Rebuttal Document: reviewer #1

Dear reviewer,

Thank you for your time, intentionality, and due-diligence in your review and consideration of our manuscript. We appreciated your wise constructive comments and supportive suggestions that will surely strengthen the integrity and effectiveness of this publication. We enjoy any opportunity to learn from and collaborate with others that value the same curiosity that led us to this moment.

Please let us know if you have any additional comments and suggestions. We look forward to continuing this process with each of you, and thank you in advance for any more time you will contribute to this project. We have responded to each of your comments below!

Warmly,

Hannah Robert and Dr. Michael Folkerts

Comments:

I. Manuscript Summary:
   A. The manuscript submitted by Robert et al. describes methodology and considerations for experiments in which monitoring of the estrous cycle in rats is necessary. The topic would be a meaningful addition to the JOVE literature and would be useful for many laboratories beginning to monitor estrous cycles while following the NIH policy on sex as a biological variable. The approach used in this manuscript includes an abundance of information, yet the inclusion of some sections distracts from the utility of the methodology as a whole and makes the manuscript more cumbersome. As such there are a number of issues in the manuscript's present form that need to be resolved prior to publication.

II. Major Concerns:
   A. Elements/sections, while presenting interesting information, do not necessarily add substantial value to the manuscript.
      1. Figure 11 is confusing and should be cut. The discussion of the data presented in Figure 11 describes the graph without adding further information about what the observed differences between these days means with regards to the analysis of estrous cycles. The authors believe this figure is important to the integrity of this publication. To clarify the

purpose and importance of this analysis tool, more information has been provided beginning with line 1311 marked by the paragraph starting with "another option when analyzing the data extracted is the creation of estrous cycle profiles…".

2. Figure 1B is similarly out of place. It does not appear to be necessary for this manuscript, especially given that it is not referenced in the text. Addressed: included A and B in the text. This figure is important to demonstrate a more comprehensive profile of the main hormones involved in the cycle, and to reduce the isolationism misconception discussed in the 'Translation to Humans' subsection of the introduction.
3. The introductory paragraph beginning "The contemporary structure and understanding…" (lines 84-95) is interesting about the history of the study of the estrous cycle. However, the manuscript would be improved by cutting the paragraph to focus the manuscript on the methodology. Addressed: cut this paragraph and refocused it to include information for the application of the protocol.
4. Additional sections should be reviewed critically to ensure their inclusion enhances the overall focus of the manuscript. Addressed.

B. Acyclicity is mentioned numerous times without being operationally defined. A key aspect of the manuscript is to aid laboratories in performing and evaluating vaginal cell samples. A definition that laboratories could use to standardize an acyclicity effect is needed. Addressed: the definition of "..when the animal is presenting an irregular progression of the estrous stages, known as acyclicity" seen in lines 1064 and 1065.
C. More information is required about the utility in analyzing the arrangements of the cells. The arrangements do not seem to be influential on the determination of the cycle phase, so their inclusion in the analysis is unclear. Addressed: added information about arrangements specific to specific stages in the 'Stage Categorization' subsection of the protocol (lines 772-919). More information can be found in Figures 2C, 5A-D, and 6A-D.
D. Operational definitions of "smidge", "modest", and "numerous" are required. Addressed: included in lines 758-763 in the 'Stage categorization' subsection of the protocol.
E. In the equipment and experiment preparation subsection 3, time of day is defined relative to a clock instead of a physiological zeitgeber. This section should be revised to describe timing of lavage relative to a change in light cycle. Addressed: changed to time relative to the light cycle.

F. Table #1 is difficult to comprehend and fails to provide information that would be useful to a laboratory using lavage to assess estrous phase. Instead, perhaps analyze the data collected to determine the following statistics for each phase of the estrous cycle: The authors of this publication did not intend to provide any statistical analyses for comparative purposes, but rather to provide examples of analysis options. This table provides another example of how data can be analyzed for later statistical analysis (lines 1939-2049) that can be used to compare ranges/values from other laboratories.
    1. Max Duration of phase
    2. Range of Duration of phase
    3. % AKE
    4. % Leu
    5. % LNE
    6. % SNE
    7. % Clumped
    8. % Evenly Dispersed
    9. % Randomly Dispersed
    10. This information would then allow an investigator to compare their assessment of phases against these to determine whether they are assigning phases appropriately.

