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: Mohammad Abdul Momin Siddique, M.Sc.	**First name(s), surname, titles of supervisor:** Prof. Dipl.-Ing. Otomar Linhart, DSc.
Title of PhD thesis: Fertilization strategies for externally fertilizing fishes	

REVIEWER:

Surname: Gomelsky	**Institution:** Aquaculture Division, College of Agriculture, Food Science & Sustainable Systems, Kentucky State University, 103 Athletic Drive, Frankfort, KY 40601 USA (502) 597-8114
Name: Boris	
Titles: Dr., Professor	**E-mail**: boris.gomelsky@kysu.edu
Please describe your professional relationship to the PhD student:	**Please describe your field of expertise:** Fish genetics and 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 studies performed in the frame of reviewed Ph.D. thesis were aimed to investigate influence of different factors (pre-incubation of eggs, sperm concentration, type of activation media and others) on fertilization rate in several freshwater and marine fish species. In the course of study the candidate used many advanced methods of analysis and novel equipment such as computer assisted sperm analysis system, cell counting using image analysis software, transmission electron microscopy and others. Results of studies have both theoretical and practical significance and will be interesting for scientists and practical fish-culturists all over the world.

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 reviewed thesis is devoted to different aspects of fish reproduction and consists of seven chapters. The Chapter 1 (General Information) presents literature review on fertilization

strategies of externally fertilizing fish and roles of different factors which influence fertilization success. The significance of different factors, such as sperm longevity, egg receptivity, activating media and pre-incubation of eggs, has been reviewed. Also, brief information on parthenogenetic development of eggs is included in the first chapter. The first chapter is concluded with the list of thesis's aims. The Chapter 2 presents published review of the structure of sturgeon eggs membranes and associated with this subject terminology. Chapters 3-6 of the thesis present results of original experimental studies, which have been performed by Ph.D. candidate with collaborators. The final Chapter 7 includes General Discussion, Summaries in English and Czech, List of Publications and some other materials.

Five of seven thesis's chapters present articles published in reputable scientific international journals (Journal of Applied Ichthyology, Animal Reproduction Science, Aquaculture Research and Theriogenology). It means that major thesis's experimental results have passed successfully journals' peer-reviewing processes. Used in experimental studies approaches and methods are chosen correctly to achieve thesis's aims. The obtained results correspond to the Ph.D. thesis objectives listed in Chapter 1.

More detailed review of basic thesis chapters is given below (in Overall Commentary of PhD thesis).

OVERALL COMMENTARY ON THE PhD THESIS

Please write comments in extent of 1-2 pages:

Chapter 1 (General Introduction) of the thesis includes review of fertilization strategies of externally fertilizing fish and roles of different factors, such as sperm longevity and egg receptivity, activating media and pre-incubation of eggs, which influence fertilization success in fish. Also, Chapter 1 included short review of parthenogenetic development of eggs. List of aims of the thesis is presented in the final part of the chapter. In general, Chapter 1 of the thesis is very informative, well-structured and is written in good scientific style. Besides text, it includes good general illustration (Figure 1) and informative tables on sperm longevity and egg receptivity in marine and freshwater fishes. Nevertheless I have several comments and suggestions:

- In my opinion it would be good to present some general information on structure of the thesis and brief list of performed studies at the final part of the chapter (besides aims).

-Chapter 1 does not contain any information on subject of Chapter 2 of the thesis. "To review of structure of sturgeon egg membranes and of associated terminology" could be included in the list of thesis's aims.

- Minor issue: I would not call parthenogenesis as 'fertilization mode' (as in aim 4) – 'development mode' would be more correct.

Chapter 2 presents review on the structure of sturgeon egg membranes and associated terminology published in the scientific journal. Although this article is not based on original experimental data it is quite valuable and will be helpful as a reference for scientists working in this area and students.

