For research article

Response to Reviewer 3 Comments

(Date of this review
03 May 2025 21:57:49)

1. Summary

We are pleased to submit the revised version of our manuscript entitled *"Exploring New Ways to Analyze Data on Spontaneous Physical Activity of Rodents Through a Weighing Balance"* for your consideration.

We sincerely appreciate the time and effort invested by the editor and reviewers in evaluating our work. Their valuable feedback has helped us improve the manuscript. We have carefully addressed all comments and made the necessary revisions. We believe that our study will be of broad interest across various fields related to animal experimentation, including veterinary science and physiology, and anticipate it will attract attention from researchers working with animal models. We are grateful for the opportunity to have our work considered for publication in *Sensors*.

With best regards,

2. Questions for General Evaluation	**Reviewer's Evaluation**	**Response and Revisions**
Does the introduction provide sufficient background and include all relevant references?	Yes	We thank you so much for the important contributions. We strongly believe that our manuscript has improved, largely due to the suggestions provided by the excellent editorial board and reviewers.
Is the research design appropriate?	Yes	
Are the methods adequately described?	Yes	
Are the results clearly presented?	Yes	
Are the conclusions supported by the results?	Can be improved	

3. Point-by-point response to Comments and Suggestions for Authors

Comments 1: *Dear authors, Move highlights to the introduction.*

Response 1: Thank you for your comment. However, we followed the journal's submission guidelines (https://www.mdpi.com/journal/sensors/instructions), which specify that the highlights should be submitted in the *Front Matter* section—together with the Title, Author list, Affiliations, Abstract, and Keywords.

Comments 2: *Mention the main quantitative results in the abstract.*

Response 2: Due to the volume of the results, which are distributed across 8 figures and 3 tables, we faced challenges in condensing them into a few words for the abstract. Instead, we opted to provide the reader with a general overview to help guide their understanding of the findings. We hope the reviewer can understand our choice.

Comments 3: *Add hyperlinks to references, tables, and figures.*

Response 3: Thank you for your comment. We understand the importance of including hyperlinks to references, tables, and figures. However, we assumed that these hyperlinks would be automatically inserted during the journal's typesetting and final formatting process.

Comments 4: *At the end of the introduction, add a paragraph describing the manuscript's structure. Add a brief description between section and subsection titles.*

Response 4: Thank you for your suggestion. To address your request, we have outlined the main steps of the manuscript's structure. The updated version of this description can be found at the end of the introduction, as shown below:

> *The manuscript is organized to guide the reader through the development and analysis of the study. It begins with a description of the construction and development of the weighing balance, followed by an explanation of the load cell arrangement and signal acquisition system. Next, we discuss how raw data is transformed into weight values through calibration and outline the experimental setup, including the rodents and SPA recordings. The methodology section continues with an introduction to the algorithm used to analyze SPA and the mathematical strategies applied, including Biesiadecki's summation method and the Mean of Weight Changes (MWC) approach. We also explore the concept of signal dispersion and its role in SPA analysis. The classification of SPA into distinct domains is explained, along with the creation of heat maps for minute-by-minute SPA domains. In the results section, we compare different mathematical strategies, present an overview of the 23-day experiment, and examine analyses across light and dark phases, as well as across different times of the day. Further analyses explore the SPA classification into domains both within hours and across all experimental days.*

Comments 5: *In Figures 1 and 2, decimal division is indicated by a comma; please correct it.*

Response 5: Thank you for pointing this out. We have corrected the decimal separators in Figures 1 and 2 by replacing the commas with dots, in accordance with the standard formatting conventions.

Comments 6: *The resolution of the figures needs to be improved.*

Response 6: We have updated all figures to higher-resolution versions to enhance visual

quality and ensure that all details are clearly visible. Additionally, we have also provided the original figures as separate files in the submission system for further reference.

Comments 7: *Please define all variables and parameters in the equations and figures in the main text.*
Response 7: We have made every effort to define all variables and parameters in the equations. However, if the reviewer identifies any specific points that remain unclear, we are more than happy to make further improvements and provide clarification as needed.

