RADCLIFFE INSTITUTE
FOR ADVANCED STUDY
HARVARD UNIVERSITY

Cambridge, MA, 7 December, 2016

Dear Dagmar,

Thank you for the opportunity to participate in the review of Aliaksandr Pautsina's dissertation which has been submitted to the Faculty of Sciences at the University of Southern Bohemia. The work, entitled "Noninvasive crayfish cardiac and behavioral activities monitoring system", aims to establish an improved technique for the monitoring of heart rate in invertebrates. The goal is to capture the complex patterns of synchronized relationships in cardiac physiology, and to explore quantitative changes that result from environmental insults. The system thus provides a biological monitoring system for the detection of toxic, waterborne substances, using crayfish as the impacted test organism.

Pautsina has indeed succeeded in this goal as he combines a thorough review of previously published solutions, considerable technical prowess in developing an integrated utility for this purpose, and a sophisticated, multidimensional analysis of cardiac signals in response to a host of environmental toxins. This study greatly enhances our ability to characterize internal, physiological parameters using non-invasive technology and thus manages to significantly advance our ability to explore the effects of physiological determinants in behavior. Representing an impressive conceptual and technical effort, the strongest element of this study, in my opinion, is the inclusion of a designed set of sensor hardware with complementary, multivariate analysis approaches. I found the inclusion of correlation matrices, color coded for capturing inter-individual variation, particularly useful.

I think the content of this work is of high interest in the field of behavioral physiology. Invertebrates have emerged as useful study models for a wide range of behavioral, genetic and physiological effects. The manuscript progresses through several levels of technical complexity in an intuitive way and I found the train of thought easy to follow. The length of the manuscript appears adequate and the dissertation is well written. I have added some editorial suggestions to the manuscript and am including this with my email. Validation for the quality of the work also derives from a number of peer-reviewed publications which are based on the work described in Pautsina's dissertation.

A strong element of the dissertation is also the quantitative section exploring variation in many physiological measures concurrently. I'd like to encourage Pautsina to consider the addition of of Multivariate analysis of variance (MANOVA) to examine treatment group differences in specific cardiac measures as a function of odor treatment. The inclusion of a canonical centroid plot for environmental effects may be appropriate for this analysis as well. Regardless of responses to this suggestion, the work is a significant body of work and a technical tour de force. I rate this dissertation as one of the best I have seen, and I strongly support his doctoral candidacy.

With best regards,

Robert Huber, Ph.D.
Professor of Behavior and Neuroscience

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Aliaksandr Pautsina

Noninvasive crayfish cardiac and behavioral activities monitoring system

Within the Ph.D. thesis are presented three original scientific publications dealing with the heart rate (HR) and behavioral activity (BA) monitoring methods.
The first work 'Noninvasive crayfish cardiac activity monitoring system' was published in the journal Limnology and Oceanography: Methods (IF: 2.252) where A. Pautsina is the first author. The second work 'Non-invasive sensing element' is presented as registered national utility model no. 27114, and A. Pautsina is again the first author. The third work 'Method of behavioral monitoring of crawfishes and/or mollusks' is presented as the Czech national patent no. 305212, and A. Pautsina is the third author.
In further, during the Ph.D. study, A. Pautsina as the first author published the work in the journal Aquaculture Engineering (IF: 1.181), was the coauthor of the book ,Bioinformatics and Biomedical Engineering' and coauthor of other two publications in the scientific journals (without the IF) that are not included in the Ph.D. thesis.

Monitoring methods of aquatic environment are presented in the Introduction of the thesis followed by the section introducing crayfish as the model group of organisms appropriate for biological monitoring and the section describing the principles of heart rate (HR) and behavioral activity (BA) methods in the crayfish. The reasons for the development of an older design method that were validated by the author and his team, and also precise objectives of the research are specified at the conclusion of introduction (25 pages in total).

Detailed technical description of the monitoring system is presented in the chapter (app. 13 pages) titled 'Noninvasive crayfish cardiac and behavioral activities monitoring (NICCBAM)'. Results of the tests are presented in the chapter 'Assessment' (26 pages) followed by general Discussion, Conclusions, References and four separate attachments including a) experimental data used for statistical analyses; b) publication in the Limnology & Oceanography: Methods; c) Czech national patent and d) registered national utility model.

The Ph.D. thesis is written clearly and logically; general information from the text of introduction is showed in the summarizing table. The text is generously complemented by

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charts, diagrams and tables, literary sources are properly cited. Submitted Ph.D. thesis is formally fully compliant.

Scientific level of the submitted Ph.D. Thesis

In the thesis is presented interesting and practical tool for monitoring of physiological and behavioral activity of aquatic invertebrates particularly crayfish. One of the main disadvantages of older invasive methodological approaches is the surgical procedure, when crayfish carapace is performed, and sensors are introduced into the body cavity that requires relatively long time period for the recovery of organism, and also it can modify behavior of observed individual. On the contrary, presented noninvasive approach is animal friendly as the sensor is attached to the surface of the carapace. The system utilizes relatively simple cardiovascular system of invertebrates (in comparison to vertebrates) for observation of functional status of an organism. Older versions of the system were innovated and enriched by monitoring of behavioral activity (BA) hence the system can be used for more precise behavioral and ethological observations, but also for monitoring of aquatic environment as suggested by authors. The opportunity to track multiple individuals with different tasks, simultaneous data record and its automatic valuation may be designated as a great advantage of innovated system.

