



# Sex differences in exploratory behavior of rats successfully performing the object-in-place recognition memory test

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## ABSTRACT

Male and female rodents display unique search strategies when exploring new and familiar environments. Sex differences are well-documented in the literature and may be observed in tasks that rely on spontaneous exploration (e.g., recognition memory tests). Therefore, we assessed patterns of male and female rat behavior in the object-in-place (OiP) test, a common recognition memory paradigm involving object-location associations. Twelve male and 12 female adult Long Evans rats were tested four times in the 1-h OiP test and exploratory behaviors were compared during habituation, sample, and test phases. Results revealed that females moved faster and farther than males, showed increased immobility frequency and reduced immobility duration, reduced outer zone mobility duration, and increased inner zone entrances, compared to males during habituations. During sample phases, female rats moved faster than males, displayed reduced immobility frequency in the inner zone, and demonstrated consistent distance travelled across repeated sessions; conversely, male rats moved less in later sessions and exhibited increased mobility frequency in the outer zone. Analyses comparing test phase behavior revealed females continued to move faster than males; however, no other sex differences were observed. These findings are consistent with previous literature highlighting unique sex differences in explorative behaviors during recognition testing. Sex differences in locomotion and mobility state behaviors may be more indicative of individual motivation and search strategy between the sexes and less indicative of recognition memory.

## 1. Introduction

Contemporary behavioral neuroscience research suggests recognition learning and memory in rodents may differ between the sexes, experimental paradigm (e.g., novel object recognition vs. radial arm maze), and length of delay (e.g., 30-min vs. 24-h) [1–6]. In particular, behavioral neuroscience paradigms were initially developed to promote reliable testing between laboratories; unfortunately, many common paradigms used today were optimized in male rodents only [7,8]. The National Institutes of Health (NIH) initiated the Sex As a Biological Variable (SABV) mandate in 2016, marking the beginning of a cultural change regarding how best to consider the influence of sex in behavioral research designs. Eight years later, uncertainty remains regarding how best to interpret recognition memory performance, particularly in light of known sex differences in exploratory behaviors in recognition

learning paradigms [9–12]. To fully account for sex differences in behavioral research, it may be necessary to re-consider how behavioral differences influence interpretation of recognition performance metrics [3–5,11–13].

Previous research suggests male rats may exhibit an advantage in object-in-place (OiP) recognition memory, particularly when delay periods are greater than 30-min; however, differences appear to diminish when estrous and environmental factors are considered [1,3–5,11,14]. In contrast, we recently reported that male and female Long Evans rats performed similarly in a carefully controlled object-in-place (OiP) test with a 60 min delay [18]. In addition, temporary inactivation or blockade of ionotropic glutamate receptors in the retrosplenial cortex impaired this memory in both sexes [18]. In light of inconsistent findings regarding sex differences in associative recognition learning, we were interested in assessing whether there were differences in the

*Abbreviations:* ANOVA, analysis of variance; NIH, National Institutes of Health; Ns, not significant; OiP, object-in-place; SABV, sex as a biological variable; S.E.M, standard error of the mean.

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behavior and search strategy of the sexes during the habituation, sample, and test phases of the OiP test, even in the face of similar discrimination abilities.

Researchers have investigated exploratory behavior in rodents, including locomotor activity during pre-test habituations and object exploration during recognition testing [13]. Given that habituation and sample phases are fundamentally different contexts in recognition tests, it is recommended to assess locomotor activity by frequency of alternations between mobility states (i.e., mobile, immobile), in contrast to total duration [13]. Evidence also suggests that male and female mice display uniquely individual behavioral phenotypes that remain consistent across repeated recognition test sessions; however, males exhibit increased variability during open field exploration, while female exploration patterns remain stable across repeated tests (estrous stage has a minimal influence on exploration behaviors) [10].

The OiP test is a spontaneous recognition paradigm that relies on rodents' innate tendency to explore unfamiliar objects and was used to assess visuospatial recognition learning behavior in male and female rats [1,15–19]. Neuroanatomical studies have thoroughly characterized brain structures and circuits supporting OiP recognition, highlighting the anterior thalamus, nucleus reunions, medial dorsal thalamus, medial prefrontal cortex, mammillary bodies, dorsal hippocampus, perirhinal cortex and retrosplenial cortex [16,18,20–27]. Interestingly, evidence suggests that females increasingly rely on hippocampal-based [allocentric] search strategies when circulating ovarian sex hormones are high (e.g., proestrous, estrous), and a striatal-based [egocentric] strategy when hormone levels are low (e.g., metestrous, diestrous) [1,3–5,8,11,28].

Given reported sex differences, the present study returned to previously published data comparing novelty recognition in male and female Long Evans rats, during four repeated OiP tests with a 1-hour delay [18]. In the present experiment, previously captured behavioral videos were re-analyzed to compare unique exploratory behavioral metrics between the sexes, during repeated OiP tests. Behavior was compared between the sexes during pre-test habituations (conducted within 24-hours of each test), sample, and test phases and we tested the following hypotheses: compared to males, (1) female rats will move faster and farther during repeated OiP habituations, sample, and test phases; (2) females will exhibit greater inner zone latency and reduced inner zone duration times; (3) female rats will increasingly alternate between mobile and immobile exploration states, in all assessed phases.

## 2. Materials and methods

### 2.1. Subjects

Adult male ( $n = 12$ ) and female ( $n = 12$ ) Long Evans rats were used (Charles River Laboratories, Kingston, NY, USA). Same-sex rats were pair- or triple-housed throughout testing in standard, ventilated polypropylene cages and left undisturbed for one week to acclimate to the vivarium. The vivarium was controlled for temperature (21°C) and humidity, and water and food (standard rat chow) were available *ad libitum* throughout experiments. Each cage included environmental enrichment in the form of Nylabones, plastic tubes, multi-level platforms, multiple water sources, and cage-mates during experiments. The light-cycle was maintained on a 12:12 h cycle (lights on at 7:00 a.m.). Following the acclimation period, an extended 7-day experimental habituation was conducted with each rat: rats were handled with cagemates for 2.5 min/day, tail marked for identification, and weighed (days 1–2), introduced to the test environment and handled 5-min/day inside the test room (days 3–4), followed by three consecutive days of box habituations in which rats explored an empty test box for 10-min/day with no objects inside. Experiments were in accordance with the ARRIVE guidelines 2.0 [29], the Canadian Council on Animal Care guidelines, and approved by the University of Saskatchewan Animal Research Ethics Board.

