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: Dmytro Bytyutskyy, M.Sc.	**First name(s), surname, titles of supervisor:** Prof. Dipl.-Ing. Martin Flajšhans, Dr.rer.agr.
Title of PhD thesis: Interrelationships between ploidy level, genome size and cell size in series of ploidy level models from 2n to 14n fish	

REVIEWER:

Surname: Benfey	**Institution:** University of New Brunswick P.O. Box 4400 Fredericton, New Brunswick, Canada E3B 5A3
Name: Tillmann	
Titles: Prof.	**E-mail:** benfey@unb.ca
Please describe your professional relationship to the PhD student: none.	**Please describe your field of expertise:** fish physiology and aquaculture, especially with respect to artificial triploidy.

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 work has been published as three papers in international peer-reviewed scientific journals. Chapter 2 is rather trivial but represents a necessary study to validate the use of tench blood as a standard for the following two chapters. Chapters 3 and 4 present interesting and novel results with respect to the relationship between ploidy level, DNA content, erythrocyte nuclear size and nuclear surface-to-volume ratio. The work is orginal and adds to our knowledge on sturgeon genetics and cell biology. (See overall commentary section for more details.)

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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 order and content of the various chapters represent a logical and practical flow, with each research chapter providing groundwork for the one that follows. The published chapters (2-4) are well written and presented; the Introduction and General Discussion are less well written but still understandable. The approaches used are appropriate and the results address the states thesis objectives. (See overall commentary section for more details.)

OVERALL COMMENTARY ON THE PhD THESIS

General Comments

This dissertation begins with a detailed and well supported review of the literature outlining the diversity and significance of polyploidy in sturgeon and the methods (with strengths and weaknesses) used to determine ploidy level in fishes (chapter 1).The following three research chapters present a logical and practical progression, beginning with a simple study to validate the use of diploid and triploid tench blood as standards for ploidy determination in other species of fish (chapter 2), continuing with a study to determine whether nucleus size is determined by nucleus DNA concentration (as measured by densitometry from Fuelgen-stained red blood cells) in sturgeon having different genome sizes and ploidy levels (chapter 3) and finishing with a study that expands on chapter 3 by including additional ploidy levels and comparing densitometry with confocal laser scanning microscopy as an alternative tool for estimating nucleus size (chapter 4). A general discussion completes the dissertation. This final chapter was disappointing; although it provides an adequate summary of the research, there is little conjecture on what it all means or any suggestion of likely future research paths to build upon this work.

The three research papers have all been published in peer-reviewed international journals, and are therefore well written and presented. The first and final chapters are less well written, although certainly understandable. I recognize that English is not the candidate's first language and find no fault with this. Of more fundamental importance is whether the candidate has demonstrated an ability to put forward testable hypotheses and has properly understood the results and interpreted them within the current state of knowledge. Chapter 2 is rather trivial, but represents a necessary step for establishing standards for the following two chapters. Chapters 3 and 4 clearly demonstrate an ability to conduct independent research, and I have no reservations in recommending that the thesis proceed to defence. I enjoyed reading the dissertation and I believe the candidate has significantly advanced the field of study.

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In the following paragraphs I outline some edits, questions and concerns pertaining to the dissertation. Many of these apply to work that is already published in chapter 2-4. I recognize that this may cause some difficulty, but my role is to review of the thesis as a single body of work rather than a collection of papers that have already been accepted through peer review.

Chapter 1

1. Near the middle of page 18 it is stated that "notable exceptions from the above scheme in triploids obtained by mating tetraploid males to diploid females". It should be noted that it is also possible to obtain triploids by mating tetraploid females to diploid males.

2. Towards the bottom of page 18 it is stated that "high growth rates cause captive animal to reach puberty earlier than their wild conspecifics … and before the fish reach marketable size". It should be noted that not all species of fish will mature before reaching market size, even with fast growth rates.

3. Section 1.3.2 begins by naming two theories (nucleoskeletal and nucleotypic), but never provides clear definitions for either.

Chapter 2

1. There is text missing at the fourth line from the bottom of page 40: where it currently states "a fish standard and induced triploid" it should state "a fish standard. The purpose of this study was to measure genome size for diploid and induced triploid".

2. Near the top of page 41 it is explained that six blood smears were made from each specimen, and then at the top of page 42 it is stated that 50 nuclei were measured from each specimen. This needs better explanation. Were 8-9 nuclei measured on each of the 6 slides to give the 50 total? What criteria were used for selecting specific cells for measurement? [These same ambiguities apply to chapters 3 and 4.]

