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Brief Report

Reassessing Sex Differences: Behavioral Analysis of Male and Female Wistar Rats in the Open Field Test

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Abstract

This study aimed to investigate sex-specific behavioral differences in adult Wistar rats using a 3-minute Open Field Test (OFT), aligning with ethical guidelines emphasizing inclusive animal research for translational validity. While previous literature suggests female Wistar rats often display greater locomotor activity and central exploration, indicative of lower anxiety, these findings are not universal due to protocol variations. Fourteen Wistar rats (7 males, 7 females), aged 12 weeks, underwent the OFT in a controlled environment, adhering strictly to ethical protocols. Behavioral parameters assessed included locomotor activity, time in central zone, rearing frequency, grooming, and defecation. Data were analyzed using Student's t-test or Mann-Whitney test, with a significance level of $p < 0.05$. Results revealed no statistically significant differences between sexes for any analyzed variable ($p > 0.05$). Females exhibited numerically higher locomotor activity (50.43 ± 10.0 vs. 45.29 ± 12.3) and rearing frequency (17.43 ± 5.56 vs. 14.29 ± 4.15), whereas males showed numerically higher grooming (2.43 ± 2.51 vs. 0.14 ± 0.38) and defecation (1.71 ± 1.5 vs. 0.43 ± 1.13). These numerical trends, however, did not reach statistical significance. The findings align with the mixed results reported in the literature concerning sex differences in anxiety and exploratory behaviors within the OFT paradigm, particularly when using small sample sizes. Methodological limitations include the sample size and the absence of estrous cycle control, though the latter was reinterpreted for its ecological validity. Despite the lack of robust differences in spatial or general activity metrics, the observed trends in grooming and defecation hint at subtle sex-specific stress reactivity. This study contributes to methodological optimization by demonstrating the applicability of a brief 3-minute OFT protocol, which reduces animal exposure to experimental stress. Furthermore, it reinforces the critical importance of systematically including both sexes in behavioral research to ensure translational validity, even when statistically robust differences are not immediately apparent. Future research incorporating larger sample sizes and comprehensive hormonal monitoring is necessary for a more nuanced characterization of sex-specific behavioral responses in stress-related paradigms.

Keywords: animal behavior; sexual differences; Wistar rats; open field test; anxiety

1. Introduction

Experimental research in psychology plays a fundamental role in understanding behavioral and emotional processes, allowing for the isolation of variables and the production of replicable evidence that supports both theory and clinical practice. Contemporary ethical guidelines for animal research, including those from the National Institutes of Health (NIH) and the Committee for the Update of

the Guide for the Care and Use of Laboratory Animals, explicitly reaffirm the necessity of including both males and females in experimental studies to ensure translational validity and avoid sex biases that compromise the generalizability of results (CLAYTON; COLLINS, 2014; NATIONAL RESEARCH COUNCIL, 2011).

The Open Field Test (OFT) constitutes a classic protocol widely used in experimental ethology, offering objective measures of locomotor activity and anxiety-related behaviors. The test consists of placing the animal in a novel arena and recording behaviors for a standardized interval, evaluating metrics such as distance traveled, time spent in center versus periphery, frequency of central entries, rearings, and defecation (VÖIKAR; STANFORD, 2021; DENENBERG, 1969).

However, protocols vary significantly between laboratories regarding illumination, arena dimensions, test duration, and cleaning procedures, substantially influencing results and hindering interlaboratory comparisons (VÖIKAR; STANFORD, 2021). Although several studies report consistent sex differences, indicating that female Wistar rats exhibit greater locomotor activity and central exploration, suggesting lower anxiety-like behavior compared to males (KNIGHT et al., 2021; SUDAKOV et al., 2021), it is important to consider that these differences may not be universal. Variations in experimental protocols and other contextual variables may result in discrepant findings, highlighting the need to investigate whether these behaviors persist under different experimental conditions.

The objective of this study is to investigate behavioral differences between adult male and female Wistar rats in the 3-minute Open Field Test, analyzing measures of locomotion, center usage, and emotionality indices, with the aim of contributing to methodological standardization and to the discussion regarding translational validity in experimental psychology.