### 2.2. Pilot experiment

A pilot experiment was conducted to assess optimal object types and arrangements for repeated OiP testing in naïve Long Evans rats of both sexes. Performance and object interaction times were compared using a variety of objects that differed in size, material, color, and pairings. Observations suggested that rats interacted more with colorful ceramic objects and less with black 3d-printed objects, regardless of sex. Findings also highlighted the importance of carefully selecting objects and object-arrangements for recognition testing, ensuring objects are equally interesting yet remain distinct from one another. Following pilot analyses, four reliable object sets were selected for the present study (Fig. 1A-B). Note a fifth novel object set (not shown) was reserved for re-tests when necessary (not shown).

### 2.3. Experimental design

We tested male and female rats on four repeated OiP tests with a 1-h delay and 5–7 days between tests (Fig. 1A). Unique object sets were used for each test session and rats were assigned to one of two identical testing boxes which remained constant throughout testing. Rats were pseudo-randomly assigned to each object set condition, such that no rat saw the same object twice (Fig. 1B). Behavioral tests and habituations were conducted between October – December, 2022 and rats were randomly assigned to test during three different times of the day (~8:00–11:00 am, ~11:00 am – 2:00 pm, or ~2:00–5:00 pm) to control for potential time-of-day effects. Habituation behaviors were assessed and averaged during the 10-min pre-test habituations conducted within 24-hours of repeated test sessions (note: only the first 5-min was assessed) (Fig. 1), 5-min sample phases (Fig. 2), and 4-min test phases (Fig. 3). In the present design, all 24 rats completed the 7-day experimental habituation protocol, including a final 10-min box habituation in the test room. All rats were habituated and recorded separately prior to the first OiP test (Session A); however, cage-mates were habituated simultaneously inside the test room (in separate boxes; only one rat recorded) for subsequent habituations (Sessions B-D). Rats were counter-balanced so that the opposite cage-mates were assessed during subsequent habituations, resulting in the following sample sizes: Session A ( $n=24$ ), Sessions B-D ( $n=12$ ).