The authors systematized information from numerous literature sources and compiled valuable tables on female fecundity and egg sizes in different sturgeon species, and on terminology on egg membranes used by different authors. Also, the article contains two illustrative figures. Here are some comments and suggestions:
- It would be desirable to note in legends whether these figures are original or after some literature sources. Also, if Figure 1 is original it would be desirable to note in legend how it was drawn (using some device for drawing directly from microscope field or from digital image).
- In description of formation of fertilization cone there was no need to cite studies in teleost fish.
- This sentence is not clear: "Sturgeon eggs need only one percent of spermatozoa to fertilize each oocyte in salmonids."

Chapter 3 of the thesis describes results of study on effects of pre-incubation of eggs in fresh water and varying sperm concentration on fertilization rate in starlet sturgeon. The authors tested 3 different duration of egg pre-incubation (0.5, 1 and 10 min); sperm to egg ratio varied in experiments from 430,000 : 1 to 430 : 1). Also, this study included investigation of eggs using transmission electron microscopy to determine changes which pre-incubation possibly causes in eggs. In general, experiments were successful and the results have been published in international scientific journal. Here are some comments:

- The authors mention in Introduction that quantification of sperm density is important for standardizing fertilization protocols and unfortunately there is no data on optimal sperm to egg ratio. In my opinion 'sperm density' and 'sperm to egg ratio' are different parameters. I am sceptical towards 'sperm to egg ratio' parameter. Density of sperm, or concentration of spermatozoa during fertilization is more important index. The number of eggs, which are placed at given spermatozoa concentration, can be different in relatively large extent and this should not influence fertilization success.

- The application of electron microscopy is not mentioned in Introduction. Therefore when the authors present in 2.4 of Materials and methods techniques of fixation and preparation of eggs it is hard to figure out for which purpose electron microscopy was used.

- It would be better to present some data on absolute numbers of eggs in experimental variants; it is mentioned only that 2 g of eggs were used in each variant.

- It seems to me that conclusion that pre-incubation of eggs for 0.5 and 1 min can enhance fertilization rate for low sperm to egg ratio is too general. As data presented in Figure 1A show, the difference in fertilization rate observed in control (0 min) and in 0.5/1-min variants is statistically significant at ratio 4300:1 but not at 430:1.

- It would be reasonable to choose for experiments much wider range of pre-incubation time taking into account that egg receptivity in sturgeons can last up to last several hours.

Chapter 4 describes results of experiments on investigation of effect of pre-incubation and determination of egg receptivity period in sea bass; also in this study formation of perivitelline space

in sea bass eggs was described. This study was clearly designed and performed; made conclusions are based on obtained results. Discussion is thoroughly compiled and contains valuable literature review on duration of egg receptivity in different fish species. I have only one comment (question):
- On which criterion the overripping of eggs was identified? It is not clear for persons who do not work with this species.

The results of investigation on effects of preincubation of eggs and type of activation medium on the percentage of eyed embryos in idle are reported in Chapter 5 of the thesis. I think that experiments were designed well, obtained data were analysed by appropriated statistical methods and results of the study are clear. I have only one comment (question):

- The authors do not explain why they used such parameter as percentage of eyed embryos in this study. Why they have not determined fertilization rate at cleavage stage; the method which is usually used in such type of studies? I see that in Discussion the authors discuss influence of used factors (duration of pre-incubation of eggs and type of activation medium) on fertilizing ability of idle eggs. However, in reality they did not provide data on fertilization rate but on proportion of live embryos at advanced embryonic stage. Apparently these parameters correspond to each other but the authors had to provide some explaining statement on this subject.

Chapter 6 of the thesis is devoted to parthenogenetic activation of eggs in starlet sturgeon. Actually I have some concerns and questions which are connected with both general introduction of this subject in the article and interpretation of the obtained data:
- I see that the authors cited Hubbs and Hubbs (1932). In that article natural gynogenesis in fish was described for the first time. In title of that article parthenogenesis was mentioned but in further publications on this subject gynogenesis became the common term applied for mode of reproduction of unisexual forms in fish. In case of gynogenesis the activation of eggs by spermatozoa is needed although embryo development is under control by chromosomes of maternal origin. Currently in fish about 15 unisexual gynogenetic fish are described, which usually have hybrid origin. I saw that in some relatively recent publications (for example, Lamatsch and Stock 2009; Sperm-dependant parthenogenesis and hybridogenesis in teleost fishes. In Lost Sex: The Evolutionary Biology of Parthenogenesis) natural gynogenesis is called by specific term 'sperm-dependent parthenogenesis'. This also show the difference between gynogenesis and pure parthenogenesis when presence of spermatozoa is not needed at all. Taking into account these considerations I think that it would be more correct either not to cite Hubbs and Hubbs (1932) or to cite it together with other citations on natural gynogenesis in fish with some explanations on difference between natural gynogenesis and parthenogenesis.

