PhD Thesis Acceptance Report
Research Discipline Council of Biological Sciences
Jagiellonian University in Kraków

Candidate's name and surname: Terence Al L. Abaquita

PhD Thesis Title: The role of heme oxygenase in the nervous system of *Drosophila melanogaster*

Thesis Supervisor: prof. dr hab. Elżbieta Pyza

Assistant Supervisor / Second Supervisor/ Co-supervisor (if applicable):……..……..

Reviewer: Jacek Jaworski

THESIS EVALUATION

1. **Scientific merit of the thesis**

a. Originality of the research *(25-200 words):*

Heme oxygenase (HO) plays an essential role in the adaptation of cells to stress conditions, especially in protecting them from excessive oxidative stress. Because of their physiology, neurons are particularly susceptible to high levels of free radicals, which can lead to aging or death if unphysiologically high levels persist. *Drosophila* is a suitable model to study HO, because unlike vertebrates, it has only one gene encoding HO, and this is regulated by cellular stress. However, previous studies on the expression and function of this gene in the fruit fly nervous system have been conducted mainly in the visual system, which is not fully representative of the rest of the nervous system due to its strong exposure to light stimuli. Therefore, the main objectives of the work submitted for my review were to determine the expression pattern of HO in the *Drosophila* brain during the circadian cycle, physiological aging, and under the influence of both known antioxidants and compounds that increase oxidative stress. In addition, the aim of this study was to investigate the effects of overproduction and knockdown of HO in the *Drosophila* nervous system on selected vital functions and the expression profiles of selected genes related to apoptosis and autophagy. Since such studies have not been performed on this scale before, I consider the subject of the work to be original and the results obtained to be a substantial extension of the current knowledge on the role of HO in the nervous system, both the expected one, i.e., protective, and the non-obvious one, i.e., leading to neuronal degeneration.

b. Scientific merit of the chapters / articles *(25-200 words):*

As I mentioned earlier, Terence Al Abaquita's dissertation is, in my opinion, of unquestionable scientific value, both for its novel topic and for the model used, which, thanks to the genetic tools available, provides a very detailed insight into the role of HO in the brain. Among the most scientifically valuable observations, in my opinion, is the demonstration that the level of HO in the brain must be tightly controlled since both underactivity and overactivity of HO lead to severe CNS dysfunction (Annex I). The second aspect that I found particularly interesting was the demonstration that as the brain ages, the changes observed during the circadian rhythm in the rhythm of expression of HO disappear (Annex II), which the author believes may be one of the reasons for the adverse neuronal changes associated with aging.

2. **Substantial merit of the thesis**
(ability to introduce the research topic and clarity of research hypotheses, the choice of research methods and statistical tools for data analysis, presentation and critical analysis of

the research data, the ability to discuss research data and the theoretical background, clarity and quality of the conclusions) (25-200 words):

I rate the merits of Terence Al Abaquita's dissertation as satisfactory. The General Introduction provides a very good compendium of knowledge about HO, the regulation of its expression, and its cellular functions. I find particularly valuable the presentation of the contexts in which HO may have protective functions or, conversely, contribute to neurodegeneration. Results and Discussion form a chapter in which the Ph.D. candidate efficiently summarizes the results obtained and their interpretation. Reading this chapter allows the reader to combine the conclusions of the two experimental papers included in the dissertation into a coherent whole. Materials and methods are included in each manuscript and are sufficient to address the objectives and are usually well and comprehensively described. At the same time, as I write in Section 4, I consider it a weakness of the critical analysis of the results obtained that changes in the levels of some selected mRNAs are overly identified with changes in cellular processes in the cell. In this context, I believe that conclusion 2 (Conclusions chapter p. 38) is not entirely accurate, at least with respect to autophagy. I also have some reservations, which I discuss in more detail in Section 4, about the statistical methods used. In most cases, I do not think this affects the interpretation of the results, but it may be important in the case of smaller differences. At the same time, there is the question of the extent to which some of the small differences that the author considers statistically significant are biologically meaningful and worthy of discussion. However, this is not only a problem of the Ph.D. candidate, but in general of many researchers in the life sciences who are forced to over-quantify processes in cells and living organisms with the limited number of observations.

3. **Layout and register**

(layout, register and the clarity of the language, the quality of the visual material etc.) (25-200 words):

The evaluated Ph.D. thesis contains all the elements according to the guidelines of the Council of the Faculty of Biology of Jagellonian University on the structure of a Ph.D. thesis prepared as a thematically coherent series of articles. However, in my opinion, a more consistent adherence to the order of chapters suggested by the Univeristy, i.e., general introduction with objectives, hypotheses, research procedure, followed by scientific articles and general discussion as a warp up would have promoted the clarity of the work more than the layout used, where original papers were attached as annexes. To make it more complicated for the reader, the supplementary materials for Annexes I and II were moved to separate annexes plus Appendices A-F were added. In my opinion, this makes it difficult for the reader to navigate. Overall, however, the style and presentation of the work are reasonably clear and logical. I have no comments on the figures included in the main body of the dissertation. On the other hand, I feel that the presentation of the results in the annexes, especially the statistical significance in the figures, is not very clear and does not really appeal to the reader. In particular, Annex 1 does not provide in the captions to the figures the statistical tests used or detailed references to what the letters used in the graphs mean from a statistical point of view. Finally, in the case of the Annex 1, the references to the relevant panels of Figure 3 were incorrectly indicated in the text. It is astonishing that such an obvious error was not noticed in a published paper that I assume was subjected to multilevel peer and editorial review. Also, in some cases error bars are missing (listed in detail in section 4).