Results of the research are supported not only by publication in the prestigious scientific journal but also by the national patent and utility model. I highly appreciate particularly the last two mentioned practical applications, because interconnection of academic research and practice is still insufficient in the Czech Republic. I consider submitted Ph.D. Thesis as challenging, high quality and with international outreach hence unambiguously recommend it for approval.

Query

In the study you mentioned that behavior of crayfish and its physiological response can individually differ. The phenomenon of behavioral syndromes has been studied for the period of last two decades and can be characterized as the complex of consistent behavioral responses of an individual across different times and situations (e.g. Sih et al. 2004; Brodin 2008; Conrad et al. 2011). For example, active individuals tended to show higher sensitivity to stress, show higher cortisol concentration in blood and need longer period for acclimatization in new environments (Øverli et al. 2005). However, this issue is not discussed in your study. Provided in the randomly selected pattern of observed animals will be predominantly present individuals of the same behavioral syndrome (e.g. according to catchment method or size selection – large individuals are usually more active) then the results of monitoring will be less objective. Sorting the experimental animals according to behavioral syndromes could precise results of your monitoring. Did you consider during the experiment to sort crayfish into some groups according to similar responses to some stimulus/factors?

Prague, January 7 2015

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To Whom It May Concern,

Review of PhD thesis by A. Pautsina. "Non-invasive crayfish cardiac and behavioral activities monitoring system"

I am keeping my comments short for two reasons, one is that most the thesis seems well-prepared and written-up and the second was the unfortunately short time frame I had for my review due to issues of communication and timing of mailing of the thesis.

Mr. Pautsina's thesis is well-written with just minor flaws in English usage that can be easily ignored. The whole presentation is easy to follow. As I am not familiar with the technology underlying the construction and testing of the presented apparatus, my comments are focused on the evaluation of the collected data from the standpoint of a biologist and potential user of the system for crayfish behavioral research and for detection of low levels of chemicals in an biological early warning system (BEWS).

1. Fig. 3.3 and accompanying text.
   - I don't understand how primary and secondary peaks on this graph were identified. It is unclear if this was done manually, which seems unlikely given the large data set displayed, or if software was used to determined the peaks' types. If a manual method definitions was used it would be good to clarify what criteria were used to distinguish between peak types.
   - It would be good to know if the errors in automated peak determination were caused by the occasional peak being misclassified among a series of correct classifications (which would be easy to fix through an additional software routine) or if there was a certain heart rhythm that caused frequent errors in a succession. Fig 3.3 suggests the second scenario. The algorithm shown in Fig. 3.3. seems to be able to distinguish the two peak types rather well, but there are areas where PHN > 0.8, and where PRSN > 1.5 AND PHN between 0.2 and 0.4 where the algorithm could be improved upon. Since misclassified secondary peaks have a large potential impact on heart rate measurements, some more information about how to best distinguish them by software would be useful.
2. Table 3.1 lists a lot of statistically significant pairwise comparisons. However Table 3.2 shows that the difference between means was often very small, e.g. less than 1% in heart rate in the before/after values for the control treatment, while Table 3.3. shows that standard deviations greatly surpassed the effect size. With a critical p(alpha) value set to less than 0.0005, I cannot see how a minuscule effect size can be statistically significant unless a gigantic sample size is assumed. The number of independent replicates for all the test clearly should be 12 crayfish; anything beyond that would be pseudo-replication (Hurlbert 1984 doi:10.2307/1942661). The same measurement taken from the same individual cannot be independent samples in a statistical sense. The accompanying publication (Appendix B) seems to acknowledge the fact that none of the presented

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before/after odor comparisons reached statistical significance (at the end of the Results section: "additional experiments with a bigger number of crayfish are required to prove that described effects are statistically significant"). This Ph.D. thesis should adhere to the same standard as the publication and not present statistical tests with inflated sample sizes.

3. This brings me to my final comment. Looking at Fig. 3.6, I was struck by how much the heart rate varied in each individual over a mere 30-minute interval- doubling of heart rates was common. A quick look at the heart rates of Crayfish 1 before odor application (appendix A) confirmed the pattern that heart rate varied by a factor of > 1.5 spontaneously. I wonder what may have caused such large variation in heart rates in absence of experimental stimuli. Movements by the crayfish are one possibility and, again, one sentence in the appended publication hints at this situation. However, it would be good if the thesis went into some detail about the causes of high spontaneous heart rate variation and if there are ways to reduce it. The whole usefulness of the system for BEWS hinges on the question if spontaneous variation can be sufficiently reduced. I myself have tried using the flick rate of crayfish antennules for detecting reactions to very low concentrations of odors (which crayfish are clearly able to do) and after weeks of trials finally had to give up because the flick rate on the individuals I used varied spontaneously over short time periods so much that it drowned out any signal but the strongest (food odor). Increasing the number of crayfish as suggested in the appended publication is not a feasible solution to the problem of signal-noise ratio in this instance. Twelve crayfish makes for an already pretty big apparatus. Restraining crayfish so they can't move may be one solution, lowering the water temperature may be another one. The thesis should discuss this issue in some detail.

I wish the candidate good luck with his thesis defense and further work on this subject.

Sincerely,

Ulrich Reinhardt-Segawa