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**Review of the Richa Joag PhD thesis entitled: "Sexual conflict in three arthropod species: experimental evolution approach"**

The PhD thesis presents results of Richa Joag research performed at the Institute of Environmental Sciences of the Jagiellonian University in Kraków, under the supervision of Prof. Wiesław Babik.

Sexual conflict, resulting from conflicting reproductive interests of males and females, is potentially a very significant, and at the same time quite intriguing, force of the evolution. Two types of sexual conflicts were recognized. Interlocus sexual conflict is resulting from the evolution of traits improving each sex fitness but encoded by different loci in the two sexes. Intralocus sexual conflict, on the other hand, occurs when phenotypic traits in males and females are controlled by shared gene loci; however, the optimal phenotypes do not coincide in the two sexes. Both forms are suggested to contribute to speciation, each in its own specific way. Although studied by a number of researchers, sexual conflict and its contribution to the evolution are still far from being fully deciphered. In the presented thesis, PhD candidate Richa Joag, describes interesting research projects, in which, advised by her supervisor, she studied sexual conflict in three arthropod species: *Drosophila melanogaster*, *Drosophila simulans* and *Rhizoglyphus robini*. To investigate both, interlocus and intralocus sexual conflict, she utilized experimental evolution approach.

Presented thesis contains general introduction followed by three chapters, each describing different aspects of performed studies and conducted on separate species.

Every chapter includes abstract, detailed introduction dedicated to a given aspect of studies, description of materials and methods, results, and discussion. One chapter contains also supplementary materials.

Chapter 1, entitled "Transcriptional changes in seminal proteins of *D. melanogaster* populations evolving in the absence of sex peptide receptor", is focused on interlocus sexual conflict. In the study, the peptide-receptor system was used to investigate the evolutionary response of males to changes in females' physiology. Seminal fluid of *D. melanogaster* male contains over 100 accessory gland proteins. One of them is sex protein (SP) that influences reproductive physiology and behavior of mated females to male benefit. The main hypothesis of the study was that males from populations where females do not express sex peptide receptor (SPR) may be selected to decrease investment in sex peptide but may compensate by increased expression of related seminal proteins, such as Ductus ejaculatorius peptide 99B (Dup99B), assuming Dup99B does not act solely by binding to SPR. To test this hypothesis PhD candidate Richa Joag, utilized experimental evolution approach involving artificial selection followed by the measurement of the expression level of genes coding for sex protein and Duo99B protein. The procedure was undertaken on selection lines and control populations of *D. melanogaster* with 4 replicates per treatment. The experimental lines carried a deletion mutation covering the entire X-linked *SPR* gene. All lines were kept for 25 generations in controlled sex-ratio and density conditions, from 26-45 generations in uncontrolled conditions, and after generation 45 sex-ratio and density were again controlled. After 55 generations virgin males were collected and dissected to prepare samples for RNA extraction. Design of this procedure, next to the selection of studied proteins, was the most crucial factor in the experiment. However, the process of switching between sex-ratio and density controlled and uncontrolled conditions is not explained. Could the PhD candidate clarify the significance of this strategy?

Performed RT-qPCR experiment revealed that the expression of *SP* gene in experimentally evolved males was significantly higher than in control males but the level of *Dup99B* did not differ. This is contrary to the prediction made by the PhD candidate.

As Richa Joag describes in her thesis, there could be several explanation for this observation. This includes existence of alternative pathways, in addition to SPR, via which sex protein can influence female physiology, lack of correlation between the level of SP gene expression and the amount of SP transferred to female in ejaculate, and positive selection acting not on the gene expression but on the protein abundance. Which of those hypotheses would PhD candidate consider as the most probable?

Chapter 2 of presented PhD thesis is entitled: "Effect of experimental evolution of *D. simulans* males under altered mating system and temperature on harm to females" and similarly to the previous one, concentrates on interlocus sexual conflict. This time, the PhD candidate examined populations evolving under altered mating systems (increased/decreased polyandry) and in standard or increased temperature. It was shown previously that evolution under elevated polyandry and increased temperature results in increased male sexual competitiveness. In her work, Richa Joag wanted to test whether evolution of increased competitiveness would lead to increase of mating cost to females in term of decreased longevity and/or productivity. This time the model organism was *D. simulans*. In the experiment flies were divided into those living in ancestral temperature (25°C) and in novel (increased) temperature (27°C). Each group was further split into fraction evolving under enforced monogamy and under polyandry. Together four different groups, each in four replicates, were used to perform the experiment. After 60 and 64 generations, for polyandry and monogamy groups respectively, populations were standardized and longevity and productivity assay was carried out. For standardization flies from all experimental groups were kept under the same conditions: intermediate temperature (26°C) and intermediate male to female ratio. After 30 generations in such conditions longevity and productivity of females was measured. Here, only males from experimental populations were used and test females were from the base population. The results showed that neither temperature nor mating system had an effect on longevity of females. However, females mated with males evolving under polyandry and in increased temperature showed higher productivity.

