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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: Effrosyni Fatira, M.Sc.	**First name(s), surname, titles of supervisor:** M.Sc Taiju Saito, Ph.D.
Title of PhD thesis: Nuclear transplantation in sturgeon eggs	

REVIEWER:

Surname: Lareyre	**Institution:** INRA, France
Name: Jean-Jacques	
Titles: Dr.	**E-mail:** jean-jacques.lareyre@inra.fr
Please describe your professional relationship to the PhD student: none	**Please describe your field of expertise:** Male reproductive physiology, Spermatogenesis, germ stem cell biology, Regeneration of genetic resource in fish

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)**:

Originality

Although the nuclear transfer of somatic cells (SCNT) has been successfully carried out in many vertebrate species, including fish, the studies described in the manuscript remain highly original because they are the first studies reporting SCNT using sturgeon somatic cells and the subsequent genetic characterization of the resulting reconstructed embryos.

Research aimed at understanding the mechanisms involved in the reprogramming of differentiated somatic cells in the purpose of supporting embryo development is a highly competitive and innovative research domain. The somatic cell nuclear transfer (SCNT) is a unique functional test that needs to be mastered to address the scientific questioning. Improving the technique remains a challenging goal in fish but once mastered, this methology could open inovative applications for sturgeon farming (production of female mnosex populations) or cryopreservation of endangered sturgeon species. The present studies used an original experimental design to demonstrate the sucess of the SCNT. Different Sturgeon species showing different ploïdy levels were chosen as donor (somatic cells of the fin) and recipient (sturgeon ovocyte) animals. This experimental design

facilitates the screening of putative truly cloned embryos that were subsequently characterized using microsatellite markers. The studies used state of the art techniques that are mastered by only few research team worldwide. Combined with the well recognized zootechnic background, there is no doublt that the research carried out during the course of the PhD program participated to maintain the research team among the top 5 laboratories capable to develop biotechnologies based on SCNT in fish.

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 manuscript includes a well-documented introduction, a clear description of the objectives, two chapters corresponding to primary research articles describing the experimental design and significance of the results, and a general discussion and conclusion.
The manuscript is well elaborate, clear and easily understandable. The different experiments are well designed to address the technical challenges of SCNT using sturgeons as animal model. All experiments followed logically and are appropriate to improve the efficiency of SCNT and embryos survival.
All results, detailed in two research articles, have been published in a peer reviewed journal (Scientific Reports) with an excellent impact factor of 4.01. In addition, the results gained from the thesis work were presented in five international conferrences as oral or poster presentations.

OVERALL COMMENTARY ON THE PhD THESIS

Please write comments in extent of 1-2 pages:

The manuscript entitled "Nuclear transplantation in sturgeon eggs" is well structured and includes a general introduction, a description of the objectives, two chapters describing the experimental design and data, a general discussion and a short conclusion.

The general introduction presents the definitions and properties of the embryonic and somatic totipotent cells and the history of the somatic cell nuclear transfer (SCNT) in different vertebrate species including fish. It also provides a synthetic and up-to-date review of the state of knowledge on the improvements and limitations of the SCNT. It would have been useful to detail the knowledge on mechanisms or genes involved in somatic cells reprogramming (Sox2, Oct4...). In addition, It would have been useful to describe the advantages and limitations of the different techniques used to cryopreserve and regenerate valuable genetic ressources in the animal kingdom including germ stem cell transplantation or induced embryonic stem cells. The fact that the mitochondrial DNA of the donor cells can not be transmitted to the offspring after nuclear transfer should be discussed because it questions the use of SCNT to faithfully regenerate genetic

ressources.