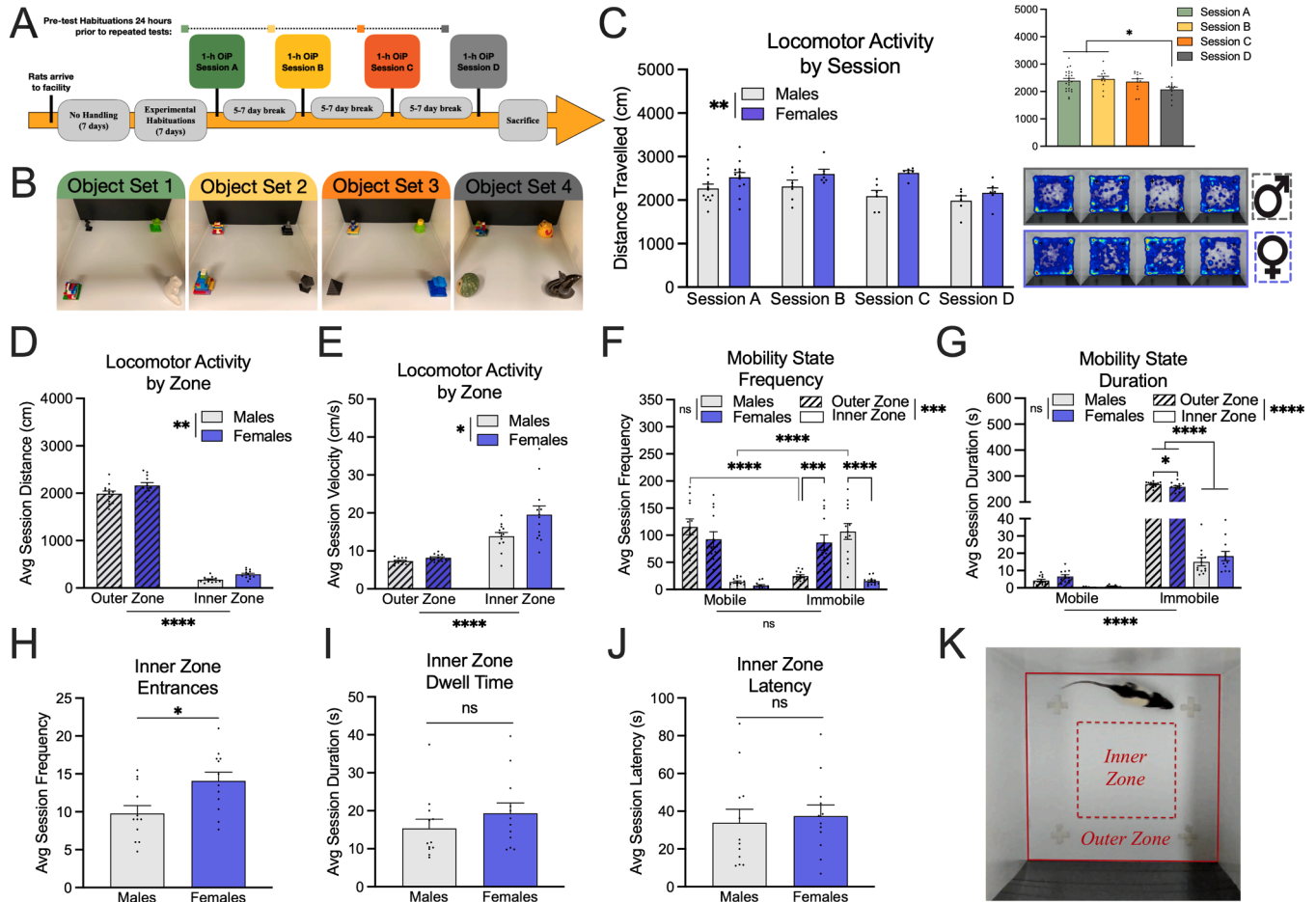
### 2.4. The object-in-place (OiP) test

Rats were habituated to an empty test box ~24 hours prior to each test day. On test days, rats return to the box for 5-minutes to explore four novel objects placed in the corners (sample phase), return to their home cage for a 1-hour delay period, then return to the test box to explore a new arrangement of the previously explored sample phase objects (4-min test phase). These methods are in accordance with previously established protocols and described in detail elsewhere [1,18]. Behavior was recorded using a ceiling-mounted camera and object interaction times (s) were initially assessed with stop watches by researchers blinded to the location of familiar and novel objects [18]. Previously reported object interaction times during OiP sample (Fig. 2B) and test (Fig. 3B) phases, are presently included for comparison with exploration behaviors.

### 2.5. Data analysis

Exploration behaviors were assessed using EthoVision XT Version 5.0 (Noldus) during four repeated 1-hour OiP test Sessions (A, B, C, D). A final re-run session was conducted following the last test to correct for minor testing errors (i.e., rat knocked object over), and included five males and three females. Data were analyzed during the first 5-min of repeated 10-min habituation phases (conducted in an empty test box within 24-h of each test session), 5-min sample phases, and 4-min test phases. The following dependent variables were assessed during each

## 5-min Habituations



**Fig. 1.** During habituations, females exhibited increased locomotion, mobility state alterations, and inner zone entrances, compared to male Long Evans rats. (A) Experimental timeline shows repeated-measures design including habituation protocol and 5–7 day breaks between repeated tests. (B) Schematic shows four object sets used for 1-hour object-in-place (OiP) tests (note the location of the black wall insert). (C) Males moved less in subsequent test sessions indicating enhanced habituation to the test arena, while female distance travelled was more reliable between sessions, particularly the first three. Rats exhibited reduced exploration between sessions A–D and B–D, regardless of sex (upper right inset). Finally, a representative heat map of habituation demonstrates enhanced female locomotor activity and reduced thigmotaxic behavior, compared to males (bottom right inset). (D) Females moved greater distances than males regardless of zone, while all rats moved farther in the outer zone. (E) Females moved faster than males and all rats moved faster in the inner zone. (F) Rats showed reduced mobility frequency in the inner zone, regardless of sex. Female rats exhibited increased immobility frequency in the outer zone and reduced immobility frequency in the inner zone, compared to males; while males showed reduced immobility frequency in the outer zone and increased immobility frequency in the inner zone. (G) Increased immobility duration occurred in the outer zone and reduced immobility duration in the inner zone, regardless of sex; while male rats were increasingly immobile in the outer zone, compared to females, while all rats exhibited reduced immobility duration while in the inner zone. (H) Female rats increasingly entered the inner zone compared to males; however, no sex differences were observed for (I) inner zone dwell time, or (J) latency to enter the inner zone. (K) Schematic of the habituation test box including inner (50%) and outer (50%) zone dimensions.

phase and averaged across repeated sessions for analysis: distance travelled (cm), velocity (cm/s), frequency and duration of mobility states in the inner (50%) and outer (50%) zones, as well as frequency entering the inner-zone, total time (s) spent in inner zones (dwell time), and inner zone latency (s) indicated the first time a subject's nose crossed into the inner zone from the outer zone. In contrast with previous findings comparing recognition learning in the OiP test [18], current analyses aimed to characterize exploration and search strategy between the sexes, during key OiP learning phases. Two-way mixed ANOVAs were computed with GraphPad Prism Version 10. We compared average distance travelled by Sex by Session (Figs. 1C, 2C, 3C), Sex by Zone (Figs. 1D, 2D, 3D), and average velocity by Sex and Zone (Figs. 1E, 2E, 3E). A Sex x Zone (inner zone, outer zone) x Mobility State (mobile, immobile) three-way mixed ANOVA was used to compare frequency (Figs. 1F, 2F, 3F) and duration (Figs. 1G, 2G, 3G) in different mobility states ("mobile" = 20–60%, "immobile" < 20% time in