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Results obtained by PhD candidate are contrary to the prediction that increased male sexual competitiveness is associated with lower longevity of females as an outcome of increased harm. These results also disagree with experiment performed by other researchers on *D. melanogaster*, where it was demonstrated that male induced harm (in terms of female longevity) was reduced when males were grown at high temperature. However, experimental evolution of males did affect productivity of females, which was increased when females mated with males evolving in polygamy and in elevated temperature. Nevertheless, increased productivity was not reached at the cost of reproductive lifespan, as it could be expected in the case of interlocus sexual conflict. Results from number of other studies, similarly to this performed by Richa Joag, also did not provide a clear evidence for interlocus sexual conflict. This could be explained in many ways but it is quite conceivable, that the response to the interlocus sexual conflict is much more complex and the study requires different experimental setting. Therefore, I would like to ask the PhD candidate, to which extend the experimental design could affect the final result?

The last chapter of presented PhD thesis is dedicated to intralocus sexual conflict, which was studied in bulb mites (*Rhizoglyphus robini*). In bulb mite there are two male phenotypes with alternative reproductive tactics and differing degrees of sexual dimorphism. These two types are: aggressive fighters and benign scramblers. The PhD candidate investigated the intralocus sexual conflict by comparing gene expression patterns of males and females from replicate lines selected for increased proportion of fighters or scramblers. Since the intralocus sexual conflict occurs over shared phenotypic traits controlled by the same loci in males and females, it was predicted that genes, which changed expression in males in response to selection on male morph, would undergo a correlated expression change in female. In this particular study Richa Joag did not carry out the experimental evolution assay on her own but took advantage of lines already selected by another researcher. However, she isolated RNA from adult females and males and performed bioinformatic analysis of sequenced transcriptomes.

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Transcriptome analysis revealed 438 genes significantly biased between aggressive male lines and scrambler lines. Majority of these genes had increased expression in aggressive line. Out of these 438 genes, 9 showed significant differential expression between females from investigated two lines. Four genes overexpressed in males from aggressive line were also biased in aggressive line females, and similarly five genes identified as biased in scramblers line males were biased also in females from this line. Nevertheless, comparison of all 10 625 genes expressed in females revealed only two deferentially expressed genes, both were present in the group of 9 genes uncovered in the analysis of 438 genes showing expression difference between males. Performed gene ontology analysis revealed that genes biased in scramblers were not over- or underrepresented for any gene ontology category but genes biased in fighters were overexpressed in several categories including, for example, ATPase regulatory activity and hydrolase activity.

Overall, results obtained by PhD candidate show that divergent selection on male phenotype in a species with alternative male morphs causes changes in patterns of gene expression in both forms of males and in females. These results are consistent with the predictions and allowed to identify candidate genes underlying intralocus sexual conflict. I have, however, a question about the nine genes identified as biased in male lines as well as in female lines. These genes were not annotated however; Richa Joag was able to identify functional domains in three of them. My question is: was any other analysis, beside domain search and automated annotations, which always have some limitations, performed in order to identify putative functions of these genes products?

From the technical point, I did not notice many problems in the presented manuscript. I found only few typos and small mistakes. However, the manuscript is poorly illustrated. More cartoons presenting obtained results and demonstrating experimental settings would be helpful. Also, a list of acronyms should be included. In addition, results presented in the chapter 3 are described in a little bit confusing way.

In summary, PhD candidate Richa Joag carried out three independent research projects to study sexual conflict in arthropod species. In all of them she applied a very

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challenging and time consuming experimental evolution approach. Difficulties in such experiments could come from many factors. Nevertheless, the PhD candidate was not afraid of this challenge. Despite the fact that the outcome of two experiments, described in chapters 1 and 2, did not support predictions, her studies are still very valuable. The results of the first one show that components of male genome respond to mutations in female, although the basal mechanism of this particular response could not be deciphered in the course of performed research. Studies presented in the second chapter, combined with results from other reports, suggest interaction between reproductive fitness of males and females evolving under elevated polyandry. Research described in the last chapter resulted in the identification of nine candidate genes underlying intralocus sexual conflict.

Altogether, Richa Joag presented in her PhD thesis interesting and original results. She also proved that she is able to design and carry out evolutionary experiments as well as correctly interpret results. Therefore, I recommend the Faculty of Biology and Earth Sciences of the Jagiellonian University to proceed with all the necessary procedural steps to confer Richa Joag a PhD degree.

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