



Confidential

Review of USB FFPW PhD Thesis

First name(s), surname, titles of the PhD student: Shakhawate MD Hossain, M.Sc.	First name(s), surname, titles of supervisor: Ing. Miloš Buřič, Ph.D.
Title of PhD thesis: The marbled crayfish: parthenogenetic invasive species as an applicable biological model	
REVIEWER:	
Surname: Breithaupt	Institution: University of Hull United Kingdom
Name: Thomas H.	
Titles: Dr.	E-mail: T.Breithaupt@hull.ac.uk
Please describe your professional relationship to the PhD student: host of internship in Jan to March 2020 in Hull	Please describe your field of expertise: Sensory ecology, behavioural ecology, crustaceans., chemical communication

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):

An excellent thesis centered on the use of marbled crayfish *Procambarus virginalis* as a model for behaviour, ecotoxicology, and biology of invasion. While the marbled crayfish, mainly due to its parthenogenetic reproduction, is an extremely dangerous invasive organism worldwide, it also promises to be very useful as a model for biological research. With this thesis the author lays the foundation towards establishing the species as a model for behavioural and ecotoxicological research. For the first time the effects of pharmacological contaminants on the behaviour of the marbled crayfish are investigated in depth demonstrating the great potential of the species for such studies. With their relatively small size, fast growth, short reproductive cycles and great suitability for behavioural bioassays, marbled crayfish offer a combination of characteristics that make them ideally suited for future applied and basic research, a fact that becomes very clear throughout the different chapters of this thesis. While he is not the first in studying crayfish interspecific interactions or effects of pharmaceuticals on behaviour, the focus on the marbled crayfish, the selection of quantitative approaches and the scientific rigor offered in all chapters of this thesis will make the work as a whole and many of its chapters important references for future studies of biology of invasions and behavioural 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, logicity 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 very well structured and very professionally written. Following an introduction that clearly defines the aims and objectives of the thesis, the literature is summarized in an authoritative comprehensive review paper. The experimental research chapters start with an analysis of the morphometric and reproductive characteristics during growth of this prospective model organism in a laboratory culture, then quantify the competitive interactions with other invasive crayfish species, and finally use the marbled crayfish for the first time as a model to study the effects of pharmacological contaminants on the behaviour of aquatic invertebrates. The chapters follow in a logical order and built on each other. Five of the papers are already published in good, internationally recognised journals. I have no doubt that the second manuscript presented in chapter 4 is also publishable in an international journal. The discussion demonstrate that the author is successful in achieving the objectives set out in the introduction. The level of detail, the scientific rigor and the comprehensive coverage of relevant literature in this thesis is impressive.

OVERALL COMMENTARY ON THE PhD THESIS

Please write comments in extent of 1-2 pages:

Chapter 1:

The introduction provides a good background to understand the aims and objectives of this study. The figure illustrating the distribution and richness of crayfish families and the table summarising the crayfish species currently invasive in Europe are very informative in this respect.

The publication "Clonal crayfish as biological model..." provides a very good comprehensive review. This is an excellent authoritative reference for research on the biology, ecology or behaviour of the marbled crayfish. I am sure it will be highly cited by any studies referring to the marbled crayfish as a model organism.

Chapter 2: A solid paper which provides important baseline information for future studies that use the marbled crayfish as model, particularly in ecotoxicological studies or general stress studies.

Chapter 3:

3.1. "Still waters run deep..." This is a good, well controlled study, demonstrating that marbled crayfish



can outcompete size-matched red-swamp crayfish, some of the most aggressive invasive species. There are quite a few typos in the manuscript and the ecological relevance is moderate due to the size difference between the two species. A comparison of size distribution of population in relation to fight outcome would have been useful. It would probably reveal that where populations meet in the field *Procambarus clarkii* would dominate. However, this paper is still an important contribution as it highlights that marbled crayfish are even more aggressive than the most invasive crayfish species currently known.