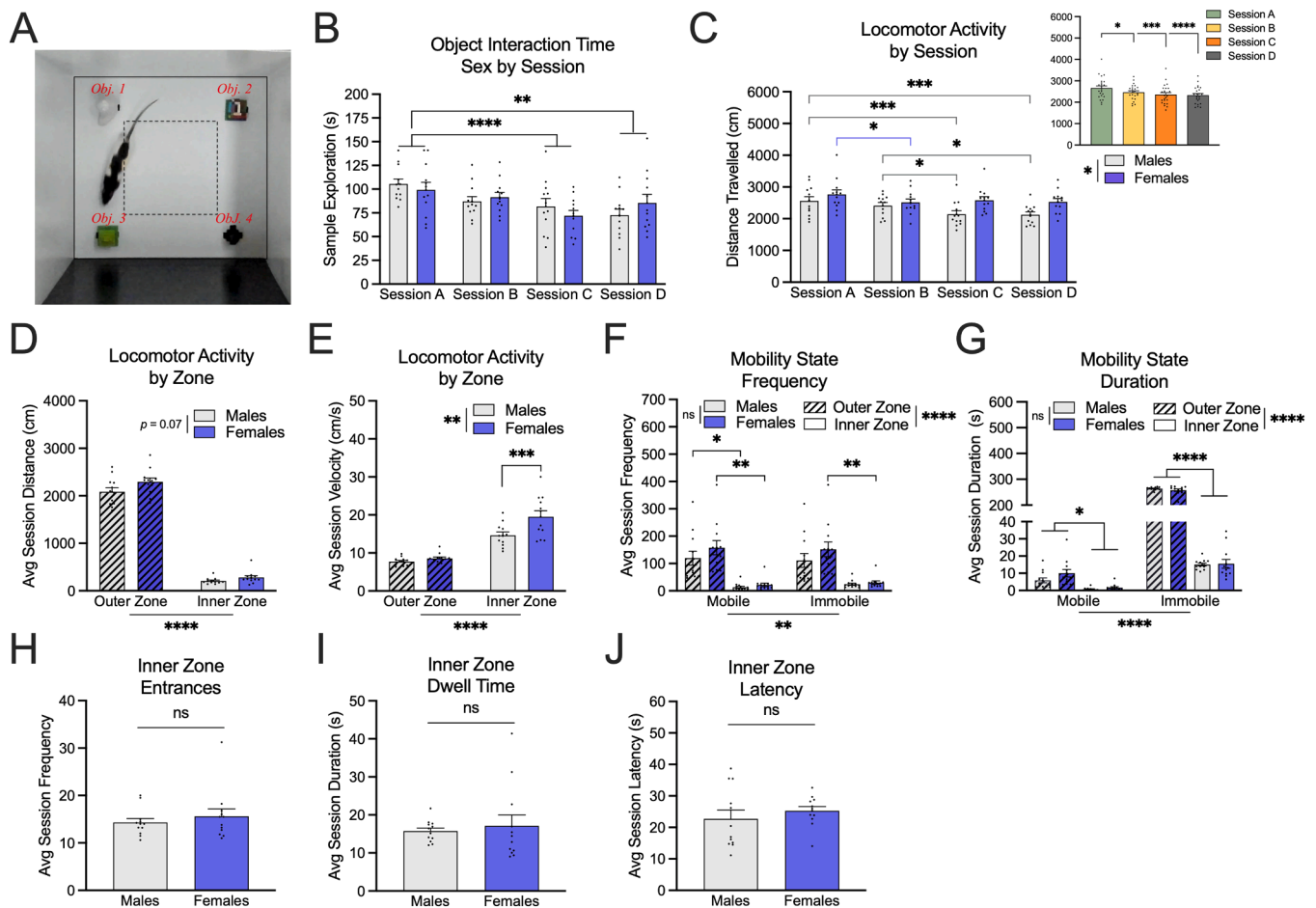
motion). Finally, unpaired t-tests were used to compare the influence of Sex on inner zone entrances (Figs. 1H, 2H, 3H), duration (Figs. 1I, 2I, 3I), and latency (Figs. 1J, 2J, 3J). *Post hoc* analyses were conducted using Tukey's multiple comparisons test unless otherwise stated. All values are presented as mean  $\pm$  S.E.M. and significance is denoted by \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ; \*\*\*\* $p \leq 0.0001$ .

### 3. Results

#### 3.1. During habituations, female rats exhibited increased locomotion, mobility state alterations, and inner zone entrances, when compared to male rats

For locomotor activity in the first 5-min of habituations, a main effect of Sex was observed ( $F(1,22) = 8.62, p < 0.01$ ) and inspection of the data revealed females travelled greater average distances compared to males.

## 5-min Sample Phases



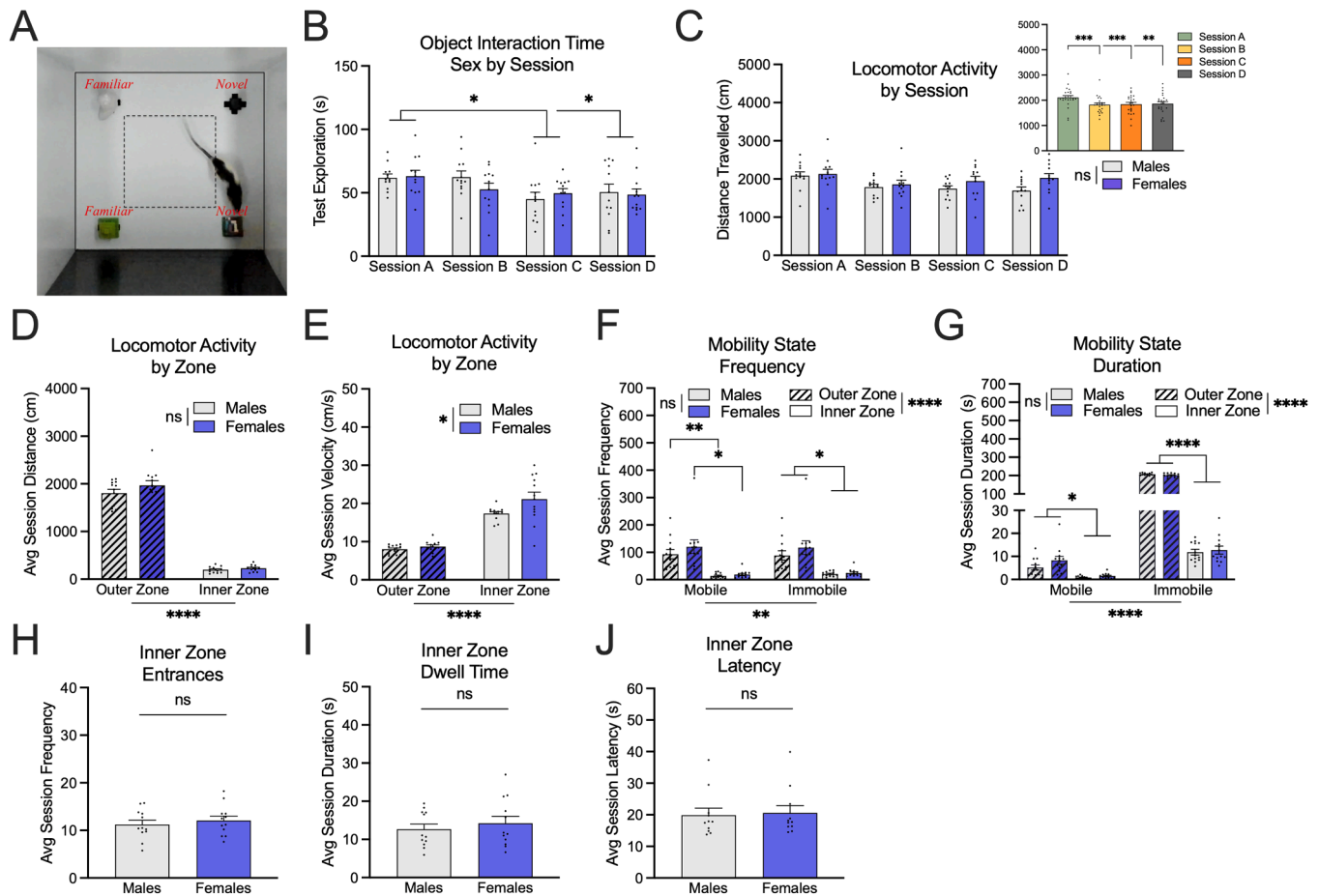
**Fig. 2.** During repeated OIP sample phases, female rats exhibited increased mobility frequency, yet consistent velocity and distance travelled, while male rats moved less with repeated sessions. (A) Schematic of the test box, including a representative object sets and their locations. (B) Rats interacted less with objects between Sessions A-C and A-D, although no sex differences were observed for object interaction times. (C) Results comparing distance travelled during repeated sample phases revealed a variety of findings: rats expectedly exhibited reduced distance travelled across repeated sample phase sessions (upper right inset), while female sample exploration remained consistently higher than males. Furthermore, female rats exhibited a slight drop in exploration duration between Sessions A-B, while female rats moved consistently between sessions. (D) All rats moved farther in the outer zone compared to the inner zone, and no main effect of Sex was observed ( $p = 0.07$ ) for distance travelled. (E) Female rats moved faster than males regardless of zone, and exhibited increased velocity in the inner zone, when compared to males. All rats moved faster in the inner zone. (F) Rats exhibited increased mobility frequency compared to immobility frequency, while male rats alone exhibited reduced mobility frequency between the inner and outer zones. Female rats exhibited reduced immobility frequency in the inner zone, compared to males. (G) Rats spent less mobility state duration in the inner versus outer zones, and increased immobility duration, compared to time spent mobile. Furthermore, rats moved less in the inner zone, regardless of sex. Comparison of inner zone behaviors revealed no sex differences for inner zone (H) entrances, (I) dwell time, or (J) latency.