3. The term "standard" is not used consistently. On page 41 it is first used as a heading to describe chicken blood and then at the bottom of the page (second-from-last line) it is used to describe a set of tench samples.

4. Given that 10 separate chicken blood samples were used, why is there no standard deviation given for the chicken data in Table 1 (page 44)?

Chapter 3

1. In the fourth paragraph of the Introduction, it is stated that "the relationship between DNA content and erythrocyte size is so far the least established in fishes". This statement needs to be qualified, i.e., recognizing that it refers specifically to vertebrates and to inter-species comparisons. There is a wealth of literature that clearly shows a relationship between DNA content and erythrocyte size within species of fish, when comparing diploids with triploids.

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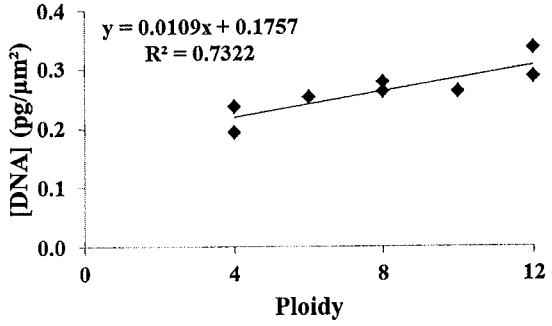
2. In the final paragraph of the Introduction, it is stated that sturgeon hybrids were included in the study, but none of the fish listed in Tables 1 or 2 are shown as hybrids. I was left wondering whether inclusion of the term "hybrid" was an error or whether it was meant to imply that the 6n and 10n sturgeon were considered to be within-species hybrids, i.e., (8n x 4n) and (12n x 8n), respectively. This becomes clear in the next chapter 4 (i.e., they are within-species hybrids between individuals of different ploidy levels), but this should be clarified within chapter 3.

3. There is a great deal of important information missing from the image analysis methods section, i.e., (i) the number of nuclei measured per fish, (ii) the criteria used for selecting specific nuclei for measurement, (iii) the number of staining kits (or batches of stain) that were used, and (iv) how the internal standards were used (added to the sturgeon slides or on separate standard-only slides).

4. There appears to be an error in Figure 1: the DNA concentrations shown for 12n Siberian and 12n Russian sturgeon (approximately 0.31 and 0.32, respectively) differ from what I calculate using the data in Table 2 (= 0.34 and 0.29, respectively). All other values appear to correspond between the figure and the data in the table: 0.19, 0.24, 0.25, 0.26, 0.28 and 0.26, going from left to right in Figure 1. Assuming the error is with the data used for this figure (rather than errors in the table), this means that the equation derived to fit the data is also incorrect.

5. Given that Figure 1 is meant to show the effect of ploidy on nuclear DNA concentration, it makes no sense to me to have the different types of sturgeon equally spaced along the X-axis independent of their ploidy, rather than grouped by ploidy. This has a profound (and incorrect) effect on the inferred relationship. If the graph is redrawn using ploidy on the X-axis and the correct values for the two 12n sturgeon, it does appear that there is a reasonable linear relationship:



[Note that in the next chapter, Figure 2 does group data by ploidy.]

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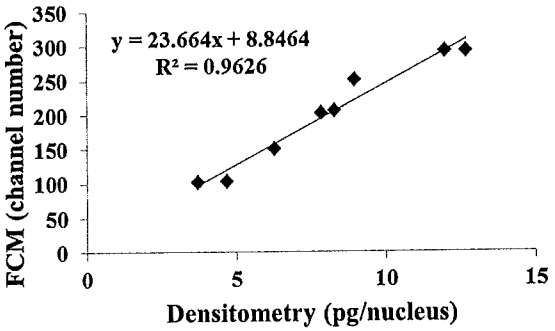
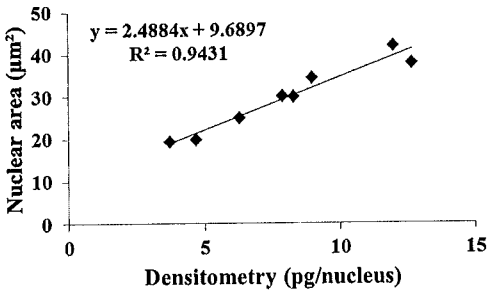
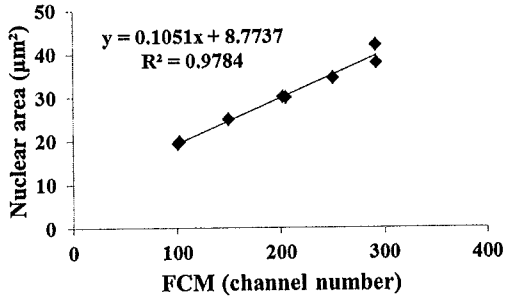
6. It is apparent from the data that have been included in this paper that there is excellent correspondence between DNA content as estimated by flow cytometry and as estimated by densitometry:



Furthermore, DNA content estimated by flow cytometry is a slightly better predictor of nucleus size than DNA content estimated by densitometry (although still excellent):





Given these observations, and the fact that flow cytometry can measure DNA content of far more nuclei within the same period of time than densitometry, what is the advantage of densitometry for this kind of study? [The ability to measure more nuclei by flow cytometry is apparent in the next chapter, where 8000 nuclei were analyzed per specimen, compared to 100 nuclei with Fuelgen image analysis.]

7. At the beginning of the Discussion, it is stated that "all authors used flow cytometry", but is this correct for Kafiani et al. (1958)? I cannot access a copy of this paper, but based on the date of publication it seems unlikely that they would have used flow cytometry.

8. Further in the Discussion (top of page 50), it is stated that "results obtained with FIA were in good accordance with data established by most other authors (Table 2), with the exception of Zhou et al. (2011)" and "the DNA contents measured in this study exhibited high similarity to those obtained by Birstein et al. (1993)". Although this is generally correct, it should be noted that the current study's value for the stellate sturgeon was closer to that of Zhou et al. (2011) than to Birstein et al. (1993).

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9. I do not understand how the values of 0.19-0.32 were calculated for DNA content "increments" (see Abstract and second-to-last paragraph of Discussion); it seems to me these values should be 0.52 and 0.22 for the tetraploid and octoploid species, respectively.

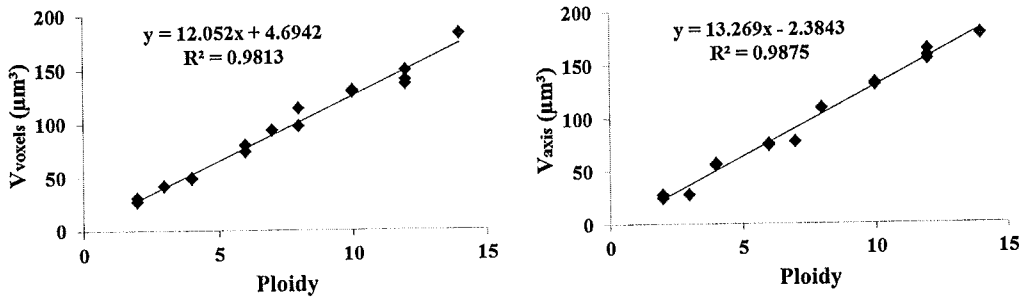
Chapter 4

1. As in chapters 2 and 3, details are lacking as to how many nuclei were measured per fish (rather than total measured per species) and what criteria were used for selecting specific nuclei for measurement.

2. In the section on CLSM (page 58), it is mentioned that "dividing cells and immature erythrocytes were excluded" from the analyses. This is unfortunate, given that previous research has shown that abnormal erythrocytes (perhaps dividing) are more frequently seen in triploids than in diploids. Are there no data that can be included in this study?

3. In the first paragraph of the Results and Discussion section, it is stated that "There seem to have been only several reports … dealing with nucleus and cell volume in sturgeon species, including Siberian sturgeon (8n), beluga (4n), Russian sturgeon (8n), and their hybrids, but no information considering other sturgeon ploidy levels." There is at least one such prior study (not cited anywhere in this dissertation), giving cell volume data for 12n and 18n: Beyea, M.M., T.J. Benfey & J.D. Kieffer. 2005. Hematology and stress physiology of juvenile diploid and triploid shortnose sturgeon (*Acipenser brevirostrum*). Fish Physiol. Biochem. 31: 303-313. (http://dx.doi.org/10.1007/s10695-005-1552-y)

4. The statement near the top of page 61 that "that nuclear volume and surface-to-volume ratio changes non-linearly with increasing ploidy level" is only partly correct. Figure 2 confirms this non-linear relationship for surface-to-volume ratio, but using the data presented in the paper it seems to me that there is very clearly a linear relationship between nuclear volume and ploidy:



This difference between what the paper concludes and what the data say needs to be explained.

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5. The observation that nucleus surface-to-volume ratio does not decrease linearly with ploidy, and that DNA therefore appears to be more densely packed within nuclei as ploidy increases, is to my mind probably the most interested and novel result to come from this thesis. It would be interesting to see more conjecture about why this might be so and what the implications are for fish having higher ploidy levels. [This same point applies in the General Discussion, towards the end of the first paragraph on page 67.]

