University of South Bohemia in České Budějovice
Faculty of Fisheries and Protection of Waters
Zátiší 728/II, 389 25 Vodňany, Czech Republic
VAT: CZ60076658
☎ +420 387 774 601 fax: +420 387 774 634 e-mail: sekretar@frov.jcu.cz

Confidential

Review of USB FFPW PhD Thesis

First name(s), surname, titles of the PhD student: Azadeh Hatef, MSc.	**First name(s), surname, titles of supervisor:** Prof. Dipl.- Ing. Otomar Linhart, DSc.
Title of PhD thesis: Sperm functions impairments and steroidogenesis transcriptomic alternations in fish exposed to endocrine disrupting chemicals	

REVIEWER:

Surname: Ciereszko	**Institution**: Polish Academy of Science Institute of Animal Reproduction and Food Research Olsztyn, Poland
Name: Andrzej	
Titles: Prof., Ph.D.	**E-mail**: acieresz@pan.olsztyn.pl
Please describe your professional relationship to the PhD student: none	**Please describe your field of expertise:** Male 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 results of the thesis have been published in peer-reviewed journals ranked quite high by the ISI Web of Knowledge (Journal Citation Reports 2010), Environmental Toxicology and Chemistry (IF = 3.03) is ranked 34 (of 193) in the category „Environmental Sciences" and 25 (of 83) in the category „Toxicology", Ecotoxicology and Environmental Safety (IF = 2.34) is ranked 38 (of 83) in the category „Toxicology", and Journal of Applied Ichthyology (IF = 0.94) is ranked 26 (of 46) in the category „Fisheries". As such, the results of the thesis were subjected to a very strict peer-review process and thus were validated according to very rigorous standards of the scientific process. In my opinion, this thesis is very well focused around the effects of endocrine disruption chemicals (EDC) on male reproduction in fish. Both detailed studies of sperm function impairments and evaluation of alteration in transcriptomics and steroidogenesis make a significant contribution to the knowledge concerning ecotoxicology. It is especially important that several studies performed by the author employed the use of EDC at environmentally relevant concentrations. Overall, the scientific importance of the thesis is very high, both for basic research (regarding knowledge on mechanisms of endocrine disruptors action on the gametes and tissues of reproductive system) and applied research (concerning evaluation of threat to aquatic organisms related to exposure to EDC). Ms. Azadeh Hatef has already established herself as an important contributor to the scientific field of fish reproduction and toxicology, her papers have been cited 48 times, and her h index is 5 (SCOPUS, June 2012).

University of South Bohemia in České Budějovice
Faculty of Fisheries and Protection of Waters
Zátiší 728/II, 389 25 Vodňany, Czech Republic
VAT: CZ60076658
☎ +420 387 774 601 fax: +420 387 774 634 e-mail: sekretar@frov.jcu.cz

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 main part of the thesis is composed of three chapters which are reprints of published or accepted papers (4) and manuscripts to be submitted (2). In the latter, information concerning target journals is lacking. Chapter 2 provides information concerning effects of EDC on sperm functions (sperm motility characteristics and morphology. Alterations of steroidogenesis of sex hormones in response to Bisphenol A and vinclozolin are presented in chapter 3. Chapter 4 demonstrates sperm impairments associated with alterations in sex steroid receptors and steroidogenesis mediating genes. The scientific part of the thesis is preceded by the introductory chapter 1, in which the author adequately reviews the topics related to theses, such as the definition and the nature of endocrine disrupting chemicals, the mechanisms of their action, characterization of male reproductive system in fish, with the emphasis on the endocrine regulations of spermatogenesis and sperm biology. Endocrine disruptors relevant to the thesis are then characterized, each paragraph is ended with few sentences providing clear justification for the study. Objectives of the study are the last part of the chapter. General discussion (chapter 5) follows the scientific part; the main achievements of the thesis are summarized and concluded. Altogether, all parts of the thesis are very well connected and subjects of the thesis are cohesive. The language of the thesis is clear.

OVERALL COMMENTARY ON THE PhD THESIS

University of South Bohemia in České Budějovice
Faculty of Fisheries and Protection of Waters
Zátiší 728/II, 389 25 Vodňany, Czech Republic
VAT: CZ60076658
☎ +420 387 774 601 fax: +420 387 774 634 e-mail: sekretar@frov.jcu.cz

Please write comments in extent of 1-2 pages:

As I mentioned above, this thesis covers a set of original topics linked together in a logical way. The thesis is written in a clear manner and the results of the thesis were already published in top peer-reviewed journal. The dissertation would obviously benefit from publication of two manuscripts in peer-reviewed journals. Basic and applied aspects of fish reproduction are well balanced in the thesis, from basic sperm motility and morphology studies to testing steroidogenesis and transcriptomic analysis in response to EDC. In vivo studies were performed with the use of environmentally relevant concentrations of xenobiotic substances. It needs to be mentioned, that Ms. Azadeh Hatef, besides of papers included in the thesis, is coauthor of 11 peer-reviewed papers. This number is impressive and confirms the excellence of her research and her competence to scientific work.
In conclusion, my overall grade of the thesis is excellent and I strongly recommend the thesis for the defense of PhD thesis.