2. Method

2.1. Animals

This study was derived from a larger experimental project conducted at Centro Universitário FMABC. Fourteen Wistar rats (7 males and 7 females) aged 12 weeks at the time of the open field test were used, obtained from the institutional vivarium. Animals were housed in groups of 3 to 4 individuals per polypropylene cage with stainless steel lids, strictly following established animal welfare practices.

Environmental conditions in the vivarium were maintained at $22 \pm 2^\circ\text{C}$ temperature, $70 \pm 5\%$ relative humidity, and a 12-hour light/dark photoperiod (lights on at 07:00h). Filtered water and balanced commercial chow were provided ad libitum throughout the experimental period.

The experimental protocol was approved by the Ethics Committee on Animal Use of Faculdade de Medicina do ABC (CEUA/FMABC), under number 04/2022, in accordance with ethical principles established by the National Council for the Control of Animal Experimentation (CONCEA).

2.2. Experimental Procedure

The open field test was conducted in a room with controlled illumination (approximately 40 lux), using a transparent acrylic arena ($80 \times 80 \times 30$ cm) with the floor virtually divided into 25 equal quadrants (16×16 cm each). Experiments were conducted between 18:00h and 20:00h, respecting the period of greatest rodent activity.

Each animal was individually positioned in the center of the arena for free exploration for 3 minutes. Between tests, the arena was rigorously cleaned with 70% alcohol to eliminate residual odors. All procedures were performed by the same trained evaluator, using personal protective equipment (gloves and protective clothing) and avoiding the use of perfumes or aromatic products.

Sessions were recorded on digital video for subsequent analysis of the following behavioral parameters: locomotor activity (number of quadrants traversed), time in central zone, frequency of rearing (elevation on hind paws), grooming (self-cleaning), and defecation (number of fecal pellets).

2.3. Statistical Analysis

The sample size of 7 animals per group was determined by statistical power calculation using G*Power 3.1.9.7, considering a large effect size ($d = 1.2$), 80% power, and 5% significance level, resulting in a minimum n of 6 animals per group, expanded to 7 for greater statistical robustness.

Data were expressed as mean \pm standard deviation. Distribution normality was verified through the Shapiro-Wilk test. According to data distribution, Student's t-test for independent samples (parametric data) or Mann-Whitney test (non-parametric data) were applied. The significance level adopted was $p < 0.05$. Analyses were performed using GraphPad Prism 9.0 software.

3. Results

Behavioral assessment was conducted on 14 animals, comprising 7 males and 7 females. Results are presented in Table 1 and illustrated in Figure 1. Statistical analysis revealed no significant differences between sexes for any of the analyzed variables ($p > 0.05$).