- I think the authors had to note large difference in natural parthenogenesis described by Chapman et al. (2007, 2008) and Robinson et al. (2011) in sharks and observed parthenogenetic activation of eggs in sturgeons. In sharks parthenogenesis is functional and results in appearance of viable progeny while in sturgeons it is abortive, incomplete and only several initial divisions of blastomeres are observed. Therefore these phenomena hardly can be compared.

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- The authors noted that after initial article by Detlaff and Ginsburg (1950) no research has been conducted on parthenogenetic cleavage development in sturgeons. In this connection I would like to note that possibly no journal articles on this subject have been published but the parthenogenetic cleavage is described as well-known phenomenon in books on developmental biology of sturgeons. In book by Dettlaff et al. (1993), which the authors cited, 'Parthenogenetic Cleavage' is subtitled part of subchapter 2.4.4 'Cleavage Defects'. Also, parthenogenetic cleavage in sturgeons is described by Dettlaff and Ginsburg (1991) in Chapter 2 of book "Animal Species for Developmental Studies (Volume 2, Vertabrates)".

- Application of linear regression analysis and graph presented in Figure 4 give some indications that there is dependence of parthenogenetic activation rate on the quality of eggs in terms of fertilization rate. However, based on data presented in Table 1 it can be seen that rates of parthenogenetically activated eggs at fertilization rates 28.7% (female 4), 44.8% (female 2), 74.1 (female 6) and 92.7% (female 5) are not significantly different. Therefore I presume that made conclusion on existence of this dependence is not based on the obtained data.

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**

June 14, 2016 Frankfort, Kentucky, USA
Date and place

Boris Gomelsky B. Gomelsky
Name and signature

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Title of PhD thesis: Fertilization strategies for externally fertilizing fishes	

REVIEWER:

Surname: Labbé	**Institution:** INRA
Name: Catherine	
Titles: Dr.	**E-mail**: Catherine.Labbe@rennes.inra.fr
Please describe your professional relationship to the PhD student: None before this thesis reviewing ;	**Please describe your field of expertise:** Gamete biology, reproductive biotechnologies, epigenetics

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)**:

This question is difficult to state on the sole basis of the information given in the manuscript, see my general comment. Momin Siddique did not emphasize clearly the practical or scientific questions his thesis was aimed to answer. I believe this will be better demonstrated during the oral defending.
Apart from this comment, the underlying idea which is to understand better the limits of egg manipulation in water or in other media prior to fertilization is an important issue for aquaculture practice. For this reason, I will say that the thesis is very interesting.

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)**:

This information is provided in my general comment.

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OVERALL COMMENTARY ON THE PhD THESIS

Please write comments in extent of 1-2 pages:

The seemingly aim of the project is to get a better understanding at oocyte handling conditions during artificial insemination in several fish species : sterlet sturgeon, sea bass and ide. Interestingly, the project has scanned fresh water and marine species, and teleostei and chondrostei infraclass and subclass whose gamete biology and fertilization conditions are diverse.