3.2 "Potential of marbled crayfish ..." This is another solid, well quantified and interesting study. The current scientific impact may not be particularly high as the calico crayfish is currently not as widely distributed as many other invasive crayfish species. However, the study will gain importance since both species appear to be a major threats to European waters for years to come. The paper is well written, provides an interesting background about history of invasions and interesting conclusions about interactions between the two invasive species. As in the previous paper, consideration of the size differences between these species in natural populations would have been desirable to evaluate the ecological impact.

Chapter 4

4.1 "Effect of pharmaceuticals..." A very good, highly relevant ecotoxicological study of the effect of environmentally relevant pharmaceutically active compounds on freshwater invertebrates. The study shows clear effects and demonstrates the excellent suitability of marbled crayfish as a model for these kinds of studies. There is a small error in the discussion where it is argued that elevated brain serotonin levels increase boldness by displaying a reduction in shelter seeking. The original study that they refer to (Fosset et al. 2014) showed the opposite effect, that elevated brain serotonin levels lead to an increase (not a reduction) in shelter (darkness) seeking.

4.1 "Combination of six psychoactive pharmaceuticals..." This still unpublished manuscript represents an important approach by using the marbled crayfish to study the behavioural response to exposure to a combination of psychoactive pharmaceuticals. Some of these compounds have been shown to occur together in freshwater systems. The authors found some clear effects on the animals to combined exposure at concentrations that were thought to be environmentally safe. The manuscript highlight the need for further studies focussing on synergistic and antagonistic effects of these compounds during combined exposure.

Chapter 5.

The general discussion presents a good summary of the work but I would have hoped for a broader synthesis and more critical analysis of the different aspects of the work. For example, the general theme of the discussion could be: "what makes a good model species?". The different chapters of the work could be analysed from that particular viewpoint. For each chapter (the culturing, the behavioural studies, the toxicological studies) the suitability of marbled crayfish could be compared to that of other crayfish species as well as to the more famous invertebrate or vertebrate model species. It is important to also highlight which where the marbled crayfish may be less suitable as a model. In this respect, perhaps the clonal reproduction and occurrence only as female may set some limits. I would also have discussed in more detail the prospects of a fully sequenced genome, which is expected to be available soon. This has been an important feature of all other famous model organisms.



However, I don't want to be too critical here.

Overall, the thesis represent a remarkable piece of work facilitating the use of marbled crayfish as a model species for future studies in behaviour, ecotoxicology, and invasion biology. It is the result of excellent rigorous research based on a comprehensive understanding of the literature. I would like to complement the author to this great, internationally influential work that will form the basis of an excellent career in science.

FINAL RECOMMENDATION

- PhD Thesis can be recommended for defence
 PhD Thesis can be recommended with reservations for defence
 PhD Thesis can not be recommended for defence

Hull, 20th December 2020

Date and place

THOMAS BREITHAUPT
Thomas Breithaupt

Name and signature



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Title of PhD thesis: The marbled crayfish: parthenogenetic invasive species as an applicable biological model	
REVIEWER:	
Surname: Petrusek	Institution: Univerzita Karlova v Praze
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Please describe your professional relationship to the PhD student: none	Please describe your field of expertise: aquatic ecology, astacology

QUESTIONNAIRE

Originality, scientific importance, perspectives and impacts of results presented in the PhD thesis for basic and/or applied research

The PhD thesis of Md Shakhawate Hossain, dealing with various aspects of the marbled crayfish biology, is at the first sight an impressive collection of five first-author papers and one unpublished manuscript (not mentioning several additional papers the candidate contributed to, but which were not included in the thesis). Upon detailed inspection, though, it seems that the quantity in this case partly prevails upon quality. It is undoubtful that the Mr. Hossain has invested a lot of effort into the experimental work, and learned how to write scientific papers. Having apparently no previous experience with freshwater crayfish, he also managed to absorb a large amount of published literature. In these aspects, his PhD studies clearly demonstrated his ability to perform research. I have to congratulate to his success in publishing repeatedly in relatively high-quality journals.

