters (note: error on page 5), three of which were experimental research (resulted in 2 publications). Here, I quickly overview the individual chapters (note more specific details for each chapter are highlighted below). **Chapter 1** was an overview of the literature with clear insights of the state of art in aquatic cryobiology. It had logical flow and provided a framework for choosing the model organisms, carp and sturgeon. One complaint was the "Aims and objectives of the study" section, could have been more elaborate with detailed predictions and hypothesis. But overall, it was a nice job. **Chapter 2**, published in Cryobiology (2016), examined how lipids impact sperm cryopreservation success. This was a rather short study (4 published pages), but was a subject worthy of investigation and relates back to the aims and objectives. **Chapter 3**, published in Theriogenology (2017), tested a rather simple concept when freezing cells. The results, however, clearly detailed the need for further standardization for aquatic cryopreservation, as for instance, they found placement of 6 or 60 straws on a raft or changing the location within a shipping dewar during freezing can have massive ramifications for cryo-success. Research **Chapter 4** was by far the most novel and analytical component of the thesis. Here, the objective was to compare the viability and proteomes of fresh and cryopreserved sperm samples before and after separation using a Percoll density gradient. Results were clearly defined and directly corresponded to the thesis objectives. Finally, **Chapter 5** gives a general discussion of the experiments and thesis. Although, a nicely written section I would have enjoyed a "*what would be the next logical research steps*" section to solidify overall comprehension. Based on the above, this thesis seems to address three separate aspects of cryopreservation (lipids, freezing, and proteins) using two different fish species. On a negative note, I believe this PhD thesis would have been much more comprehensive if it focused strictly on one aspect (say understanding more defined impacts of lipids OR proteins) of cryopreservation, or even addressing the above aspects on one species rather than two. I would like to hear the students take on this last issue.

OVERALL COMMENTARY ON THE PhD THESIS

Please write comments in extent of 1-2 pages:

Below, I highlight more specific comments (C) and ask questions (Q) for each of the thesis chapters.

Overall comment: Information on replication should be more elaborate in the thesis. i.e. how many times were each dependant variable measured per male?

Chapter 1:

Q. Who is your favorite scientist in the field of cryopreservation? Who do you think has made the best strides to advance the field?

Q. It appears that efforts in cryopreservation research (Fig 1) go in "up and down" cycles. Can you explain this phenomenon?

Q. What are major differences that impact cryopreservation successes between different taxonomic groups. What taxonomic group has the most successful sperm quality post-thaw?

Q. Aims and objectives: It would be nice to have the fish species of thesis specified in this section. Please add, if possible. And can you define specific hypothesis based on your research?

Chapter 2:

C. Abstract: I believe you have to be a little more specific on what the "groups" are and make the abstract

slightly more quantitative.
C. Introduction: The first sentence is a run-on sentence. It may be nice to correct grammar throughout.
Q. Methods: What were some basic physiological traits of the males' milt, i.e. pH, osmolality, concentration? Do you think this would impact cryopreservation success?
Q. Methods: What were the broodstock fed? Considering this study is examining lipids, don't you feel this is important to include? Where are the lipids coming from in the sperm?
Q. Methods – sperm activation: Is a 1:100 activation solution too concentrated How many cells were visualized on the screen for quality analyses?
Q. Methods – cryopreservation: This needs to be more repeatable, i.e. what was the equilibrium time with extender before freezing? Why not mention more details on the size of your Styrofoam box, especially considering that is a major highlight of Chapter 3. How were the straws sealed? Etc., etc.,
Q. Methods - Why did you choose 40% and <10% as the experimental grouping of high and low-quality sperm? Why not 60% and 5%, for instance?
Q. Methods - Stats: Curious why you consider n = 25 a small sample size, as to be that is a nice sample size. Can you elaborate? Also, please justify the use of non-parametric test, based on this sample size.
Q. Results: Why did you express your fatty acids as a percentage and not i.e. mg/g dry mass?
Q. Results – First paragraph under Figure 2: Why do you think these results are occurring?
Q. Results – Table 1: Again, why not express these results as mgFA/g dry mass?
Q. Discussion – You mention future studies are needed to explain the variability you observed. Can you please elaborate on how you would do these future studies?
Q. Would you anticipate different results if you used a different cryopreservation procedure?
Chapter 3:
Q: Introduction – Second to last paragraph. You mention importance of raft and freezing conditions, hence, why I am curious you did not elaborate in Chapter 2.
Q: Methods – Stats section: Why would you consider 10 a small sample size and why does it require the use of a nonparametric test? Also confused by your statistical approaches in the section "The VCL, VAP, BCF..... with $P < 0.05$". Can you please provide more details? Also, curious why you did not compare the slopes of the lines between the treatments (Fig. 4, Table 2) using a multiple comparison of slopes method, i.e. GLMMCSLO, (Proc GLM, SAS Institute).
Q. Results – Fig 3. Why is there limited variability for Raft6 in Panel B. Please speculate.
Q. Results – Fig 4: The raft with 60 straws had lower sperm quality. Although you did not measure these traits, can you speculate what this treatments sperm morphology would look like compared to the other treatments (i.e. about head, midpiece and flagella)?
Q. Results – Table 2: Only 2 decimals are needed for R2. P for R2 is incorrect. The P value is testing the slope of the line and whether it is zero.
Q. Would you anticipate different results if you used a different cryopreservation procedure?
Chapter 4:
Q. Very nice study – I really enjoyed reading this chapter. Perhaps, I missed it, but should you not mention how many cells were recovered after separation and how many cells were included for each of the steps? Did you lose a lot of cells from the separation process?
Introduction – bottom of page 33: Which cell separation technique is best for fish and why?
Q. Methods – first paragraph page 34: Should this not be in the objectives paragraph?
Q. Methods – cryopreservation: Make it more repeatable. There are details missing.
Q. Methods – Stats: Same question on "due to low number of samples, a nonparametric ....." . Please elaborate on this method.
Q. Results – Table 3: Confused on your slope and intercept comparisons. Can you justify this methods?
Q. Table 3: So, all treatments decreased at a similar rate (i.e. no differences in slopes) but had higher or

lower initial sperm quality (i.e. differences in intercepts). Can you please explain these findings?
Q. Would you anticipate different results if you used a different cryopreservation procedure, i.e. DMSO at 5-20%? How do you anticipate this would impact protein changes?
C. Pages 45-48: Beautiful figures and tables. Can you add the function of the proteins for Tables on page 47-48?
Q. Page 49: What does the sentence "Furthermore, IPA demonstrated..... abundant proteins" mean?
Q. Discussion: Would you not agree that it is also important to learn about poor quality cells so that we can make poor quality cells high quality cells?
Q. Page 52 – top paragraph: How does cell sorting actually happen? Why do cells of certain quality migrate to different layers?
Q. So you found differences in proteins from the different groups. Great!! Now what would be the next steps to improve cryopreservation for this species?
Chapter 5:
Q: Lipids were shown to impact sperm quality. Thus, what would you need to manipulate to improve the lipid quality of the sperm for cryopreservation?

I enjoyed reading this thesis and I hope my comments will help improve the quality of the thesis. Best wishes in the future.

Ian,

FINAL RECOMMENDATION

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☒ **PhD Thesis can be recommended for defence**
☐ **PhD Thesis can be recommended with reservations for defence**
☐ **PhD Thesis can not be recommended for defence**

.....16 July 2018.....
Date and place

Ian A.E. Butts.....
Name and signature

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Title of PhD thesis: Applied aspects of fish sperm cryopreservation	

REVIEWER:

Surname: Horvath	**Institution:** Department of Aquaculture Szent Istvan University H-2100 Godollo
Name: Akos	
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Please describe your professional relationship to the PhD student:	**Please describe your field of expertise:**

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 thesis is definitely an important one as it does not pretend to develop new protocols for fish sperm cryopreservation (there are confusingly many of those, anyway) but rather attempts to bring out the best of them, trying to define key parameters that either characterize or define good post-thaw survival in terms of motility or fertilizing capacity. Thus, the contents of this thesis are obviously beyond the state of the art of this field. Experiments in chapters 2 and 4 help to determine post-thaw sperm quality. The use of Percoll separation for optimal sperm quality in chapter 4 adds a very good practical aspect to the work and its combination with proteomics improves our understanding of the changes that occur during freezing/thawing. Chapter 3 presents a very practical and very useful approach to improving cryopreservation outcomes. It shows how problems that seem so difficult to solve depend on minor details that are easy to improve and yet, they matter so much. Thus, I am convinced that the contents of the thesis are important from both scientific and practical points of view.