FINAL RECOMMENDATION

XX 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 16, 2014 (Fredericton, Canada)
.....
Date and place

Tillmann Benfey
.....
Name and signature

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Review of USB FFPW PhD Thesis

First name(s), surname, titles of the PhD student: Dmytro Bytyutskyy, M.Sc.	**First name(s), surname, titles of supervisor:** Prof. Dipl.-Ing. Martin Flajšhans, Dr.rer.agr.
Title of PhD thesis: Interrelationships between ploidy level, genome size and cell size in series of ploidy level models from 2n to 14n fish	

REVIEWER:

Surname: Ráb	**Institution:** Ústav živočišné fyziologie a genetiky AV ČR Rumburská 89 277 21 Liběchov
Name: Petr	
Titles: Prof. Dipl.-Ing., DSc.	**E-mail:** rab@iapg.cas.cz
Please describe your professional relationship to the PhD student: None	**Please describe your field of expertise:** Fish genetics, cytogeneticsand taxonomy/biodiversity research

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)**:

Sturgeon species are important not only because of their conservation, wide distribution and economic issues but also because of their evolutionary age, phylogeny and genome evolution due to multiple genome duplication events. All acipenseriform fishes are highly or critically endangered and hence mostly dependent on various conservation efforts. Thus all available data about acipenseriform fishes are of high importance and polyploidy issues due to their significance are one of the most important. Submitted Thesis reports on detailed methods of ploidy measurements, specifically genome and cell sizes, topics which are, as shown in Introduction, not fully explored yet. The findings in Thesis are therefore very important both for basic and applied polyploidy research as well as for practical use in sturgeon aquaculture.

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)**:

Submitted Thesis are based on three published/accepted papers, two in Journal of Applied Ichthyology, one in Cell Biology International, and thus independently peer-reviewed. Introduction briefly but clearly overviews issues of fish polyploidy in evolution and in more details issues of polyploidy in acipenseriform fishes, both evolutionary and induced. Then in desirable details describes methods used to determine ploidy levels, namely karyotyping, flow cytometry, Feulgen image densitometry, molecular genetic tools and erythrocyte geometry, the last focus of Thesis. Aims are consistently formulated and fulfilled. Discussion is desirably formulated as comments on observations in papers include in Thesis, in more details reports on issues of sturgeon hybridization and intermediate ploidy levels and their detection. The important and original finding is obviously non-exponential relationship between DNA content and ploidy level in higher ploidy levels. Thesis are well and logically structured, though English language as well as some formulations used is uneven in some places.

OVERALL COMMENTARY ON THE PhD THESIS

Please write comments in extent of 1-2 pages:

Submitted Thesis represents undoubtedly significant contribution to issue of determination of ploidy level in fishes and in sturgeons specifically. In details, Thesis studied relations between ploidy level, genome size and cell size in series of ploidy from 2n to 14n individuals of tench and namely pure and hybrid sturgeons. The important and original finding is obviously non-exponential relationship between DNA content and ploidy level in higher ploidy levels. This observation opens new direction on DNA structure package. The text of Thesis is clearly and logically formulated though English is awkward in some places. I do not comment on some typos in the text. I have small objections as follows.

i) The term Habitat in Fig.1 is erroneously used. Correctly, distribution/geographical range, sturgeon habitat is marine + fluvial, potamic, riverine (= large rivers) or fluvial only
ii) The sentence "The chromosome numbers of Acipenseriformes in most cases are clear, but ploidy level status remains unresolved" What is the meaning?
iii) The statement in sentence "In addition, some microchromosomes of Acipenseriformes are probably accessory chromosomes (B-chromosomes); the number of such chromosomes may be subjected to polymorphism (Vasil'ev, 1985)" was not confirmed by recent cytogenetic studies. Micros are true chromosomes and likely gene and repetitive sequences dense elements. It is important to use recent literature.
iv) Again, in the sentence "In such species as, *Acipenser multiscutatus, A. dabryanus, A. desotoi, Scaphirhynchus albus, S. suttkusi, Pseudoscaphirhynchus hermanni, P.*

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fedtschenkoi, Psephurus gladius, situation still remains unclear." Data are not entirely correct. All shovelnoses of both genera are paleotetraploids, Chinese paddlefish is also paleotetraploid, *A dabryanus* is paleooctaploid, *A. multiscutatus* likely also, *A. desotoi* is junior synonyme (ortography *de sotoi*) of *A. oxyrhinchus*, which paleotetraploid. These findings came from both cytogenetics and molecular genetic studies.

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**

26. 5. 2014 Liběchov
Date and place

Petr Ráb.....
Name and signature