Elaboration of the PhD thesis, objectives of the work and deliverables

The introductory and concluding parts of the thesis frame well the main part, which consists of five published papers and one unpublished manuscript. These are arranged in four chapters, one with a review connected to the general introduction, one self-standing, and two chapters comprising two parts each, on aggressive interactions of marbled crayfish, and its use as an ecotoxicological test animal. This arrangement makes sense, and simplifies the orientation in the thesis.

A thesis of such extent and structure would fulfil all criteria for a successful PhD defence at any major European university. There are, however, some shortcomings in individual papers, including a particularly



major flaw in the statistical evaluation of experiments on aggressive interactions, which substantially weakens conclusions of those two papers.

Despite my critical comments highlighted below, I find the volume of work performed and included in the presented thesis acceptable. The student has certainly managed to learn various methods of scientific work, generated valuable data, and wrote numerous papers that were accepted in the peer-reviewed journals. I thus recommend the thesis for defence but I urge the candidate to reflect the problems with the statistical analysis. I hope his colleagues involved in a similar research will take more care when preparing their publication to improve their quality and avoid critical mistakes.

I hope to be able to discuss the presented research with the candidate at the thesis defence.

OVERALL COMMENTARY ON THE PhD THESIS

Please write comments in extent of 1-2 pages:

The general introduction of the thesis is relatively short, as it is to a large extent replaced by a review published in *Biologia* (chapter 1.2). It is an extensive chapter that deals with various aspects of the marbled crayfish as a model organism, with an impressively long list of references. Unfortunately, the review is not in all aspects entirely up-to-date, some of the statements are incorrect, outdated or with inappropriate references¹. Often it is unclear when the statement deals with crayfish in general (and the reference refer to different crayfish species) and when it is specifically about marbled crayfish². Overall, however, the review is informative and highlights important features that make marbled crayfish an interesting model organism. I would like to point out, however, that the clonal reproduction of this taxon (repeatedly highlighted throughout the thesis) is not only a benefit for the experimental work but also a certain drawback. In fact, I find the generalization potential of ecotoxicological experiments on clonal organisms very limited (and I would like to hear from the candidate some reflection about these potential limitations, and how to overcome them).

The chapter 2 on morphometry, size at maturity and fecundity of marbled crayfish, published in *Zoologischer Anzeiger*, is a descriptive paper bringing some basic data on biology of the target taxon. It is not very easy to read, and some expressions remain unclear³ or contradictory. I was not convinced with the section on the condition factor as an indicator of “wellbeing”. There is no explanation why that particular formula and threshold value should have a biological relevance, and the reference in use (Weya et al. 2017) is not helpful – it is a very poor paper on crayfish from Papua, and gives no explanation either.

¹ For example, it has been known for many years that the so-called “tens rule” in invasion biology is not a rule at all, and most organisms do not follow the 1/10 ratio. Highlighting this (p. 845) is like breaking into an open door. Description of the evidence for triploidy (p. 842) is highly confusing, references at p. 847 do not refer to crayfish plague in Japan, etc.

² Example (one of many): “Independent marbled crayfish juveniles, at stage III, occasionally leave the mother to move freely and forage in the vicinity (Vogt and Tolley 2004). Juveniles return to the mother’s abdomen after short excursions or if disturbed (Ameyaw-Akumfi 1976; Figler et al. 1997) with the help of species-specific brood pheromones (Little 1975) released by the female.” The first sentence is indeed about marbled crayfish but the following one refers to studies on other species (as obvious also from the year of publication).

³ For example, the statement from section 3.3 (Fecundity) “Marbled crayfish first spawned at a weight of 0.73 g (2.19 ± 1.38) when carapace length was 14mm (19.7 ± 3.7) and total length 31.5mm (42.8 ± 10.8)” is not properly explained. I suppose the numbers before parentheses indicate the minimal values observed in the whole dataset – but it is just my guess.