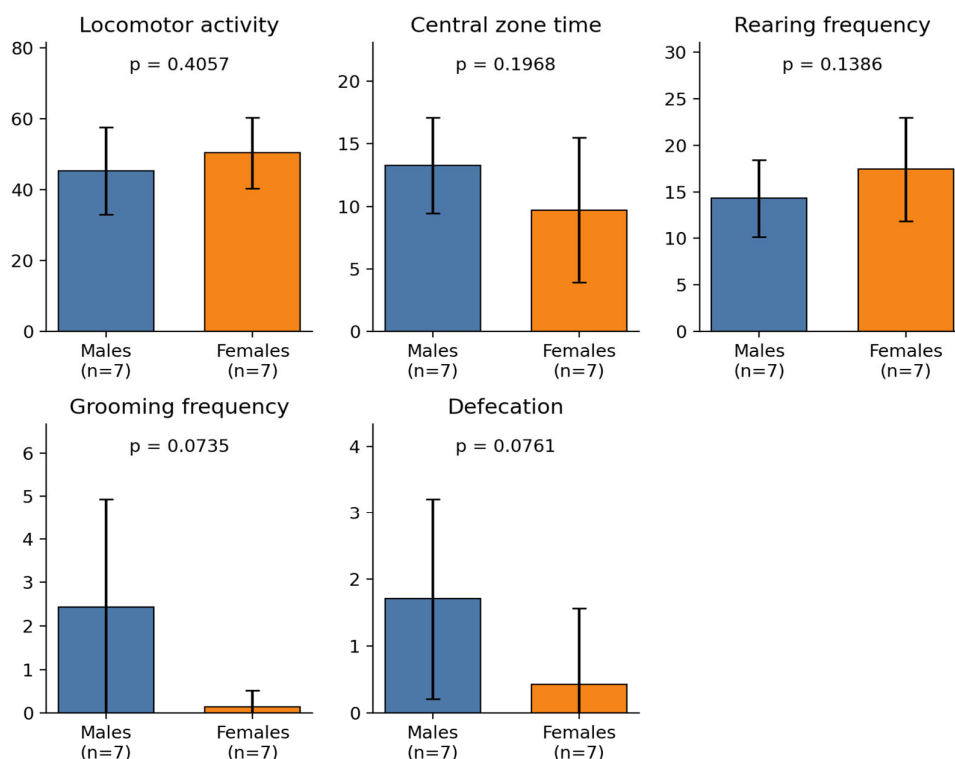


Figure 1. Behavioral parameters in male and female Wistar rats during the Open Field Test. Data are presented as mean \pm standard deviation. No significant differences were observed between sexes for any measured parameter ($p > 0.05$). Fonte: elaboração própria por Julius AI (2025).

Male animals exhibited a mean locomotor activity of 45.29 ± 12.3 , while females displayed slightly higher values (50.43 ± 10.0). The Mann-Whitney test demonstrated no significant difference between groups ($p = 0.4057$), indicating that general exploratory activity levels remained similar between males and females under the established experimental conditions. This finding suggests that both sexes presented comparable patterns of movement and horizontal exploration of the experimental environment.

Regarding time spent in the central zone, females remained in this region for 9.71 ± 5.77 seconds, while males stayed for 13.29 ± 3.82 seconds. Analysis using Student's t-test showed no significant

difference between groups ($p = 0.1968$), indicating that both sexes exhibited comparable levels of spatial anxiety-related behavior in the experimental paradigm employed. The central zone represents an area of greater exposure and vulnerability, serving as an indicator of anxiety in behavioral tests.

Females presented higher rearing frequency (17.43 ± 5.56) compared to males (14.29 ± 4.15). Statistical analysis demonstrated no significant difference between groups ($p = 0.1386$; Mann-Whitney test), indicating that both sexes exhibited similar patterns of vertical exploratory behavior. Rearing behavior, characterized by vertical positioning on hind limbs, constitutes an important indicator of environmental exploration and investigation.

Grooming frequency was numerically higher in males (2.43 ± 2.51) compared to females (0.14 ± 0.38), though without statistical significance ($p = 0.0735$; Mann-Whitney test). Grooming represents an important behavioral indicator of anxiety and stress states in rodents, frequently observed in situations of conflict or emotional discomfort. The absence of significant differences indicates that both sexes presented similar behavioral responses related to stress during the experimental period.

Males exhibited a greater number of defecation episodes (1.71 ± 1.5) relative to females (0.43 ± 1.13), without statistical significance ($p = 0.0761$; Mann-Whitney test). Defecation is frequently utilized as a physiological indicator of anxiety in behavioral tests, reflecting autonomic nervous system activation in response to environmental stressors. Results demonstrate that both sexes presented comparable physiological responses to experimental stress, not evidencing significant sexual dimorphism in reactivity to the testing environment.

Table 1. Behavioral parameters in the Open Field Test: comparison between male and female Wistar rats.