Comments 8: *The article does not include any calculations or reasoning to justify why 10 animals per group (n = 20) are sufficient for the statistical analyses performed. "Newly weaned mice" are mentioned, but the inclusion criteria are not clearly defined, nor is it reported whether animals were discarded or for what reasons. A better description of the selected animals is needed.*
Response 8: The reviewer raised an important concern, and a robust response is necessary to meet their expectations. Indeed, in this study, we did not perform a sample size estimation to test our hypotheses. This study is part of a larger research project investigating various outcomes, including the impact of housing space on physiological markers and the deleterious effects of obesity. Not all outcomes are directly related to the present analysis. Specifically, to validate the use of the apparatus developed for the broader project, we initiated the study with control animals to assess the reliability of the equipment in monitoring SPA. Moreover, in accordance with the principles of the 3Rs and the ethical and appropriate use of laboratory animals, the Institutional Animal Care and Use Committee approved the proposed sample size based on previous studies conducted by our research group (Scariot, Manchado-Gobatto et al. 2019, Polisel, Beck et al. 2021, Scariot, Manchado-Gobatto et al. 2021, Scariot, Gobatto et al. 2022, Scariot, Manchado-Gobatto et al. 2022). These prior studies demonstrated satisfactory results related to SPA in rodent models and served as the scientific foundation for the current report. From this perspective, we opted to use a sample size consistent with our earlier work, which had shown high statistical power and methodological reliability. Nonetheless, we fully acknowledge the reviewer's concern and recognize this point as a limitation of our study. A corresponding statement has been added to the discussion section.

Comments 9: *The density per animal in the cages varies between groups. This introduces an additional variable that is not controlled separately from cage size.*
Response 9: Thank you for your insightful comment. We intentionally designed the study so that the floor area per animal would differ between the large cage (267.9 cm^2) and the small cage (49.5 cm^2) to allow more space for movement in the larger cage. Our goal was to stimulate SPA by providing animals in the large cage with an environment that more closely resembles natural conditions, where rodents have the freedom to explore and move. In contrast, limited space in smaller cages imposes a sedentary lifestyle, which can lead to physiological adaptations that differ markedly from those observed in animals given greater environmental space.
We chose not to equalize housing density across cages because doing so would have introduced other confounding issues. To make it easier to understand, let's say that in the

small cage, each mouse has 49.5 cm^2 of space. If we wanted to keep the same amount of space per mouse in the large cage, we would have to place 54 mice inside it, even though the large cage is much bigger. This would equalize the space per animal, but it would introduce a severe imbalance between the groups (10 mice in the small cage vs. 54 mice in the large cage) and create confounding effects related to social density and overcrowding. Therefore, for these reasons, we preferred to fix the number of animals at 10 per cage in both conditions to keep the number of social interactions relatively constant between groups. We believe our approach was the best option to provide sufficient space for stimulating SPA without introducing social order issues.

Comments 10: *The proposed system is not compared to another reference method, which weakens claims about its validity. The system is inferred to be "robust and valid." However, validation is limited to detecting expected differences between large and small cages or between light/dark phases, representing a circular analysis.*

Response 10: Thank you to the reviewer for this insightful comment. Comparing the system to an another reference method would indeed strengthen claims about its validity, and this remains an important goal for future work. However, we respectfully clarify that our validation approach is not circular. In circular analyses, hypotheses are typically derived from the system's output itself, which would result in the system being validated by the very data it generates. In contrast, our hypotheses were based on well-established theoretical backgrounds and findings from the literature, not derived from the system's data. Specifically, we predicted that the system would detect two known patterns of SPA: (1) increased activity during the dark phase and (2) higher activity in larger cages. By confirming these patterns, which are already expected based on the literature and biological principles, we can assert that our balance demonstrates construct validity.

Comments 11: *No confidence intervals or effect sizes are reported (except once with Cohen's d). This prevents assessment of the true magnitude of the differences.*

Response 11: We understand the reviewer's concern and agree that presenting confidence intervals is an important approach. However, considering the current layout and number of tables, we would like to inquire which specific results the reviewer considers most appropriate for the inclusion of this information. As our study encompasses topics from different fields, one of the challenges we aimed to address was ensuring the accessibility of the methods and results to professionals from various disciplines. To this end, careful attention was given to the presentation of the study. We believe that including confidence intervals in all applicable tables might detract some readers from the main findings. Therefore, we respectfully ask the reviewer to indicate which results would benefit most from the inclusion of confidence intervals.