I have two questions that I would like the candidate to consider:

- Is the length-fecundity relationship (as shown in Fig. 6) indeed best explained by linear function? Can there be at the same time linear relationships (with non-zero slopes) between F3P a total fecundity and between %F3P and total fecundity (Fig. 7)?
- Relative fecundity (eggs per mm CL) differed significantly between maiden and repeatedly reproducing females. I suppose a similar difference might be seen between smaller and larger females in general, and reflects the non-linear relationship between body length and biomass. Is that assumption correct? Were the maiden and repeatedly spawning females of comparable size compared?

The **chapter 3** consists of two papers on experimental evaluation of aggressive interactions of marbled crayfish with other cambarid invaders. The experiments themselves were performed adequately. However, the statistical analysis of the resulting data in both papers suffers from a critical flaw that affects the interpretation of the results. The chi-square tests to evaluate whether the difference in the tendency to dominate between the two species is significant are inappropriate. This requires binomial test (or a modification to account for ties, i.e., pairs where the dominance was not established). When the key results of the experiments (assessing who becomes dominant) are appropriately evaluated, there is no combination in which the marbled crayfish is significantly more successful than the other tested species. This is not surprising considering the rather modest numbers of interacting pairs (10 and 19 in the chapter 3.1, and 18 and 17 in the chapter 3.2). The general trends and some other observations indicate that it is plausible that marbled crayfish would indeed be highly successful in these interactions. Unfortunately, the present results do not provide an unambiguous evidence, and thus the papers are overinterpreted. Even if not considering this issue, the expressions in the chapter 3.1. tend to overemphasize the patterns. For example, in the abstract it is stated there that "Premature marbled crayfish dominated in more than 75% pairs," though it was the case in only 7 pairs out of 10. Similarly, the dominance in female-female pairs was not 100% in favour of marbled crayfish, as in 5 out of 8 pairs there was no clear dominance established.

I have one specific question for that chapter:

- In the Methods and Discussion, it is written that an automated analysis of the recordings of the aggressive interactions were used ("Moreover, two observation methods (including visual and automatic software evaluation) were used to see deeply to the patterns accompanied with dominance establishment.") There is no explanation what was automatically evaluated. Can you provide this information?

The **chapter 4** deals with effects of pharmaceuticals on marbled crayfish. One part was already published in the Aquatic Toxicology, the other is included as an unpublished manuscript. The experiments testing the effects of environmentally relevant concentrations of two chemicals in chapter 4.1 seem properly performed and analysed. I am not convinced that the duration of the experiments when the animals were exposed to the chemicals (7 and 21 days) was long enough to consider the observed effects to reflect "chronic exposure". Similarly, the proper assessment of the effects on life history traits would require much longer experiments; it is particularly the behavioural traits that seem relevant in the experiments such as this.

The unpublished manuscript (**chapter 4.2**) dealing with a complex mixture of six chemicals seems more ambitious. It is a pity the we cannot directly compare the effects of individual chemicals and the mixture (it seems they have been reported mostly in separate publication), but even the comparison of exposed and control animals and the contrasting effect of the shelter presence is interesting. I do have some suggestions how to the manuscript could be improved (such as combining Fig. 1 and 2 together; analysing the data on



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animals with and without shelter in a single analysis that would also reveal the interaction effects, etc.). I am ready to provide some more detailed comments on this manuscript upon request in case the version included in the thesis has not changed meanwhile substantially.

What I find particularly insufficiently explained is the comparison of individuals with and without developed glair glands – there is a detailed table (showing the values) but no test results (except information that the effect is not significant) and – most importantly – there is no information about the actual number of individuals with or without the developed glands. It thus unclear how balanced the groups in comparison were.

FINAL RECOMMENDATION

- PhD Thesis can be recommended for defence
 PhD Thesis can be recommended with reservations for defence
 PhD Thesis can not be recommended for defence

Prague, February 11, 2020

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Date and place

prof. Adam Petrušek

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Name and signature