A main effect of Session was also observed ( $F(3,30) = 4.32, p < 0.05$ ) and *post-hoc* analyses revealed reduced exploration occurred between Sessions A-D ( $p < 0.05$ ) and B-D ( $p < 0.05$ ) (Fig. 1C, upper right panel inset). No Sex by Session interaction effect was observed ( $F(3,30) = 0.86, p = 0.47$ ) for average distance travelled in the habituation sessions (Fig. 1C). When distance travelled was compared between inner and outer zones, a main effect of Sex was observed ( $F(1,22) = 9.31, p < 0.01$ ) and inspection of the data revealed females moved farther than males. A main effect of Zone was also observed ( $F(1,22) = 1915, p < 0.0001$ ) with rats traveling less distance in the inner versus outer zone, regardless of sex. No Sex by Zone interaction was observed ( $F(1,22) = 0.52, p = 0.47$ ) for average distance travelled during habituations (Fig. 1D). When velocity was considered by zone, main effects of Sex ( $F(1,22) = 7.09, p < 0.05$ ) and Zone were observed ( $F(1,22) = 46.17, p < 0.0001$ ), without a significant interaction ( $F(1,22) = 3.36, p = 0.08$ ) (Fig. 1E). Thus, comparison of distance travelled and velocity during the first 5-min of habituation sessions conducted prior to repeated test sessions, revealed

that female rats moved faster than males regardless of zone, and all rats moved faster in the inner zone, regardless of sex.

When mobility state frequency was compared by sex and zone, no main effect of Sex was observed ( $F(1,22) = 3.17, p = 0.09$ ), however a main effect of Zone was observed ( $F(1,22) = 19.65, p < 0.001$ ) and inspection of the data revealed increased mobility state frequency in the outer habituation zone, compared the inner zone. No main effect of Mobility State was observed ( $F(1,22) = 0.06, p = 0.81$ ), although a Sex by Zone interaction effect was observed ( $F(1,22) = 17.15, p < 0.001$ ) and *post-hoc* analyses revealed reduced female immobility frequency in both the inner ( $p < 0.0001$ ) and outer ( $p < 0.001$ ) habituation zones, compared to male rats. A Mobility State by Zone interaction effect was also observed ( $F(1,22) = 107.60, p < 0.0001$ ) and *post-hoc* analyses revealed reduced mobility frequency occurred in the inner zone ( $p < 0.0001$ ). Finally, a Sex by Zone by Mobility State interaction effect was observed ( $F(1,22) = 77.91, p < 0.0001$ ) and *post-hoc* analyses revealed males exhibited increased mobility frequency in the outer zone ( $p <$

## 4-min Test Phases



**Fig. 3.** Male and female rats displayed equivalent object interaction times and performance during repeated 1-hour OiP tests, although female rats moved faster compared to male rats. (A) Schematic showing the OiP test box, including representative locations of the familiar and novel objects. (B) Rats interacted less with objects between Session A-C and A-D, although no sex differences were observed. (C) Results comparing distance travelled during repeated OiP test phases, revealed rats explored less during test phases, regardless of sex (upper right inset). (D) Rats moved farther in the outer zone compared to inner zone, regardless of sex. (E) Female rats moved faster than male rats, while all rats moved faster in the inner zone, when compared to outer zone velocity. (F) Panel shows mobility frequency between mobile and immobile states during test phases. Rats exhibited increased immobility frequency compared to mobility frequency and reduced mobility frequency in the inner zone vs. the outer zone. (G) Reduced inner zone mobility and increased immobile durations were observed during OiP test sessions, regardless of zone; while reduced mobility state duration occurred while in the inner zone. Comparison of inner zone behaviors revealed no sex differences for inner zone (H) entrances, (I) dwell time, or (J) latency.