Minor corrections that can be used to improve the text are listed below.

Title – male fish should be indicated because females were not studied in the thesis
P5 "sex steroidogenesis" is not fortunate term.
P5 the title of 6th MS is different that the title on pages 59
P5, 4.2. typographical errors should be corrected (hyothalamus , are release)
P5, 4.2 eSRS22/CA should be defined
P10 the paragraph concerning testis morphology is rather short. I would add at least the figure presenting main structures and cells of the testis
P13, L3 I would not express production of DEHP in pounds
P13, figure 2 I would rather provide formula for mercury chloride
P14 6. Objectives of the present study
P87, L7 estradiol?
P115, L7 concentration
There is no need to introduce abbreviations several times, for example E2 is introduced on pages 10 and 116, 17,20β-P is introduced on page 10 and 117, Vtg is introduced on pages 12 and 118 (on page VTG is also used, in addition to Vtg), StAR is introduced on pages 11 and 119, P450scc is introduced on pages 11 and 119, etc. Please carefully check all abbreviations used in the chapter 1 and 5.
P117 third sentence is unclear
P117, L12 luteinizing
P118, third paragraph Hatef
P118, fourth paragraph E2 concentration was unchanged? Next sentence is unclear (E2 induced E2?)
L120 Hg effects are not mentioned in concluding paragraph; results of in vitro studies are not mentioned
Concluding paragraph 3rd line dose
L133 Niksirat et al. 2007 rainbow

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**

Olsztyn, 6 June 2012.....
Date and place

Name and signature

University of South Bohemia in České Budějovice
Faculty of Fisheries and Protection of Waters
Zátiší 728/II, 389 25 Vodňany, Czech Republic
VAT: CZ60076658
☎ +420 387 774 601 fax: +420 387 774 634 e-mail: sekretar@frov.jcu.cz

Confidential

Review of USB FFPW PhD Thesis

First name(s), surname, titles of the PhD student: Azadeh Hatef, MSc.	**First name(s), surname, titles of supervisor:** Prof. Dipl.- Ing. Otomar Linhart, DSc.
Title of PhD thesis: Sperm functions impairments and steroidogenesis transcriptomic alternations in fish exposed to endocrine disrupting chemicals	

REVIEWER:

Surname: Kestemont	**Institution:** Research Unit in Environmental and Evolutionary Biology – Department of Biology Faculty of Science University of Namur
Name: Patrick	
Titles: Prof. Dr	**E-mail**: patrick.kestemont@fundp.ac.be
Please describe your professional relationship to the PhD student: I don't have any relationships with the PhD student	**Please describe your field of expertise:** Fish physiology and biochemistry, with a special emphasis on fish reproduction and endocrinology, aquatic ecotoxicology, aquaculture of freshwater 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)**:

The manuscript of Azadeh Hatef constitutes an original contribution to the scientific knowledge regarding the effects of different endocrine disrupting chemicals (EDCs) on the reproductive physiology of fish, with a special emphasis on male steroidogenesis and sperm characteristics. The study includes a variety of scientific and technical approaches using the recent methods in fish physiology and endocrinology, combining in vitro and in vivo experiments as well as molecular, cellular, physiological and morphometric variables. The results are substantial, innovative and provide new ideas and hypotheses in the field of fish ecotoxicology research. Focusing on sperm function impairement is of special interest as many researches have been done so far on the effects of EDCs on the female reproductive system while the scientific literature in male reproduction is rather scarce. Beyond a descriptive observation of testis and sperm function impairement, the work is also investigating the mechanisms of the disruption induced by different chemicals, which open new research perspective in ecotoxicology.

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 consists of 5 chapters and has been prepared as a compilation of several articles that are published or submitted for publication in peer-reviewed journals, complemented by an introduction and a general discussion, plus additional parts (summaries, list of publications, training and supervision plan and curriculum vitae of the candidate). As a general comment, the thesis represents an important piece of work and all experiments have been conducted in a proprer way. The author has applied several techniques that are very well controlled in routine by the host laboratory but has also developed new approaches based on training stages performed in different laboratories abroad and collaboration with many partners. The results are relevant and sound, and the author can be congratulated for an impressive scientific production since 4 articles are

University of South Bohemia in České Budějovice
Faculty of Fisheries and Protection of Waters
Zátiší 728/II, 389 25 Vodňany, Czech Republic
VAT: CZ60076658
☎ +420 387 774 601 fax: +420 387 774 634 e-mail: sekretar@frov.jcu.cz

published or accepted for publication yet and two others are under submission. This is definitely an excellent output for a PhD thesis.