4. **Critical notes**

At the outset, I would like to point out that I do not have many critical comments and those listed below do not significantly affect my positive evaluation of Terence Al Abaquita's dissertation.

Annex I. Abaquita *et al.,* 2021

1. My main point is about identifying changes in the expression of selected genes with actual biological processes in the cell, especially such complex processes as apoptosis or autophagy.

This applies both to the correlation of *ho* expression with the expression of *hid* or *atg5* and to the expression levels of some of the genes studied under conditions of *ho* overexpression or knockdown. At many points in the publication, the Candidate puts an equal sign between changes in gene expression and changes in the processes involving the products of these genes. In fact, some of the data published in the Annex II where functional studies have been performed show that it is possible to alter gene expression without altering the level of the process under study (e.g., apoptosis), which is discussed in detail in the publication attached in Annex II. Thus, the conclusions drawn in many places in the publication are not supported by data from functional assays showing changes in the processes studied and represents an oversimplification and overinterpretation of the actual data obtained. Based on the data presented in the paper, all that can be said is that the mRNA levels of the genes studied change depending on the conditions used. On the other hand, how the conditions studied, e.g., overproduction of HO, affect apoptosis or autophagy cannot be deduced.

2. In my opinion, the changes in *ho* expression during LD12/12 are minimal (Fig. 1a) and even if they are statistically significant, I am not convinced to what extent they are actually biologically significant.
3. Strains with HO overproduction and knockdown were used for the publications in Annex I. Unfortunately, no evidence was provided that these manipulations led to the expected changes in *ho* expression. Therefore, the results obtained should be interpreted with caution (especially in the absence of the observed changes). However, it is worth noting that for at least two lines such data can be found in Annex II.
4. The Candidate mentions in Materials and Methods that nonparametric tests were used for the analyzes. At the same time, the figure captions clearly state that 3 biological replicates of the experiment were performed. This number is not sufficient for any test I know of to assess whether the distribution is normal or not. Furthermore, some of the literature advises against using the Mann-Whitney test with $n < 5$. I also doubt that the M-W test should be used without adjustment when comparing the experimental group to the control group when there was more than one experimental group in the experiment (Figure 3). In the case of the analysis shown in Figure 3, it might have been appropriate to use the one-sample t-test, which is routinely used in many publications to analyze RT-qPCR results. In contrast, in Figures 4 and 5, where there are two variables-time and treatment, perhaps a two-way ANOVA would be a more optimal test. In summary, although in most cases the differences are such that the used test should not change much, in some cases, e.g., Figure 3c for ZT16, the statistical results obtained seem implausible.
5. For some of the plots, the standard deviations for some time points are not given, e.g., Figure 1a ZT4, ZT13 (only + part of SD), Figure 2a ZT4 10-d (only - part of SD), Figure 2b ZT20 20-d, Figure 4a ZT8 curcumin, Figure 4b ZT16 curcumin, Figure 4d ZT1 curcumin, Figure 4e ZT16 curcumin, and some points in Figure 5.

Annex II. Abaquita *et al.,* 2023

1. In the case of this publication, I stand by my comments on the statistical analyzes and the lack of SD in some places in the graphs and the overidentification of changes in gene expression with cellular processes. However, it should be appreciated that in the case of the publication in question, the Candidate performed functional assays for apoptosis. It is unfortunate that he did not also perform tests for autophagy.
2. In Supplementary Fig. 1, it is not clear to me why the qRT-PCR results were given only for the control lines and not for the experimental lines? Regarding the analysis of WB, I miss information on how many times the experiment was performed and a quantitative analysis of HO levels in each line. Moreover, in this experiment, the levels of HO in the experimental lines were compared with those of CS and not with those of the parental lines. Why?

5. **Final grade** (justification *25-200 words):*

Despite the few critical comments, I rate the dissertation as good. It is an interesting and extensive study, carried out with appropriate methods. As a result, completely new and original insights into the role of HO in the physiology and pathology of the brain have been obtained.

I, hereby, declare that the reviewed PhD thesis by **Terence Al L. Abaquita** meets the criteria pursuant to art. 13.1 of Act of 14 March 2003 on Academic Degrees and Academic Title and Title in the Arts (O.J. no 65 item 595 as amended) and request that the Research Discipline Council of Biological Sciences of the Jagiellonian University in Kraków accepts **Terence Al L. Abaquita** for further stages of doctoral proceedings.

YES/~~NO~~

Ja, niżej podpisany stwierdzam, że recenzowana rozprawa doktorska **Terenca Al L. Abaquita** spełnia warunki określone w art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2018 r. poz. 1668 z późn. zm.) i wnioskuję do Rady Dyscypliny Nauki biologiczne Uniwersytetu Jagiellońskiego w Krakowie o dopuszczenie **Terenca Al L. Abaquita** do dalszych etapów postępowania ws. nadania stopnia doktora w dziedzinie nauk ścisłych i przyrodniczych w dyscyplinie nauki biologiczne.

TAK/~~NIE~~

I, hereby, request that the thesis is accepted with distinctions. Justification *(25-200 words)*

~~YES~~/NO

Jacek Ryszard Jaworski

Elektronicznie podpisany przez Jacek Ryszard Jaworski
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INFORMATION FOR THE REVIEWER:

1. Information on requirements concerning PhD thesis structure: http://www.wb.uj.edu.pl/en_GB/stopnie-tytuly/doktoraty
2. A digital copy should be sent to:
nauki.biologiczne@uj.edu.pl

A duly signed original should be sent to:

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