0.0001) and reduced mobility frequency in the inner zone ( $p < 0.0001$ ), in contrast with female rats which exhibited reliable mobility state frequency between habituation zones ( $p > 0.99$ ) (Fig. 1F; Bonferroni *post hoc*s). When mobility state duration was analyzed by sex and zone, no main effect of Sex was observed ( $F(1,22) = 2.04, p = 0.17$ ). A main effect of Zone was observed ( $F(1,22) = 4006, p < 0.0001$ ) and inspection of the data revealed rats showed increased mobility state duration in the outer zone, regardless of sex. A main effect of Mobility State was also observed ( $F(1,22) = 16,240, p < 0.0001$ ) with rats exhibiting increased immobile duration, compared to time spent mobile, regardless of sex. A Sex by Mobility State interaction was also observed ( $F(1,22) = 4.96, p < 0.05$ ) and *post-hoc* analyses revealed that only male rats demonstrated increased immobility duration in the outer habituation zone, compared to female rats ( $p < 0.05$ ). A Zone by Mobility State interaction effect was also observed ( $F(1,22) = 2835, p < 0.0001$ ) and *post-hoc* analyses revealed reduced immobility duration occurred in the inner zone, compared to outer zone ( $p < 0.0001$ ) (Fig. 1G; Bonferroni *post hoc*s). In summary, female rats were increasingly immobile in the outer zones and less mobile in the inner zone, while exhibiting overall reduced time in the outer zones, compared to males.

Finally, inner zone entrances, dwell time, and latency were compared between males and females using unpaired t-tests. These comparisons revealed females entered the inner zone more frequently in habituations ( $t(22) = 2.75, p < 0.05$ ) (Fig. 1H), while no differences were observed for average dwell time ( $t(22) = 1.08, p = 0.29$ ) (Fig. 1I), nor latency time ( $t(22) = 0.38, p = 0.71$ ) (Fig. 1J), during pre-test habituation sessions.

### 3.2. During repeated OiP sample phases, female rats exhibited increased mobility frequency, yet consistent velocity and distance travelled, while male rats moved less with repeated sessions

For object interaction time during 5-min sample phases, no main effect of Sex was observed ( $F(1,22) = 0.002, p = 0.97$ ). However, a main effect of Session was observed ( $F(2,6,58.1) = 7.66, p < 0.001$ ) and *post-hoc* analyses revealed decreased object interaction time occurred between Sessions A-C ( $p < 0.0001$ ) and A-D ( $p < 0.01$ ). No Sex by Session interaction was observed ( $F(3,66) = 1.53, p = 0.21$ ) for object interaction time during the sample phase (Fig. 2B). For distance traveled during OiP sample phases, a main effect of Sex was observed ( $F(1,22) = 4.19, p$

= 0.05) and inspection of the data revealed female rats moved more than male rats. A main effect of Session was also observed ( $F(3,66) = 10.05, p < 0.0001$ ) and *post-hoc* analyses revealed reduced distance travelled occurred between Sessions A-B ( $p < 0.05$ ), A-C ( $p < 0.001$ ), and A-D ( $p < 0.0001$ ), regardless of sex (Fig. 2C, upper right panel inset). A Sex by Session interaction effect was observed ( $F(3,66) = 2.85, p < 0.05$ ) and *post-hoc* analyses revealed that females only moved less distance between Sessions A-B ( $p = 0.05$ ), while male rats moved less between Sessions A-C ( $p < 0.001$ ), A-D ( $p < 0.001$ ), B-C ( $p < 0.05$ ), and B-D ( $p < 0.05$ ) (Fig. 2C, main panel). When distance travelled was compared by Sex and Zone, no main effect of Sex was observed ( $F(1,22) = 3.64, p = 0.07$ ); although, a main effect of Zone was observed ( $F(1,22) = 1675, p < 0.0001$ ) and further investigation revealed reduced distance travelled in the inner zone, compared to outer zone. No Sex by Zone interaction effect was observed ( $F(1,22) = 2.09, p = 0.16$ ) for distance travelled during sample phases (Fig. 2D). When velocity was considered by zone, a main effect of Sex was observed ( $F(1,22) = 8.33, p < 0.01$ ) with female rats moving faster than male rats. A main effect of Zone was also observed ( $F(1,22) = 105.9, p < 0.0001$ ), with further investigation revealing that increased velocity occurred in the inner zone, compared to outer zone. Additionally, a Sex by Zone interaction effect was observed ( $F(1,22) = 5.32, p < 0.05$ ) and *post-hoc* analyses highlighted that female rats moved significantly faster in the inner zone, when compared to male rats (Fig. 2E). Taken together, results suggest that females maintained consistent distance travelled between repeated OiP sample phases, whereas males increasingly habituated to the sample phases, indicated by reduced distance travelled in subsequent sample phases, compared to females. Furthermore, females demonstrated enhanced velocity in the inner zone, compared to males.

Analyses comparing mobility state frequency by sex and zone, revealed no main effect of Sex ( $F(1,22) = 1.31, p = 0.27$ ). A main effect of Zone was observed ( $F(1,22) = 46.86, p < 0.0001$ ) and further inspection highlighted that reduced mobility frequency occurred in the inner zone, compared to the outer zone, regardless of sex. A main effect of Mobility State was also observed ( $F(1,22) = 8.50, p < 0.01$ ) in which rats exhibited increased immobility frequency, compared to mobility frequency during sample phases. A Mobility State by Zone interaction was observed ( $F(1,22) = 372.4, p < 0.0001$ ) and *post-hoc* analyses revealed reduced mobility frequency occurred in the inner zone, compared to the outer zone ( $p < 0.01$ ). Finally, a Sex by Zone by Mobility State interaction effect was observed ( $F(1,22) = 14.86, p < 0.0001$ ) and *post-hoc* analyses revealed that only females exhibited reduced immobility frequency in the inner zone, compared to the outer zone ( $p < 0.01$ ) (Fig. 2F; Bonferroni *post hoc*s). When mobility state duration was analyzed by sex and zone, no main effect of Sex was observed ( $F(1,22) = 0.03, p = 0.87$ ). However, a main effect of Zone was observed ( $F(1,22) = 11,208, p < 0.0001$ ) and inspection of the data revealed increased mobility state duration in the outer zone, compared to inner zone, regardless of sex. A main effect of Mobility State was also observed ( $F(1,22) = 5156, p < 0.0001$ ) and further inspection revealed rats spent more time immobile, compared to mobile, regardless of sex. A Zone by Mobility State interaction effect was observed ( $F(1,22) = 4117, p < 0.0001$ ) and *post-hoc* analyses revealed reduced mobility ( $p < 0.05$ ) and immobility ( $p < 0.0001$ ) durations were exhibited in the inner zone, compared to outer zone, regardless of sex. No additional interaction effects were observed (Fig. 2G; Bonferroni *post hoc*s). These results suggest males and females demonstrated comparable exploration strategies in the sample phase, when comparing mobile versus immobile behaviors.

