Rebuttal Document: reviewer #2

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. In this manuscript, Robert et al. described a reproducible, standardized, and cost effective approach to monitoring the estrous cycle of female Sprague Dawley (SD) adolescent rats. They describe the sample collection process by means of vaginal lavage, procedures for data categorization into proestrus, estrus, metestrus, and diestrus. They describe 4 categorizing determinants of vaginal fluid condition, cell type(s) present, cell arrangement, and cell quantity at time of collection to identify the estrous phase. They provide excellent graphic examples of satisfactory and unsatisfactory samples, and a distinction between cyclicity and acyclicity. Finally, the authors suggest interpretive and organizational practices of the data.
   B. This is an excellent methodological manuscript that is highly relevant for studies in many areas of biology. This reviewer has only minor suggestions that the authors may want to address.

II. Major Concerns:
   A. None.

III. Minor Concerns:

A. Line 40: define the meaning of "Greek oistros." Addressed: added ‘gadfly or frenzy’ to line 46.
B. There are sentences that are difficult to understand without more context. For example: line 42, what does "realities of biased investigators and of the female body" mean? Also, line 49-50: "because while hormonal cycles are routine and beneficial processes, they are surrounded by hazardous stigma—perpetuated by misconceptions." What is the hazardous stigma related to hormonal cycles? Addressed: line 47 (previously line 42) now reads; “...however, the unconscious biases of investigators and of accurate interpretations regarding the female body have evaded the scientific community”. Line 58 (previously line 49-50) now reads; “*thirdly,* because while hormonal cycles are routine and beneficial processes, they are surrounded by hazardous stigma that will be elaborated upon in later sections”.
C. Lines 516 and 518 state age of 28 and 34 days. In line 529, ages of 11 and 21 days are stated. Addressed: now reads “day 11 (45 days of age) and 21 days (55 days of age)”.
D. Although this is a methodological manuscript, it could be beneficial for the readers from many different areas if the authors briefly discuss or recommend when monitoring the estrous cycle is critical, and when it might not be required. There are sex differences that are unrelated to hormonal fluctuations. Historically, researchers have focused on male studies only because the addition of females would "make publication more difficult." The monitoring the estrous cycle and analyzing the data by the estrous cycle phases are significant concerns for many researchers. Addressed: included in the ‘Fundamental Definitions and Uses’ subsection of the introduction.
E. Another topic that could be briefly addressed is when in an experiment the vaginal lavage could be done. For example, vaginal lavage prior to a behavioral experiment, e.g., anxiety-like behavior or alcohol drinking, would likely affect the behavior outcome. The inverse might be true as well. We agree that this would be a wonderful addition, and it is something that was considered in the writing process. The concept is addressed in the ‘Equipment and experiment preparation’ protocol subsection, in step 3.1. As it is developing, there is not a consensus on when the timing of the swabbing, as it relates to experimental intervention, is appropriate. The research team at UCLA is investigating this now.