Variable	Males (n=7)	Females (n=7)	Test	p-value
Locomotor Activity	45.29 ± 12.3	50.43 ± 10.0	Mann-Whitney	0.4057
Central Zone Time	13.29 ± 3.82	9.71 ± 5.77	t-test	0.1968
Rearing Frequency	14.29 ± 4.15	17.43 ± 5.56	Mann-Whitney	0.1386
Grooming Frequency	2.43 ± 2.51	0.14 ± 0.38	Mann-Whitney	0.0735
Defecation	1.71 ± 1.5	0.43 ± 1.13	Mann-Whitney	0.0761

4. Discussion

Current findings demonstrate no statistically significant differences between males and females in the Open Field test (all $p > 0.05$), with trends approaching significance for grooming and defecation ($p < 0.10$). This pattern aligns with the literature reporting mixed results in anxiety and exploration variables within the Open Field paradigm, where sex differences do not always emerge robustly, particularly in small samples under varied methodological conditions (Prut & Belzung, 2003; Walsh & Cummins, 1976).

Locomotor activity was slightly higher in females, though not statistically significant. This finding is frequently described in the literature, as females may exhibit greater baseline activity and exploration without necessarily reflecting lower anxiety levels (Prut & Belzung, 2003; Seibenhener & Wooten, 2015). The concurrent increase in rearing behavior among females, also non-significant,

suggests a tendency toward greater vertical and investigative exploration, consistent with a more pronounced exploratory strategy in this sex. However, since time spent in the central zone did not differ between groups, anxiety levels appear comparable between sexes within the specific context of this study.

Time spent in the central zone showed no group differences, converging with reports that this measure is sensitive to multiple experimental factors, including lighting, arena dimensions, operational definition of "center," and test duration. These factors may fail to capture subtle differences when the context is not strongly anxiogenic (Prut & Belzung, 2003; Seibenhener & Wooten, 2015). Session duration and prior animal handling strongly modulate the propensity to explore the center, potentially attenuating sex-based contrasts.

The trends toward increased grooming and defecation in males ($p \approx 0.07$) are ethologically consistent with greater stress reactivity in this group. Grooming represents a classic marker of emotional regulation and may increase under states of arousal and anxiety, while novelty-induced defecation serves as a historical indicator of "emotionality" and autonomic activation in the Open Field (Hall, 1934; Walsh & Cummins, 1976). Although these trends do not reach conventional significance, their convergent direction provides functional coherence, suggesting that under current conditions, males may have experienced slightly greater stress burden than females, while locomotor and spatial measures remained similar.

Several methodological limitations warrant consideration in interpreting these results. First, the sample size of seven animals per group, though calculated a priori, may be considered limited for detecting small to moderate effects in behavioral tests. It is important to note that these data were obtained from a previous study, and animal ethics committees advocate for using the smallest possible number of animals (3Rs principle), balancing scientific rigor with animal welfare. Effect size estimates and confidence intervals should accompany p-values for more informed interpretation in future studies.

Second, estrous cycle control was not implemented in females, potentially introducing additional variability, as hormonal fluctuations can influence anxiety and exploration markers (Marcondes et al., 2002). However, this limitation may be reinterpreted as a characteristic that mimics natural reproductive timing randomness, reflecting conditions closer to real biological variability and increasing the ecological validity of findings. Furthermore, autonomic and affective behaviors such as defecation and grooming may capture stress reactivity dimensions not immediately reflected by spatial or general activity metrics, reinforcing the value of a multivariate approach.

The obtained results hold relevant translational implications for biomedical research and the development of neuropsychiatric therapies. The absence of robust differences between sexes in locomotor and spatial measures, combined with divergent trends in autonomic markers (defecation) and behavioral stress indicators (grooming), suggests that emotional reactivity may manifest in a sex-specific manner even when general exploratory activity remains comparable. This dissociation has direct implications for preclinical studies of anxiolytics and antidepressants, since therapeutic efficacy may vary between males and females not only in magnitude but also in the affected behavioral domains (Kokras & Dalla, 2014). The historical underrepresentation of females in rodent studies has contributed to biases in translating experimental findings to human populations, where anxiety disorders present twice the prevalence in women (Altemus et al., 2014). Therefore, the systematic inclusion of both sexes in behavioral protocols, even in the face of null results or non-significant trends, constitutes a methodological imperative to increase external validity and clinical applicability of findings.