The following two chapters described experimental data.
Chapter 2 described the comparison of different extender solutions for egg preservation and the survival rates of the reconstructed embryos generated following single cell nuclear transplantation. The results showed that numerous articial saline solutions wee suitable to maintain egg quality for 90 minutes at least. The injection of a single somatic cell in sturgeon ovocytes produced embryos that mainly survived until the begining of the gastrulation. However, no molecular characterization was detailed to confirm whether the embryons were true clones. Only one embryo that survived until feeding was genetically characterized using polymorphic microsatellites. The results demonstrated that the embryo was tetraploid, gynogenetic and isogenic demonstrating that it was not originating from a nuclear transfer but rather from gynogenesis induced by the absence of the first two divisons during early development. This unique case report has been published in Scientifc Reports.

Chapter 3 investigated whether injection of multiple somatic cells increased embryo surving and SCNT efficiency. Data indicate that the injection of multiple somatic cells significantly increased embryo survival until the begining of gastrulation. In addition, one truely cloned embryo was detected when multiple cells of Russian sturgeon were injected in sterlet eggs. The statistical significance of this result should have been diccussed due to the low numbers of surviving embryos in the different groups. I do not understand why more than 4 different alleles were detected with AfuG_54, Afu_68, and ACIG_35 microsatellites using Ruissian genomic DNA. This comments in no way diminish the intesrest of the study since the latter contitutes the first report of a cloned embryo in sturgeons.

Experiments described in chapers 2 to 3 were logicaly connected, well conducted and included technological achievements.

The last section of the manuscript discussed the main data presented in chapters 2 and 3 with a critical analysis of the limitations of the methodology used, interesting comparative analyses and directions for future investigations. In addition of the proposed investigations, I would recommend to standardize the cell type used for SCNT.

The strong point of the PhD studies is the originality of the fish model used and the experimental design allowing a rapid pre-screening of reconstructed embryos. Only few animals survive after SCNT and only one truly cloned embryo survived until the gastrulation stage. The weakness of the study is that the somatic cells used from the sturgeon fin are likely heterogenous (epithelial or fibrobalstic cell types and different tissue origins (smooth muscle cells, endothelial cells, dermal cells, nerve cells...). The heterogeneity of the donor somatic cells may have contributed to the low effiency of the SCNT. There is maybe a need to standardize the donor cells before any evaluation of the cell cycle stage or epigenetic status suitable for cell reprograming. Moreover, there is no demonstration that viable and fertile adult sturgeons can be generated using SCNT, a prerequisite prior to the development of applications including the regeneration of

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endangered populations.

The data presented in the thesis manuscript offer new possibilies of continuing or extending the research. Possible extensions of these researches could be to improve the sucess rate of intraspecific and interspecifc SCNT using multiple somatic cell nuclear transfer, removing cell plasma membranes, extending the incubation time after SCNT, and studying cell damages...

There is no doubt that the present studies open new lines of research to improve SCNT in sturgeons and survival of the reconstructed embryos.

I would like to congratulate Effrosyni Fatira for her technical desterity, amount of work, the quality of the thesis manuscript and the clarity of the objectives and statements.

Based on the quality of the experimental data, the pertinence of the discussion and reflection on the significance of the different findings, I do think that Effrosyni Fatira deserves the title of Doctor in Biological Sciences.

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**

December 31st 2019 at Rennes (France)

.....

Date and place

Jean-Jacques LAREYRE

.....

Name and signature

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Title of PhD thesis: Nuclear transplantation in sturgeon eggs	

REVIEWER:

Surname: Goto	**Institution:** SEFREC, Ehime University, Japan
Name: Rie	
Titles: Dr.	**E-mail:** goto.rie.me@ehime-u.ac.jp
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 PhD thesis entitled „Nuclear transplantation in sturgeon eggs" written by Ms Effrosyni Fatira is a first report of somatic cell nuclear transplantation in Actinopterygii. Nuclear transplantation in teleost has been restricted to model species such as medaka, zebrafish and other cyprinids due to technical difficulties with teleost eggs. The thesis focuses on the development of the basic embryological techniques of nuclear transplantation, donor cell preparation, recipient oocyte preparation and a microinjection method for an inter- and intra-species cloning methodology for sturgeon. It demonstrates an efficient way to producing nucleocytoplasmic hybrids by transplanting multiple somatic cells rather than single somatic cells. These findings have been published as two papers in the international journal of a reputable organization. Although research on nuclear transplantation in sturgeon is challenging due to the difficulties of manipulating un-transparent eggs, this PhD thesis develops a new cloning methodology and proposes it to be a feasible method for the conservation and aqauculture of sturgeons. Moreover, the thesis shows one of the specimens resulting from somatic cell nuclear transplantation possessed only the donor nuclear genotype while others possessed both the donor and the recipient genotype. Even though the mechanism of displacement of the recipient's nucleus has not been demonstrated, this finding is of significant importance for basic research on nuclear transplantation in fish. Therefore, I recommend this PhD thesis for defense.

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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 was prepared according to the instructions and contains four chapters, including two published papers. The researvh objective, methods and results are clear and comprehensive. The structure and format of the chapters are appropriate. However, a few issues remain for the finalization of the thesis. First, even though the active voice is becoming more acceptable in scientific papers, it is better touse less colloquial language, Second, in the Introduction, I would suggest explaining the signifikance and differences between inter- and intra-species somatic cell nuclear transplantation from the perspective of bilogy and aquaculture. The thesis claims that inter-species somatic cell nuclear transplantation, such as that between Russian sturgeon and the starlet, is a feasible method for the conservation of sturgeons. However, mitochondrial DNA is not conserved by interspecies somatic cell nuclear transplantation. Third, the Discussion section contained a couple of parts that were similar to paragraphs in Chapters 2 and 3. Please check page 5 of the Instructions, which mentions avoiding repetition in the discussion of material cited in the individual publication outputs in the previous chapters. I would suggest explaining what is needed for further studies in this line of research on sturgeons and how to use the cloning methodology for the practical conservation and aquaculture of sutrgeons.

OVERALL COMMENTARY ON THE PhD THESIS

Please write comments in extent of 1-2 pages:
P4
• Please check that title of Chapters 2 and 3 reach the right margin. Maybe it would be better to force a line break to align these two.
• Is there any reason for using bold font for "General Discussion", "English Summary", etc?
P6
• The fourth paragraph seems somewhat sudden. Please rephrase the sentences.
• The heading "History of the animal cloning technique" should be placed on the next page.
P7
• The sentence "In the triploid nuclear transplants, endogenous..." is unclear. Please rephrase it.
P8
• "carassius" should be italicized.
• Should "oocyte donor" be "oocyte recipients"?
• In the last paragraph, you described the duration until reproduction of each sturgeon. However, I do not understand your message in this paragraph. Can you expect to shorten the duration until reproduction if you use the starlet as a recipient for the Russian sturgeon iSCNT?
P9

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• Please change to "the preparation of the **recipient** oocyte".
• I do not understand your meaning in the sentence "The fin can be harvested even before the ..."
• Do you need to keep "and" between "mitochondria" and "its small DNA"?
• Please delete "(Le Bail et al., 2010)" since you have already mentioned it above.
P10
• Even though it is well-known, I think it is better to explain the normal process of fertilization in fish.
P57
• I think it is better to include a brief summary of your work in the second paragraph. In several sections, you insist that you established the SCNT in sturgeon. However, you do not have next-generations to prove nuclear transfer to the germline. Therefore, I recommend that you clearly demonstrate the achievement of this study and identify tasks for the future study.
P60
• I feel that the paragraph before the "Conclusions" comes suddenly. Please rephrase it. Furthermore, it would be better to explain how you apply the cloning technique to selective breeding programs for sturgeon conservation or aquaculture.
P61
• I think "1" is a by-product of this study. Therefore, it might be better to place it after "2".

FINAL RECOMMENDATION

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

January 10, 2020
Date and place

Rie Goto
Name and signature