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: MiaoMiao Xin, M.Sc.	**First name(s), surname, titles of supervisor:** Prof. Dipl.-Ing. Otomar Linhart, DSc.
Title of PhD thesis: The role of some proteins in freezing fish sperm	

REVIEWER:

Surname: Butts	**Institution:** Auburn Univ, Sch Fisheries Aquaculture & Aquat Sci USA
Name: Ian AE	
Titles: Dr.	**E-mail:** iana.e.butts@gmail.com; iab0007@auburn.edu
Please describe your professional relationship to the PhD student: No relationship	**Please describe your field of expertise:** Reproductive Physiology, Aquaculture, Hatchery Science, Gamete cryopreservation, Sexual selection

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 MiaoMiao Xin, as titled, examines applied aspects of fish sperm cryopreservation with the goal of improving freezing/cryopreservation techniques, to assist the sustainability of aquaculture. More specifically, the student conducted two research chapters. In research **Chapter 3** the sperm proteome was compared before and after freezing in sterlet sperm; while **Chapter 4** worked on deciphering how antifreeze proteins at different concentrations impact sperm kinematics and membrane viability. **Chapter 1 and 2** were extremely through reviews on "**Molecular and subcellular cryoinjury of fish spermatozoa and approaches to improve cryopreservation" and "sperm vitrification**". Finally, **Chapter 5** provided a nice overview of the work. For dependant variables, MiaoMiao Xin measured sperm performance using computer assisted sperm analyses system, live/dead viability using florescent probes, and did some novel protein analyses. As a combined unit, these are solid approaches for fish spermatology, especially the 2D-gel electrophoresis protein work. Together, this applied/basic science work shall improve our ability to freeze/thaw cells, for not only fishes but other organisms.

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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 MiaoMiao Xin consisted of five chapters, two of which were experimental research (resulted in 2 publications), and another chapter which resulted in a review paper. Here, I quickly overview the individual chapters (note more specific details for each chapter are highlighted below). **Chapter 1**. Is a general introduction to the thesis in terms of sturgeon biodiversity and conservation, cryopreservation process, sperm vitrification, damage to sperm during the cryopreservation and antifreeze proteins as sperm cryoprotectant. Here, the goal of this research has been highlighted, and overall, it was a nice job. **Chapter 2** was a "mini review" published in *Theriogenology* (2017) aiming to discuss the basic procedures of vitrification of fish sperm, the current progresses in vitrification application for fish spermatozoa, compare the advantages and disadvantages of vitrification, and some recommendations for future research. Although, this review has focused more on teleosts rather than sturgeon species, overall it is a nice review that is published in a good journal. **Chapter 3**, published in *Animal Reproduction Science* (2018), was by far the most novel and analytical component of the thesis. Research **Chapter 4** was also a solid cryopreservation study. Here, the objective was to compare sperm kinematics and viability from sperm samples frozen with different anti-freeze proteins at different concentrations. Results showed that these proteins were different among the treatments, and a few treatments did not differ from the fresh control. **Chapter 5** gives a general discussion of the experiments and thesis. Although, a nicely written section I would have enjoyed a more of a "*what would be the next logical research steps*" in cryopreservation section to solidify overall comprehension. Based on the above, this thesis seems to address aspects of cryopreservation (freezing and proteins) using an important model species. On a negative note, I believe this PhD thesis would have been much more comprehensive if it had more research studies and measured more variables related to molecular and cellular damage as mentioned in the intro chapter. I would like to hear the students take on this last issue.

OVERALL COMMENTARY ON THE PhD THESIS

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Please write comments in extent of 1-2 pages:

Please make sure to use common name and scientific name (in *italic*) of species for the first time, then just the common name of species through the text.

CHAPTER 1

Since you mention sperm cryoinjury goes beyond fertilization and impaired embryonic development, it would be nice if you discuss a section/paragraph in terms of embryonic development or larvae quality resulted from frozen-thawed sperm.

page 7, paragraph 3: You already mentioned that Sterlet is a vulnerable species, so here it should be highlighted that sterlet is selected for conservation and maintaining biodiversity as well as a model fish in sturgeon species.

page 17, paragraph 3: The headline for this paragraph is "Damage to the cytoskeleton, flagellum, and spermatozoon motility" so the text should start with cytoskeleton first, then flagellum and last sperm motility.

Fig. 1. Please use arrow or some indication to show the schematic structure foe sperm cell, such as head, etc.

page 22, paragraph 2: Please add a paragraph in terms of seminal plasma enzymes as well.

CHAPTER 2

Page 42, "2. Vitrification approaches". Can you specify during vitrification how controlling temperature and equilibration time could reduce the chemical toxicity of cryoprotectants?