Additionally, the differential sensitivity of behavioral parameters to sexual dimorphism observed in this study reinforces the need for multidimensional approaches in preclinical evaluation of neuroactive compounds. While traditional measures such as locomotion and time in center may not capture subtle differences, complementary ethological markers (grooming, defecation, rearing) can reveal clinically relevant patterns of emotional reactivity. This perspective aligns with contemporary precision medicine initiatives, which recognize biological sex as a critical modulator

of pharmacological responses and neuropsychiatric disease trajectories (Shansky & Woolley, 2016). In translational contexts, failure to consider sex as an experimental variable may result in undertreatment or inadequate treatment of specific populations, perpetuating mental health disparities. Thus, the findings of this study, although derived from a basic behavioral paradigm, contribute to the conceptual framework that supports the implementation of regulatory guidelines requiring balanced sex inclusion in biomedical research, from preclinical phases through clinical trials (Clayton & Collins, 2014).

Despite these limitations, the present study holds significant scientific relevance. While sex differences in exploratory behavior constitute a widely researched topic, this work demonstrates the applicability of a three-minute protocol in the Open Field test, contributing to methodological optimization and reducing animal exposure time to experimental stress. Additionally, the results support contemporary discussions regarding the imperative need for female inclusion in behavioral tests, as advocated by international guidelines (Beery & Zucker, 2011). The absence of robust differences in locomotor and spatial measures, combined with convergent trends in stress markers, reinforces that sex should be systematically considered as an experimental variable, even in the face of null results, as subtle reactivity patterns may become apparent with greater statistical power and confounder control.

5. Conclusions

The observed pattern indicates possible subtle differences in stress reactivity between sexes, without evidence of robust differences in spatial anxiety or general exploratory motivation under the tested conditions. These findings align with the body of literature describing mixed results in Open Field studies and underscore the necessity for experimental designs with greater statistical power, estrous cycle control or stratification, and rigorous methodological standardization to elucidate sexual nuances in anxiety models. Future research should incorporate larger sample sizes and comprehensive hormonal monitoring to better characterize sex-specific behavioral responses in stress-related paradigms.

Author Contributions: Davi Vantini: Conceptualization and study design; data analysis and interpretation; manuscript writing; critical review of intellectual content; project administration; funding acquisition. Giuliana Petri: Data collection and acquisition; statistical analysis; methodology development; critical manuscript review. Jose Ramos dos Santos: Data collection and acquisition; animal care and handling; statistical analysis; critical manuscript review. Glaucia Luciano da Veiga: Study supervision; methodology validation; critical manuscript review; ethical oversight. Thais Moura Gascón: Data collection and acquisition; behavioral assessment; statistical analysis; critical manuscript review. Ingrid Bertollini Lamy: Data collection and acquisition; behavioral testing; statistical analysis; critical manuscript review. Jamili Rasoul Salem de Souza: Data collection and acquisition; experimental procedures; statistical analysis; critical manuscript review. Sidnei Celso Corocine: Data collection and acquisition; technical support; statistical analysis; critical manuscript review. Fernando Luiz Affonso Fonseca: Study supervision; conceptualization; critical manuscript review; final approval; senior oversight. All authors have read and approved the final version of the manuscript and agree to its submission for publication. Each author has made substantial contributions to the conception, design, execution, or analysis of the study, and all have participated sufficiently in the work to take public responsibility for appropriate portions of the content.

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Institutional Review Board Statement: The experimental protocol was approved by the Ethics Committee on Animal Use of Faculdade de Medicina do ABC (CEUA/FMABC), under number 04/2022, in accordance with the ethical principles established by the National Council for the Control of Animal Experimentation (CONCEA). All procedures were conducted in strict compliance with national and international guidelines for animal welfare. The authors declare no conflicts of interest related to this study. As this research exclusively involved animal models, informed consent from human participants was not applicable. The study was carried out with

full ethical transparency and appropriate institutional oversight, ensuring scientific integrity and adherence to all applicable regulations concerning the use of animals in research.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author(s).

Conflicts of Interest: The authors declare no conflict of interest.

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