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: Abdul Rasheed Khanzai Baloch, M.Sc.	**First name(s), surname, titles of supervisor:** Assoc. Prof. Dipl.-Ing. Martin Pšenička, Ph.D.
Title of PhD thesis: Utilization of CRISPR/Cas9 and Germ Cells labelling technique for surrogate production in sturgeons	

REVIEWER:

Surname: Nóbrega	**Institution:** São Paulo State University Brazil
Name: Rafael Henrique	
Titles: Prof. (Associate Professor)	**E-mail:** biorhn@yahoo.com.br or rafael.nobrega@unesp.br
Please describe your professional relationship to the PhD student: No relationship.	**Please describe your field of expertise:** Fish reproduction, spermatogenesis, cell and molecular biology and comparative endocrinology

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 current thesis is original and presented relevant scientific data for sturgeon reproduction and biotechnology. The thesis developed protocols to generate sterile sterlet sturgeon (*Acipenser ruthenus*) and germ cell labeling using, respectively, CRISPR/Cas9 system and nanoparticles. These methods will improve/optimize germ cell transplantation using sterlet sturgeon as recipients to surrogate the production of long/late maturing sturgeon species. When achieved, this approach will have a great impact on accelerating sturgeon maturation, and consequently allowing conservation and restoration of self-sustaining sturgeon populations considered as critically endangered species by IUCN. Therefore, the current thesis shows scientific relevance with exciting perspectives to surrogate the production of sturgeons.

In the Chapter 2, PhD candidate reviewed the Dead end (Dnd), a germ-cell specific protein, which is necessary for primordial germ cell (PGC) specification and migration in several vertebrates. Dnd binds to several germ cell specific mRNA preventing their degradation from miRNA. The review, published in Fish Physiology and Biochemistry (IF = 1,729), covers the roles of Dnd protein in several teleosts and highlight the use of *dnd* KO strategies (MO, CRISPR/CAS9) to deplete recipient's endogenous germ cell as suitable hosts for germ cell transplantation. Chapter 3 is presented as published article at "Animals" (IF = 1,832) and showed that *dnd1* KO in sterlet sturgeons by CRISPR/Cas9 generate germ cell free recipient's for germ cell transplantation and surrogate production. Finally, Chapter 4, presented as manuscript, demonstrates the feasibility to label PGCs using iron oxide nanoparticles, providing interesting possibilities to study cell-NPs interaction, PGC isolation using magnetic field or to apply hyperthermia for host sterilization purposes. Overall, the current thesis generated important data that will support further studies to optimize and improve germ cell transplantation in sterlet sturgeon as means to surrogate the production of long and late maturing sturgeons.

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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 thesis is well structured (Chapter 1 (Introduction)/, Chapter 2, Chapter 3, Chapter 4, Chapter 5 (General discussion and Summary), and it is in agreement to the Phd thesis guidelines of the University of South Bohemia (Czech Republic). This reviewer feels that some parts of the main text/chapters/general discussion are redundant and repetitive and could be summarized/shortened, such as characteristic of sturgeons (living fossils), Dnd roles and sex determination which has not even been addressed in this thesis. The chapters followed a logical order, although the review paper about Dnd (Chapter 2) could be inserted in the Chapter 1 (Introduction). In the Introduction (just before the Aims/Objectives), the PhD candidate could write a paragraph about the importance/relevance of his work and highlight the contribution of the proposed methodology to the field. All objectives were accomplished; although Chapter 2 is not a deliverable, only Chapters 3 and 4 are considered direct deliverables of this thesis. Only deliverable Chapter 3 was published ("Animals" - Impact factor = 1,832). Chapter 4 is presented as manuscript. Overall, the results presented here are in fully agreement with the objectives proposed by this PhD thesis. The general discussion should be shortened and carefully revised by an native English speaker. Please, see minor comments bellow.

OVERALL COMMENTARY ON THE PhD THESIS

Please write comments in extent of 1-2 pages:

The thesis *"Utilization of CRISPR/cas9 and novel germ cells labeling technique for surrogate production in sturgeons"* by the PhD candidate **Abdul Raasheed Khanzai Baloch** is structured into 5 Chapters. The PhD candidate presents new data and methods that will certainly optimize germ cell transplantation using sterlet sturgeons as recipients for surrogate the production of long/late maturing sturgeon species. This thesis is of scientific relevance for sturgeon reproduction and biotechnology. Minor comments and suggestions are listed below:

Introduction

- A careful revision of grammar and English should be done throughout the text.
- Please, pay attention to the gene and protein nomenclature used in fish and other vertebrates. For fish: *dnd* (gene); Dnd (protein);
- In general, Figure captions (legends) should be better explained and described, such as the Figure 01. What is the relation of E-cadherin? This was better explained in the Chapter 2 but it is sort of vague in the Introduction;
- Some content could be resumed or even removed, such as sex determination and differentiation. The objective of the thesis was not related to sex determination;
- Page 10: There is a paper (https://academic.oup.com/biolreprod/article/69/4/1142/2712600) from Goro's group

correlating the success of germ cell transplantation with the age of the recipient: *"One possible explanation for this phenomenon is that the donor PGCs acquired immune tolerance because they were transplanted before the onset of cellular immunity. In fact, the immune system is relatively immature at the time of hatching in a number of fish species [21]."*

- Page 13: tracRNA
- Provide better importance and relevance of the work. Highlight the main contributions to the field;

Chapter 2

- As suggestion, authors could provide a Table to summarize the existing *dnd* found in fish and its roles.