This work led to 5 publications which are provided in the manuscript. The first one, published after the first year of the thesis, is a review of the egg envelopes in sturgeon species, with an interesting survey of the diversity in egg fecundity and size among species, and a useful nomenclature table where all the usage names for the different egg envelopes are given. The second publication deals with the effect of pre-incubation of Sterlet eggs in water prior to fertilization at various sperm/egg ratios. This publication showed that Sterlet egg ability to be fertilized after up to 10 min in water was not altered provided that the sperm/egg ratio remained high. Some discussion of this work might be missing, or at least I did not find it: what relationship is there between the sperm/egg ratio tested in the work, and the aquaculture practice (with 1/200 dilution). Besides, the amount of coelomic fluid remaining with the eggs (2 g eggs into 8 mL water) is not discussed, although the potential deleterious effect of the coelomic fluid is introduced. What would happen with coelomic fluid of bad spawns, more concentrated with yolk of broken eggs? The third publication shows how the same experimental line was conducted with sea bass eggs. In this work, the dilution of the eggs with seawater was completely different from the Sterlet experiment (10 mL eggs + 5 mL sea water). This kind of dilution did not impair egg fertilizing ability if fertilization was performed within the first 1 min of incubation. Longer durations impaired the fertilization rates. Interestingly, the authors observed the same sensitivity trend with fresh and overripe eggs. The 4th publication deals with Ide eggs pre-incubation in solutions with different osmolalities (80, 150 and 200 mOsm/kg), for up to 2 min. It could be shown that the solutions at the 2 highest osmolality provided the best fertilization rates after egg pre-incubation for 2 min. The last publication dealt with the relationship between egg quality and parthenogenetic activation upon fertilization. The study of 6 different spawns led to build a significant negative correlation between the egg ability to be fertilized and egg ability to parthenogenetic activation.
The manuscript ends up with a short discussion of the work. This summary cannot stand for a general discussion, but more for a short summary of the main conclusion that can be drawn form each publication in the manuscript. It is relevant and precise. The last paragraph of this discussion gives some general items open to debate, and they are very interesting. However none of these items were elaborated enough: relation between egg receptivity and sperm motility duration,

volume of ovarian fluid, egg activation strategy etc.

The individual quality of each of these publications is fair: the experimental protocols are straightforward, the number of replicates carefully set, and the statistics well performed. What is less mature in the manuscript is the general picture brought by the merging of all the experiments. It is understandable at this stage, because Momin Siddique had to work hard to get all these papers published. I do not require that the manuscript be changed at this stage. However, before the defense, Momin Siddique should work on this integration task. Below, I am giving some advices that Momin may use to prepare his defending:

1) The 3 research papers in the manuscript core dealt with egg manipulation during artificial insemination, not with animal strategies. This should be made clear to be sure of the target of the work: aquaculture, not ecology. In that sense, the title may not be completely relevant. But this may deserve some discussion that Momin Siddique is free to open.
2) The choice of the 3 species and that of the experimental protocols for each species is not explained clearly (or I missed it). In sterlet, one additional parameter is the sperm/egg ratio. In sea-bass, one additional parameter is the egg quality (ripening). In ide, one additional parameter is the composition of the egg incubation solution. Besides, all 3 species were studied with different egg/medium ratios, and with different incubation time. A general strategy in relation with the specificity of each species should be given. It can be aquaculture concerns, biological or practical concerns.
3) The link between the review paper on egg envelopes and the 3 following manuscript is not clear. It is the same with the last one on parthenogenetic activation. It is one very demanding task in thesis defending, but also one of the most intellectually challenging/exciting: putting together some seemingly scattered information. And if no logical thread can be found, it is better to skip the information of the first and last paper for the thesis defending. This lack of link is really emphasized in the manuscript by the way the aims of the thesis are presented: 4 titles without justification.

To summarize my comments, the experimental work achieved by Momin Siddique is fair and relevant for aquaculture practice, and the publications show the ability of Momin Siddique to disseminate his work. There is an obvious lack of general picture of the work in the manuscript, but considering the effort in publication (5, which is high), it does not require any rewriting of the manuscript. Momin Siddique was involved in two collaborative work, one with Daniel Zarski in Poland and one with Christian Fauvel in France, both leading to publications. He has attended several courses in relation with his need to develop his project, and this demonstrates a true will for improving and learning. He has given 3 oral presentations at international conferences.

For all these reasons, I recommend that Momin Siddique be allowed to defend his thesis. It is expected from him that at this occasion, he can show his ability to emphasize better his contribution to the scientific community and aquaculture community.

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FINAL RECOMMENDATION

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

Rennes June 23rd 2016
.....
Date and place

Catherine Labbé.....
Name and signature

Catherine LABBÉ