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, logicality 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 structure of the thesis is typical of those produced in Vodňany, they seem to follow a uniform, straightforward style that is very logical and help the reader to make sense of the work. The thesis consists of 5 chapters of which the last one is oddly numbered as Chapter 6. These chapters include a general introduction which is not very long but contains all necessary information to introduce the reader to the topics discussed in the thesis. This is followed by three chapters that are basically either papers that have already been published (Chapters 2 and 3) or represent a manuscript that will hopefully be submitted soon. Finally the last chapter gives a general discussion and other compulsory items such as summaries in English and Czech languages, acknowledgements, lists of publications, etc. The only item that seems a little out of place is the general discussion as each chapter is also discussed separately, however, the referee understands that this is a mandatory item required by the PhD school. Otherwise the thesis is unproblematic in all aspects.

OVERALL COMMENTARY ON THE PhD THESIS

Please write comments in extent of 1-2 pages:

While the referee generally agrese with the contents of the general introduction, there are two parts that are problematic:

1. On page 9 (last paragraph), the author writes that „the inability to cryopreserve fish female gametes could be compensated by androgenesis". In principle it is true, however, androgenesis is plagued with a problem that prevents its use on any scale higher than experimental: high level of homozygosity of the produced progeny which results in mass mortalities in emryonic and post-embryonic age. Therefore the efficiency of androgenesis is very low. Personally, the referee refuses to believe any published result that shows hatch rates higher than 5% in experiments on androgenesis – and survival rates to sexual maturity will be counted in a few individuas out of a million fertilized eggs. A much better, more efficient way to compensate for the inability to cryopreserve female gametes is actually practiced at the host institution of the candidate and it is the cryopreservation and transplantation of early germ cells.
2. On page 10 (last paragraph), the author writes that „the absence of specific techniques and standardization in the developed cryopreservation protocols" are the factors that inhibit applicability of sperm cryopreservation to aquaculture practice. Well, no they are not. The factor that inhibits applicability of sperm cryopreservation is that the aquaculture industry simply does not need it. It is applied only in niche areas and for the most part from public funding, not as a profit-oriented business. It is sad to admit but when – and if ever – there

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will appear a need for the use of cryopreserved sperm in the aquaculture industry, the industry will commit its own efforts to standardize protocols and methodologies.

In chapter 2, the author stated that two freezing protocols were followed in this set of experiments which mostly differed in the composition of the extender. However, later the results are not presented or discussed according to the freezing protocol but according to samples exhibiting high or low post-thaw motility. What was the reason for that? Were the results obtained with the two protocols absolutely identical? Also, were there any fertilization results to support the idea of good or poor sperm quality? This is a very sensitive topic in the species discussed in this thesis.

Chapter 3 is the referee's absolute favorite. Indeed, it solves a very problematic issue that the referee himself has faced when freezing large volumes of sturgeon sperm, although the outcomes were a little different. In the referee's experiments, and increased number of straws did not cause a uniform reduction of post-thaw motility but rather a high variation in post-thaw motility among straws. What would the author recommend for those who need to use the cooling box with a floating raft for freezing large volumes of sperm?

Again, the use of Percoll gradient to separate surviving spermatozoa from damaged ones is ingenious and the referee hopes to see it soon in a published form. The application of Percoll for sperm separation continues to amaze the referee inspite of it being already published on common carp sperm 8 years ago. The results are expecially spectacular considering the ratio of motile cells in the crypreserved group before and after separation. A minor mistake is that the ratios of cells in the text (page 41, top two rows) do not match those presented in Table 2. What was the reason for that? Also, the referee would be very much interested if the rate of improvement in motility values is also reflected in the fertilizing capacity? It is obvious from the manuscript that fertilization trials were not part of this experiment, however, did the author carry out any fertilization experiments with separated sperm later?

FINAL RECOMMENDATION

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

30th July, 2018, Budapest, Hungary
Date and place

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Ákos Horváth PhD