Chapter 3

- Did the authors characterize/sequence the target mutation? In addition, there is no mention if F0 generated is homozygous or heterozygous for the mutation;
- Authors should provide qPCR analysis to evaluate *dnd* mRNA levels in control and CRISPR/Cas9 injected fish. This data would support the reduced number of PGCs induced by *dnd* KO;
- What is "Treated" in Figure 3?
- The number of PGC is decreased but there was no significant difference between CRISPR/Cas9 and MO injected fish. Considering that both fish are sterile, what are the advantages of using CRISPR or MO? The authors should have addressed this issue in the paper;
- What about the histological analysis of the juvenile gonads?
- Are *dnd* KO all male? Gene expression analysis could be done using male or female specific genes;
- What about the expression of maternal *dnd*? Could the maternal *dnd* induce the formation of fewer PGC in the mutants? This should be addressed in the paper. Moreover, the combination of CRISPR/Cas9 + MO should be interesting to eliminate maternal *dnd* effects;
- It is not clear how authors suggested/proved the mosaicism in the F0 injected fish.

Chapter 4

- Concentrations of nanoparticles should be provided;
- Figure 1: these graphs are not clear. Have statistical analysis been done? What is the fertilization rate? Provide better explanation for the Figure captions.
- Page 52: Label each Figure;
- Discussion should be rewritten, concise and focused.
- If there is a difference in the PGC number between FITC control and FITC/IONs, could FITC/IONs be responsible to induce germ cell loss? How the authors could explain/prove the reduced number of PGC in FITC/IONs? Or is there a late migration of the cells?
- If the FITC/IONs labeling reduced the number of PGC, why it could be used to label

PGCs/germ cells?

- The sentence: "Labeling of germ cells in vivo by using IONs can be beneficial to study the interaction of IONs with cells precisely, that will in-turn to help how to treat tumors by enhanced generated heat locally with minimal damage to nearby cells or tissues" - please provide a better explanation of how studies with germ cells can help to treat tumors if the cells are different and the context is also distinct;
- Some advantages of the germ cell labeling with nanoparticles are not properly highlighted in the manuscript, such as the sorting for germ cell transplantation.
- Interesting manuscript but still preliminary. More data should be done; different concentrations, *in vivo* assays, toxicity among others.

Chapter 5/General Discussion

- This chapter should be summarized, shortened and carefully revised. Gene and protein nomenclature should be revised throughout the text.
- Moreover, conflicting information is provided here: "This species (sterlet) usually matures from 2 to 3 years (males); while the females take from 3 to 4 years.". According to previous Chapter (page 33), time of maturation was 3-7 years for males, and 5-9 years for females. Please, check this conflicting information.
- Several parts are redundant and repetitive, such as the description of sturgeons as living fossils, Dnd roles among others.
- There are no conclusions or perspectives derived from the thesis. This could be included as final paragraph;

English summary

- It should be shortened and revised as well.

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**

02/07/2019, Botucatu (São Paulo, Brazil)
Date and place

Rafael Henrique Nóbrega
Name and signature

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REVIEWER:

Surname: Hongxia	**Institution:** Beijing Fisheries Research Institute Beijing, China
Name: Hu	
Titles: Prof.	**E-mail:** huhongxiazh@163.com
Please describe your professional relationship to the PhD student: Similar in research field	**Please describe your field of expertise:** Reproductive Physiology and breeding of sturgeon

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)**:

In this thesis, Dnd1 protein was described as a molecular marker of PGC and its application in detail. Dnd1 was knocked out using CRISPR/Cas9 to achieve sterility in sterlet and the gene edited fish has the potential to be the host for surrogate production. It was also the first time to label PGCs of sturgeon by IONs. The topic of this thesis is attractive, explicit and useful. The pioneering research lay an important foundation for conservation of endangered sturgeon.

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The paper has a reasonable structure, clear logic, correct format and reasonable design of experimental scheme. Knocking ou Dnd1 in germ cell and labeling PGCs by IONs are closely related to surrogate production of sturgeon which is the objective of the PhD thesis. The experiment results were reliable and the published papers was quite high-quality.

OVERALL COMMENTARY ON THE PhD THESIS

Please write comments in extent of 1-2 pages:

This thesis has reached the level of doctoral. But there are still some questions need to be explained or corrected.

1、What is the basis for selecting these three sgRNA/Cas9 concentrations in table1 of Chapter 3, please add it in discussion if it is possible.

2、In Chapter 3，since the results about elimination of germ cells by three methods (CRISPR/Cas9，antisense morpholino oligonucleotide and UV irradiation) are compared, it should be elaborated the other two method in the introduction. All three methods are effective, but which one is more operable and valuable in application?

3、Please describe briefly the principle of the specific combination of IONs and FITC-dextran, or provide references in introduction of Chapter 4。

4、In the description of figure01 in 4.3.1, is it the same thing labeled "different groups" in A with "different females" in B, C and D?

5、In 4.3.2, since the number of PGCs is counted, it would be better to list the numerical values in each group.

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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**

July.10.2019. Beijing
.....
Date and place

Hongxia HU 胡红霞
.....
Name and signature