The weak aspect of the thesis is that the whole manuscript does not really appear, at this stage, as a comprehensive thesis but rather as a succession of articles within a general scientific domain. The introduction is (too) brief and describes the general framework of the thesis, with a short description of the male reproductive system in fish and the presentation of the selected chemicals tested as well as the thesis objective. The author has decided to separate the presentation of the different chapters according to the approach used: first in vitro studies, then in vivo ones. To some extend, this is quite logical but it does not facilitate the reading and comprehension of the whole results for a given chemical. For example, the effects of Bisphenol A are presented and discussed in chapter 2.2, chapter 3.1 and chapter 4.1. These results are separated by sub-chapters dealing with other chemicals and approaches. I would have preferred to read all the results obtained from the investigation on one chemical, using different approaches and endpoints. There is no absolute need to re-order the chapters and sub-chapters, but, at least, some short connections between chapters should be written, facilitating the reading of the thesis and linking the outputs of one chapter to the introduction of the following one. The rationale for working on different chemicals and different fish species is not very clear and should be better explained/justified in the introductive chapter: some chemicals have been applied on perch with the analysis of some variables while some other chemicals have been tested on goldfish, using other variables. Comparisons between chemicals are thus rather difficult, and relevance of some biomarkers is sometimes questionable. More comments on this matter are given in the next section on my evaluation report.

University of South Bohemia in České Budějovice
Faculty of Fisheries and Protection of Waters
Zátiší 728/II, 389 25 Vodňany, Czech Republic
VAT: CZ60076658
☎ +420 387 774 601 fax: +420 387 774 634 e-mail: sekretar@frov.jcu.cz

OVERALL COMMENTARY ON THE PhD THESIS

Introduction
As mentioned in the previous comments, the thesis would benefit from a more extended general introduction. The effects of pollutants on the male reproductive system is really short and could be more detailed, particularly regarding the existing knowledge in terms of mechanisms of action of pollutants on testis and sperm biology. Similarly, the author has chosen four chemicals. These chemicals are shortly presented with regard to their effects on mammal or fish reproductive system, but there is no real state of the art concerning the mechanisms of action of these chemicals. What do we know exactly, and what don't we know at the initiation of this thesis. A compilation of the existing literature summarized in a table would be helpful. The general physico-chemical characteristics of each chemical should be described, including the degradation rate in different environmental conditions, the toxicity of metabolites, etc. The author provides only few information about these chemicals, but this is essential in toxicology.
I have not found a general objective of the thesis. The author has described the specific objectives of the in vitro approach and those of the in vivo ones, but not the general objective itself. What was the rationale to investigate the effects of several chemicals by different approaches on different species? This should be clearly written. A thesis must be more than a compilation of different articles dealing with the same topic, and this should be presented at the end of the general introduction, as well as in the general conclusions and perspectives (which are lacking at this stage).

Chapter 2 : in vitro approach
This chapter has been properly conducted and results are already published in international journals. The chapter 2.1 is particularly interesting and combined a very nice set of in vitro experiments. As this article is published yet, changes are not possible. I am surprised by the high concentrations of $HgCl_2$ used in this experiment. Are some of these concentrations close to environmental values or close to values found in seminal fluid from environmentally contaminated fish? The author has used different approaches, making this paper an excellent contribution to our knowledge and debate about the relevance of in vitro ecotoxicology. Well done!
The chapter 2.2 is rather limited in terms of information provided. I am surprised that the paper has been published on the basis of nominal doses of BPA only. This comment is valid for some other chapters of this thesis. The author should be aware that nominal doses are no longer accepted in high quality toxicology and ecotoxicology journals. Actual doses, based on reliable measurements, are needed The author used the CASA system which is of course a technology well controlled by the host laboratory. CASA is usually providing a huge amount of complementary parameters and this is not clear why the author has limited the presentation of data to the percentage of motility and the velocity of sperm only. The experimental design used in chapter 2.1 should have been applied also in this chapter 2.2 making the paper more informative. Aside the criticism evoked above regarding the different criteria used in the different articles (making the comparison of results rather difficult), my concern is that the author has decided to present the original printed version of the articles in the thesis, but this makes the readability of the text really difficult (very small characters!).