Finally, inner zone entrance frequency, dwell time, and latency were compared between males and females using unpaired t-tests. These comparisons revealed no main effects of Sex for inner zone entrance frequency ( $t(22) = 0.74, p = 0.47$ ) (Fig. 2H), dwell time ( $t(22) = 0.46, p = 0.65$ ) (Fig. 2I), or latency ( $t(22) = 0.81, p = 0.43$ ) (Fig. 2J).

### 3.3. Male and female rats displayed equivalent object interaction times and performance during repeated 1-hour OiP tests, although female rats moved faster compared to male rats

Previously published findings looking at the same data set, observed no sex differences in OiP recognition retrieval [18]. In light of equivalent performance, exploratory behavior was further investigated here. Analyses comparing object interaction time during repeated 1-h OiP test phases (4-min), revealed no main effect of Sex ( $F(1,22) = 0.21, p = 0.65$ ); however, a main effect of Session was observed ( $F(3,66) = 4.27, p < 0.01$ ) and *post-hoc* analyses revealed decreased object interaction time occurred between Sessions A-C ( $p < 0.05$ ) and A-D ( $p < 0.05$ ). No Sex by Session interaction effect was observed ( $F(3,66) = 0.80, p = 0.47$ ) for object interaction time during repeated test phases (Fig. 3B). For distance traveled during OiP test phases, no main effect of Sex was observed ( $F(1,22) = 1.75, p = 0.20$ ); although a main effect of Session was observed ( $F(3,66) = 8.26, p < 0.0001$ ) and *post-hoc* analyses revealed reduced distance travelled occurred between Sessions A-B ( $p < 0.001$ ), A-C ( $p < 0.001$ ), and A-D ( $p < 0.01$ ) (Fig. 3C, upper right panel inset). No Sex by Session interaction was observed ( $F(3,66) = 1.99, p = 0.12$ ) for distance travelled during OiP test phases (Fig. 3C). When distance travelled was compared by Sex and Zone, no main effect of Sex was observed ( $F(1,22) = 1.83, p = 0.19$ ); however, a main effect of Zone was observed ( $F(1,22) = 977.1, p < 0.0001$ ) and inspection of the data revealed reduced distance travelled occurred in the inner zone, compared to the outer zone. No Sex by Zone interaction effect was observed ( $F(1,22) = 1.50, p = 0.23$ ) for average distance travelled during test phases (Fig. 3D). When velocity was considered by zone, a main effect of Sex was observed ( $F(1,22) = 4.81, p < 0.05$ ) with female rats exhibiting increased velocity, compared to male rats. A main effect of Zone was also observed ( $F(1,22) = 130, p < 0.0001$ ) and inspection of the data revealed increased velocity took place in the inner zone, compared to the outer zone. No Sex by Zone interaction effect was observed ( $F(1,22) = 2.57, p = 0.12$ ) for average velocity during repeated OiP test phases (Fig. 3E). In summary, test phase exploration behaviors were consistent between males and females during OiP test phases, with the exception that female rats continued to move faster in the inner zone, compared to males.

Analyses comparing mobility state frequency by sex and zone, revealed no main effect of Sex ( $F(1,22) = 1.03, p = 0.32$ ); however, a main effect of Zone was revealed ( $F(1,22) = 34.32, p < 0.0001$ ) with reduced mobility in the inner zone, compared to the outer zone. A main effect of Mobility State was observed ( $F(1,22) = 9.43, p < 0.01$ ) and investigation of the data revealed a small increase in immobility frequency, compared to mobility frequency, regardless of sex. A Mobility State by Zone interaction effect was observed ( $F(1,22) = 199.9, p < 0.0001$ ) and *post-hoc* analyses revealed reduced mobility frequency occurred in the inner zone, compared to the outer zone. No additional interaction effects were observed (Fig. 3F; Bonferroni *post hoc*s). When mobility state duration was compared by sex and zone, no main effect of Sex was observed ( $F(1,22) = 0.03, p = 0.86$ ). A main effect of Zone was observed ( $F(1,22) = 10,740, p < 0.0001$ ) and investigation of the data revealed reduced mobility state duration occurred in the inner zone, compared to the outer zone. A main effect of Mobility State was also observed ( $F(1,22) = 5553, p < 0.0001$ ) suggesting rats spend more time immobile than mobile during test phases, regardless of sex. A Zone by Mobility State interaction effect was also observed ( $F(1,22) = 4434, p < 0.0001$ ) and *post-hoc* analyses revealed reduced mobility ( $p < 0.05$ ) and immobility ( $p < 0.0001$ ) durations occurred in the inner versus outer zones. No additional interaction effects were observed (Fig. 3G; Bonferroni *post hoc*s). Thus, test exploration was consistent to sample phase behaviors, with both sexes showing comparable mobile and immobile behavior during OiP test phases.