Comments 12: *There is no evidence of verification of compliance with the assumptions of homogeneity of variances or discussion of possible additional transformations beyond the inverse normalization.*

Response 12: We appreciate the reviewer's comment regarding the absence of a formal assessment of variance homogeneity. This is indeed a valuable consideration when evaluating the robustness of statistical models. In our case, we chose not to apply standard tests of homogeneity (such as Levene's or Bartlett's), as we felt that the assumption of equal variances across groups was less central within the context of our analytical approach. First, our data were subjected to normal score transformation (also known as inverse normal transformation), a robust approach specifically used to address issues related to non-normal distributions. Transformation procedures are known for standardizing the distribution by mapping the data to a normal score scale, thereby stabilizing the variance (i.e., minimizing heteroscedasticity) (Kendler, Gardner et al. 2011, Roux, Fraïsse et al. 2014). Moreover, ANOVA remains relatively robust to violations of homoscedasticity (Lix, Keselman et al. 1996), especially when group sizes are equal or nearly equal, as was the case in our experimental design. Therefore, considering (1) the statistical robustness provided by the transformation, (2) the balanced sample sizes across groups, and (3) the exploratory nature of the biological data involved—we respectfully opted not to include formal homogeneity tests. We are grateful for the reviewer's observation, which has allowed us to discuss in a high level the rationale behind our analytical choices.

Comments 13: *Table 2 lacks statistical analysis due to non-normality. Nonparametric methods could have been used for at least an exploratory comparison between hours.*

Response 13: Upon revisiting the analysis and exploring alternative procedures, we aimed to identify a test capable of evaluating the two main effects in our factorial design. However, we found that the Kruskal–Wallis test was not suitable, as it is inherently limited to one-way comparisons (Chan and Walmsley 1997). Thus, we found the rank transformation to be the most accessible alternative (Conover and Iman 1981). Although not a purely nonparametric test, this approach is useful for data that violate parametric assumptions. To implement it, the entire set of observations is ranked from smallest to largest—assigning rank 1 to the smallest value, rank 2 to the next smallest, and so on up to the maximum value, which receives the highest rank. The ranked data can then be analyzed using standard parametric methods (for example, a two-way factorial ANOVA). This enables us to evaluate both the effect of housing and the effect of domain simultaneously.

Comments 14: *How signals are synchronized or how precise temporal alignment between cells is ensured is not specified. The conversion of raw signal to weight units is explained, but the calibration standard errors are not detailed.*

Response 14: Data synchronization between scales was not an issue, as each raw data sample was recorded with a timestamp accurate to the microsecond. We have now provided a more detailed explanation of the estimation error and included the paragraph below to address the reviewer's comments.

The Standard Error of the Estimate (SEE) was calculated using the calibration data from the load cells. It

was determined by taking the root mean value of the residual deviations between the observed and predicted measurements, adjusted for the degrees of freedom. The analysis yielded an SEE of approximately 0.4 grams across the three load cells, indicating that the predicted masses deviate from the actual known values by only 0.4 grams. This low level of error confirms that the system has satisfactory sensitivity to detect subtle weight variations in small animal models.

Comments 15: *Variability in sampling frequency (unstable 40 Hz). If not properly controlled, this can introduce artifacts. MWC mitigates this, but its impact on longitudinal studies is not analyzed.*

Response 15: We acknowledge that differences in sampling frequencies can primarily affect the SWC, and we appreciate this insightful comment. All balances were built using the same hardware, resulting in similar sampling frequencies; thus, differences between systems should be minimal. However, some variation both between and within systems is expected.

To improve the accuracy and precision of the systems, we adopted the following strategy: We assumed that the minute with the lowest SPA within a day reflects the time when the animals exhibited minimal movement. Therefore, the SPA recorded during that minute likely represents the system's baseline bias for that day. Consequently, we subtracted the value of this minimum activity from all minute-by-minute SPA values recorded on the same day. This correction enhances the accuracy of each system and reduces discrepancies between systems across the study period. Furthermore, it improves the consistency of measurements within each system across different days. We have included these methodological details in the manuscript to strengthen the technical rigor of the data analysis section.

References

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