G. Figure design and clarity could be improved.
    1. Scale bars are necessary for each microscope snapshot. They are especially necessary as the text describes evaluating samples at multiple magnifications. As these images are zoomed in to allow for visualization of the cells, there would need to place a scale bar on every image and they would not be equivalent to one another. Instead, we have input the approximate diameter of the cells on the last line of the figure legends.
    2. The placement and typeface of figure subpanels is not consistent. Addressed.
    3. Some figures include images that are unclear and/or may be protected from republication by copyright. The images that are unclear due to protection from republication by copyright will be purchased and replaced once these figures have been approved by the review team to ensure they will be used for the final publication.
    4. Figures with subpanels could be improved by presenting as a single multipanel figure rather than multiple separated images. For example, Figure 5 could be 3 images per row and 4 rows. Addressed.
    5. Figure 6 includes 2 "A3" subpanels. Addressed.

6. Figure 8 or the figure caption need to define "Ext Diest" and "Ext Est" Addressed: added as “extended diestrus” and “extended estrus” to lines 1095 and 1096.
7. The tables in Figure 8 and 9 are identical yet different conclusions are described in the text. Addressed: the correct figure has replaced what was previously called “Figure 8”.
8. Figures 8 and 9 could be combined to the same figure with 2 subpanels (A & B). Addressed.

H. Section 6 would be improved by reordering the discussion of stages to match the order presented in the rest of the manuscript (i.e. Figure 1, 2B, and/or 5). Addressed: changed order in of Figure 10A-D to follow stage progression of diestrus, proestrus, estrus, metestrus to match that in previous figures and changed order of representative results text to follow.

III. Minor Concerns:

A. Line 529 indicates the rats used for these experiments were euthanized at age 11 or 21 days, however, the rats arrived at 28 days old. Revise to accurately identify the length of the experiment. Addressed: line now reads “day 11 (45 days of age) and 21 days (55 days of age)”.

B. Ensure figure references in text match the figures. For instance, throughout section 2, cell-types examples are referenced in Figure 2C, yet Figure 2C highlights cell arrangements. Figure 2B focuses on cell types. Addressed.

C. Line 349 requires a space to separate 4.2 from 4.3. Addressed.

D. Review and revise the following contradictions: Addressed: revised to read “..3 categorizing determinants—cell type(s) present, cell arrangement, and cell quantity (Figure 2A–D). While the vaginal fluid condition component was not included in this study, it is recommended to include this as a 4th categorizing component, and further information on this aspect can be found in the reference list[16].

1. Line 75-76: "In this study, the stages were identified through components of the vaginal canal, named the 4 categorizing determinants—vaginal fluid condition, cell type(s) present, cell arrangement, and cell quantity."
2. Line 77-79: "While the vaginal fluid condition component was not included in this study, further information on this aspect can be found in The Rat Estrous Cycle Revisited: a quantitative and qualitative analysis"

E. Review and revise the following for accuracy:

1. Line 40: "The rodent estrous cycle (from Greek oistros) has been utilized throughout history". I do not believe the rodent estrous cycle has be used throughout history. Estrous cycle across species perhaps. I would like to

see this statement altered to be more clear about its claims or include a citation to show the historical usage of the rodent estrous cycle. Addressed: “The rodent estrous cycle (from Greek oistros; gadfly, frenzy) has been identified as an essential indicator of wellness”.

F. The word "data" is in the plural form with the singular being "datum". Please review throughout to ensure any verbs and pronouns agree. Addressed: corrected subject/verb agreement.
G. Line 181-184 is unclear. Addressed: the statement now reads “Ensure that stress is reduced as much as possible with the use of an acclimation period, as stress can disrupt proper reproductive system functioning. However, do not overcompensate by attempting to completely eliminate it, as a moderate amount of stress is beneficial to the animals’ well being” (lines 335-338).
H. Line 441 reads "smudge" rather than "smidge" Addressed.