Inner zone entrance frequency, dwell time, and latency were compared between males and females during repeated OiP test phases using unpaired t-tests. Comparisons revealed no main effects of Sex for

inner zone entrances ( $t(22) = 0.64, p = 0.53$ ) (Fig. 3H), dwell time ( $t(22) = 0.69, p = 0.50$ ) (Fig. 3I), or latency time ( $t(20^*) = 0.22, p = 0.43$ ) (\*note: one male and one female session were removed as statistical outliers; Fig. 3J).

#### 4. Discussion

We found sex differences in exploratory behaviors in the OiP test. During habituations, female rats moved farther and faster regardless of session (Fig. 1C) or zone (Fig. 1D-E), and spent less time immobile (Fig. 1G), compared to males. Interestingly, females increasingly entered the inner zone during habituations (Fig. 1H); however, inner zone duration (Fig. 1I) and latency (Fig. 1J) were similar between the sexes (Fig. 1I-J). During sample phases, male rats moved less in later sessions, whereas female distance travelled remained consistent from session to session (Fig. 2C). Furthermore, female rats again demonstrated increased distance travelled (Fig. 2D) and moved faster than male rats regardless of zone, particularly in the inner zones (Fig. 2E). No sex differences were observed during test phases (Fig. 3C-D), although increased female velocity remained consistent (Fig. 3E). Previously observed sex differences in mobility state and inner zone behaviors were also not observed during test phases, further suggesting that altered locomotor behaviors during habituation phases, and to a lesser degree sample phases, diminished during test phases.

Taken together, findings highlight nuanced behavioral differences between the sexes in 1-h OiP testing. Females reliably moved faster and farther than males, habituated slower (when compared by distance travelled), and spent less time immobile, which may be indicative of an anxiety-like phenotype. However, males and females showed comparable inner zone duration and latency throughout experiments, suggesting that enhanced female locomotion may indicate a unique difference in exploration strategy, yet insufficient as a performance or anxiety-like metric during recognition testing. While we hypothesized female rats would exhibit enhanced locomotor behavior and increasingly alternate between mobility states (e.g., darting-like behavior) during test phases, results suggest both sexes behaved similarly during test phases, specifically for object interactions, performance, and inner zone behavior. Of note, females exhibited increased test phase velocity, suggesting that speed and mobility state may be reliable metrics to observe behavioral sex differences in recognition tests.

Females often demonstrate a more circuitous, redundant search strategy compared to males; however, this strategy may represent an effective approach to minimize exposure to threats and predators [7,9,30]. This idea is supported by evidence highlighting that sex differences in time to complete a water-maze task, were eliminated by habituating animals to the environment beforehand [31]. Our findings are consistent with this observation (Fig. 1C inset: representative heat map) highlighting that, while females reliably moved faster and farther than males, differences did not predict test phase behaviors or 1-hour OiP performance. Thus, one approach to mitigate possible sex-dependent behaviors during OiP testing is to conduct thorough habituations. The inclusion of 7-day handling and habituations, three 10-min box habituations prior to the first test day, and additional 10-min habituations prior to each subsequent test session, may have effectively alleviated the confounding of threat- and stress-based exploration strategies with interpretation of performance. However, it is worth noting, even with extended habituations in the current design, female rats appear to accumulate information in a different manner than males, with increased locomotor and darting behavior, whereas males moved slower and less distance, including consistently less exploration in repeated sessions, compared to female rats. This might suggest female rats are increasingly engaged with their environment demonstrating divergent context-based motivational differences, opposed to cognitive capacity or recognition performance.

OiP performance is highly vulnerable to stress effects: previous data suggests infusion stressors are enough to impair OiP performance in

male and female rats, regardless of treatment [18]. Female rats appear to increasingly alternate between mobility and immobility states, while male rats appear increasingly methodical and adjust their behavior as test contexts become more familiar. Female rats appear to behave differently in 1-hour OiP testing, showing enhanced locomotion and consistent engagement with their environment across repeated test sessions (independent of anxiety-like behavior and cognitive capacity). Other researchers have reported sex differences in spatial learning and memory, highlighting a male advantage may emerge in hippocampus-dependent spatial learning tasks (e.g., radial arm maze), to encode allocentric spatial cues, whereas females increasingly rely on a striatum-based learning strategy to encode egocentric landmark cues. While these two strategies appear quite different, it is not immediately clear which approach would be most beneficial in recognition learning, particularly given the associative recognition requirements in the OiP test (e.g., visual-spatial), particularly integration between allocentric and egocentric perspectives. Thus, the presence of the black wall insert in these experiments may have provided an additional allocentric reference for promoting short-term visuospatial recognition and may help to alleviate reported sex-differences in the 1-h OiP task. Future research is necessary to further characterize how estrous phase and circulating sex hormones influence both behavior and cognition in male and female rats.

#### 5. Conclusion

This study compared male and female exploration behaviors in repeated 1-hour OiP test sessions. Despite comparable performance and object interaction time during OiP testing, these results confirm sex-dependent exploration strategies emerge during short-term OiP testing but they are not indicative of recognition performance or ability to discriminate between familiar and novel visuospatial associations. Females consistently moved faster and farther than males, demonstrated persistent locomotor activity between sessions, and spent less time immobile during all phases of OiP testing. While this may be indicative of enhanced anxiety-like behavior, female rats demonstrated comparable inner zone behaviors suggesting that differences in exploration may represent unique differences in search strategy, as opposed to a valid anxiety metric. Despite correctly predicting female rats would exhibit increased immobility and alternate between mobility states more, both sexes performed similarly in terms of recognition performance, object interactions, and inner zone behaviors during repeated OiP test phases. These observations suggest that, while female rats display a more circuitous and redundant search strategy that may be increasingly effective to mitigate environmental threats, these differences do not translate to differences in cognitive performance. Thorough habituations may help to alleviate sex-differences in performance, while persistent behavioral sex-differences may indicate different motivations between male and female rats when interacting with novel objects and environments.

#### Ethical Statement

Experiments were conducted in accordance with the standards of the Canadian Council on Animal Care and the University of Saskatchewan Research Ethics Board (AUP #: 20220041).

#### Declaration of Competing Interest

The authors have no competing interests to declare.

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### Data Availability

Data will be made available on request.

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