Page 42, "2.1. Development of traditional vitrification solutions". Please specify how salinity of an extender can play an important role in sperm protection?

Page 42, "2.1. Development of traditional vitrification solutions". Good post-thaw fertility. Isn't that Good post-thaw motility?

Page 42, "2.1. Development of traditional vitrification solutions". Are all permeable cryoprotectant toxic to sperm cell? I believe some permeable cryoprotectants are less toxic to sperm cell such as methanol.

Table 2: I wish the authors have used "Thawing" instead of "Warming rate"

Page 45, "4.4. The potential danger of disease transmission".
Has there been any report for fish regards to the risk of disease transmission during sperm vitrification?

CHAPTER 3

Very difficult to read when printed as PDF. I had to look it up on Web of science.

Objectives paragraph. Since this is a PHD thesis I would be curious to know what your hypothesis would be for the experiment.

Methods: 2.2; Are these the same 7 males as Chapter 4?

Methods: 2.2: CASA methods are not really repeatable. What was your dilution ratio, coverslip, slides, etc., How many activations per male? Etc., etc., This, all needs to be detailed to make it repeatable. Same comments as for 2.4 with lack of repeatability for cryopreservation techniques. Also is section 2.5 missing?

Fig.4 Very nice figures. Now we need to do this with corresponding gene expression ☺ Next studies!

Very nice informative Table 2. Great efforts.

CHAPTER 4

Larviculture not larva-culture

Objective paragraph: Since this is a PhD thesis, I would be curious to know what your hypothesis would be for this experiment.

Why were those specific antifreeze compounds chosen? Why did you choose those specific antifreeze protein concentrations?

Sperm cryopreservation: We always talk about lack of standardization. Thus, were samples held at 4oC before dilution in extender? How were the straws sealed? How many straws per treatment? Where the straws placed directly on ice for 10 min? What was the size of the Styrofoam box? How long were the samples remaining in liquid nitrogen? etc.,

Sperm motility: How many cells were analyzed in the frame during CASA? What was the sperm to activation media dilution rate?

Statistical analysis: This should be a two-way ANOVA model: Terms Antifreeze type and Antifreeze concentration and interaction term. Please discuss why you took a one-way ANOVA approach.

Results: Sperm motility: I suggest next time you report least square means standard error not SD – this is correct way to do it statistically. I have same comment for sperm plasma membrane integrity.

Your argument about motility evaluation at the end of page 1532 on the right is not really valid as all treatments where treated the same way, I assume. Thus, sure "out of focus and track collisions " can cause issues but it would be relative across all treatment.

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Conclusions: I think it would also be interesting to not only work on fertility but also morphology using SEM/TEM to really decipher what is happening at the cellular level to decrease membrane viability, etc.,

CHAPTER 5:

I believe it is nice to have "future research ideas on how to improve sperm cryopreservation" and maybe some "future hypotheses" that you would like to test to make you think more critically about future directions for gamete cryopreservation research. Besides that the general summary chapter was very nice.

Best wishes and good luck,

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**

...1 July 2019, AL, USA.....
Date and place

...Ian Butts.....
Name and signature

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Title of PhD thesis: The role of some proteins in freezing fish sperm	

REVIEWER:

Surname: Shelton	**Institution:** University of Oklahoma USA
Name: William L.	
Titles: Prof., Ph.D.	**E-mail:** wshelton@ou.edu
Please describe your professional relationship to the PhD student: Interaction during my appointment as visiting Scientist at USB in 2015.	**Please describe your field of expertise:** Management of Fish Reproduction

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 conceptual nature of this thesis is of the highest caliber; it provides significant advancement in the science of gamete cryopreservation. The knowledge should have high value in both basic and applied fields.**

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OVERALL COMMENTARY ON THE PhD THESIS

The investigation was well conceived and it was conducted efficiently. The thesis is organized logically in the sequence of the study organization. The text is well articulated and clearly expressed the findings. The writing style is direct and concise and with correct English construction.

I have only a few minor comments (see separate edits in English Summary):

1. **Following the General Introduction (p 5-11) is an article which is *in press;* the title on p 12 appears to be the end of the References for the previous section. It should be more clearly separated**
2. **In the text of this section, most scientific names should be in italics – a few are, but most are not.**
3. **The first sentence in this section (p 16) „...spermatozoa require a plenty of ATP ....“ this should probably be ...plentiful...**
4. **On page 19, paragraph 2 – there are misplaced hyphens (spe-cies & perox-idate) – probably these are software glitches?**

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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**

11 June 2019
.....
Date and place

William L. Shelton
.....
Name and signature