Chapter 3 : in vivo approach
The chapter 3.1 is a logical follow-up of chapter 2.2 and provides interesting data on the reproductive physiology of male goldfish contaminated in vivo with BPA. The first question is why performing the research on goldfish and not on perch? This should be explained somewhere in the thesis. Large species specific sensitivities have been shown in the literature for many chemicals. Actual concentration of BPA has been measured here. The author observed a decrease in BPA concentration between 1 and 48h after chemical addition, and concluded of a possible bioaccumulation in fish tissues. Why not a simple degradation of BPA with time? We don't have any information, neither about BPA stability in water, nor about possible production of metabolites that have not been assayed in this study. Some shortcomings should be avoided: no mortality does not mean that the doses of BPA were not toxic, but simply that the author has not worked close to the acute toxicity level. Some additional indicators should have been informative, such as the effects of BPA on sperm volume and spermatocrit. The plasma VTG level increased with dose and time of BPA, but not the E2 concentration. While it is well-known that E2 is responsible of VTG production in the liver, the author stated this result but did not discuss it. As the article is already published, no changes can be made here but discussion about the absence of E2 increase could be done in the general discussion section.

University of South Bohemia in České Budějovice
Faculty of Fisheries and Protection of Waters
Zátiší 728/II, 389 25 Vodňany, Czech Republic
VAT: CZ60076658
☎ +420 387 774 601 fax: +420 387 774 634 e-mail: sekretar@frov.jcu.cz

The chapter 3.2 investigates the effects of a fungicide on sperm quality and steroidogenesis in male goldfish. This article is under submission. The authors claim that they have performed a comprehensive assessment of VZ toxicity in goldfish, which is definitely not the case as the study only includes some sperm quality parameters and few steroid assays (11KT and E2, but not T which is the precursor of the two former steroids) and one time point. Again here, VZ has been used on a nominal basis, without measurement of the actual concentration. This limits the possibility of publication in high quality journals. This chapter provides a rather limited set of data while the discussion is, from my point of view, very speculative and should be reduced substantially. The same question is raised here again: why not measuring the same set of markers as for the other compounds? Table 1 should be revised as VZ was not ranging between 1 and 100 µg/L, but 100 to 800 µg/L.

Chapter 4: physiological and transcriptomic approaches
These two chapters are providing interesting data combining physiological and molecular approaches, leading to a better knowledge of the mechanisms of action. As for the chapter 2.1, the set of data provided in chapter 4.1 is rather large, including several time points (which is very relevant when transcriptomic data are used) and the discussion is largely supported by the results obtained, justifying the publication of this work in a high quality journal such as ETC. I do not have major comments on this chapter 4.1. I am wondering why the authors have not added steroid measurements that should have been useful to support the diagram (fig. 8).

Chapter 4.2 deals with another chemical, not investigated before in this study. The rationale for this research strategy should be betterjustified, and a short introduction added before the chapter itself. Compared with the previous chapter, chapter 4.2 is suffering from several weaknesses which make the whole paper less interesting: no actual concentration of DEHP (only nominal doses), only one time point, no T level analysis (an interesting value is the ratio 11KT/T versus E2/T, suggesting differential pathways in the steroid biosynthesis).

General discussion
Except some syntax and orthographic errors that should be corrected throughout the text, the discussion is generally well written and places the obtained results within the existing literature. The author has chosen to discuss her results by following the same framework as the one used for the presentation of the results in the different chapters, first in vitro, then in vivo and finally the transcriptomics ones. I would suggest to better integrate all these data in a more comprehensive way instead of separating the general discussion in so many sub-sections. At this stage, the discussion appears quite redundant to what has been written before in each chapter and sub-chapter, while the methodology used (and those not used) is not discussed. There are many other variables that could have been used and the general discussion is the right place to compare the outputs of the thesis with results obtained from other approaches and variables. All experiments have been conducted with adult fish. What could be the effects of these chemicals on fry, juveniles or immature fish, in which steroidogenesis and internal steroid conditions are less present, making the fish more sensitive to disruption of their future reproductive status. Sperm has been extensively analysed, but what could be the effects of these chemicals on the other stages of spermatogenesis. Histological approach would be useful in this respect.

Conclusions and perspectives should be added at this end of the thesis. Based on her knowledge and acquired results, what could the author suggest as further investigations on this topic.

Assuming that these changes or improvements are done properly, I recommend the public defence of this thesis and congratulate the candidate for the quality of her manuscript.

University of South Bohemia in České Budějovice
Faculty of Fisheries and Protection of Waters
Zátiší 728/II, 389 25 Vodňany, Czech Republic
VAT: CZ60076658
☎ +420 387 774 601 fax: +420 387 774 634 e-mail: sekretar@frov.jcu.cz

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

Namur, 7/06/2012

Patrick Kestemont