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Object Geometry and Passive Sitting Influence the Measurement of Discrimination in the Displaced Object Recognition Task

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ABSTRACT

Background: The Displaced object recognition (DOR) task is a widely used behavioral paradigm for evaluating spatial recognition memory in rodents. Standard protocols assume that any physical proximity to an object reflects active cognitive investigation; however, behavioral topographies such as passive sitting on objects may confound exploration metrics depending on object geometry.

Methods: Fourteen male C57BL/6 mice were tested in a DOR paradigm using either climb-resistant conical objects or flat-topped cylindrical objects. The discrimination index (DI) was quantified for both subjects using either raw or corrected (after mathematically subtracting the time that animal was passively sat on the subject) data.

Results: In familiarization and displacement-test phase, mean raw and corrected DI did not differ between cone and cylinder groups. However, comparing the raw data revealed a significant difference between familiarization and displacement test in the cone, but not cylindric group. Upon subtracting sitting time and calculating the corrected data, there was a significant difference between familiarization and displacement test in the cylindric group, too. There was a strongly and positively correlation between raw DI and the sitting time.

Conclusion: Object geometry significantly altered the behavioral repertoire of rodents. Flat-topped cylinder objects that facilitated passive sitting accounted for over 68% of the variance in raw DI during the test phase. Calculating the corrected data may unmask the real spatial memory in rodents.

Keywords: Object Geometry, Passive Sitting, Displaced Object Recognition Task.

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Introduction

One-trial spontaneous exploration tests exploit the rodent's innate drive to investigate novelty without requiring external reinforcement or aversive conditioning [1, 2]. Among these paradigms, the novel object recognition (NOR) and displaced object recognition (DOR) tasks are gold standards for assessing non-spatial and spatial memory, respectively [3, 4].

While the NOR task evaluates the capacity to discriminate between familiar and novel item features—relying heavily on perirhinal cortex circuitry [5, 6]—the DOR task specifically probes memory for spatial location, recruiting a distributed network that fundamentally integrates the dorsal hippocampus, medial prefrontal cortex, and parahippocampal structures [7–9].

In a typical DOR protocol, animals are exposed to two identical objects in fixed positions during a sample (familiarization) phase. Following a retention delay, one object is displaced to a novel location during the test phase [1, 10]. Spatial memory is operationally quantified via a discrimination index (DI), defined as the preferential exploration of the displaced object relative to total exploration time [3, 11]. Central to this paradigm is the assumption that proximity-based contact reflects active information gathering [4, 10]. Standard scoring protocols define exploration as pointing the nose toward the object at a distance ≤ 2 cm or touching it with the vibrissae or paws [1, 11].

However, the physical affordances of test stimuli are frequently overlooked [3, 4]. Flat-topped or horizontal surfaces allow rodents to mount, sit, or perch on the object [4]. If an animal perches on an object while grooming, scanning,

or resting, automated tracking systems and non-rigorous manual observers may record this time as active exploratory contact. This creates a risk of confounding motor, thigmotaxic, or postural behaviors with mnemonic performance [3, 10].

The primary objective of the present study was to systematically examine the methodological impact of passive sitting on DOR performance by comparing sharp-apex conical objects (which physically preclude sitting) against flat-topped cylindrical objects. We hypothesized that passive sitting on cylindrical stimuli creates an artifactual bias in raw discrimination scores, which obscures individual variance and generates misleading behavioral correlations.

Materials and Methods

Animals

Fourteen adult male C57BL/6 mice (body weight: 20–25 g; 3–4 months of age) were precured from the Tarbiat Modares University, Tehran, Iran. Animals were housed in standard polycarbonate cages in groups of 3–4 under controlled temperature (22± 2 C) and relative humidity (55± 10%) with an automated 12:12-h light/dark cycle (lights on at 07:00). Standard rodent chow and municipal tap water were provided *ad libitum*. All procedures complied with national and institutional ethical guidelines for animal care and use.

Apparatus and Objects

Testing was conducted in an open-field arena (50×50×35 cm) constructed of smooth, water-proof MDF with a non-reflective paint (Fig. 1). Two distinct object geometries were tested: 1) Cone group (n = 7): Heavy glass conical objects with a pointed apex (base diameter: 6 cm; height: 10 cm; steep slope) physically preventing the animal from mounting or sitting. 2) Cylinder group (n = 7): Heavy ceramic cylindrical objects with a flat circular top (diameter: 6 cm; height: 10 cm) permitting resting and perching postures.

Objects and the arena floor were cleaned thoroughly with 70% ethanol between trials to eliminate olfactory cues.

Behavioral Protocol

The DOR protocol consisted of four consecutive phases: a) Habituation: Mice explored the empty arena for 10 min daily over two consecutive days; b) Familiarization (Sample Phase): On day 3, two identical objects (either two cones or two cylinders) were positioned in adjacent corners (10 cm from the walls). The mouse was placed in the center and allowed to freely explore for 10 min; c) Retention Interval: The mouse was returned to its home cage for a 60-min inter-trial interval; d) Displacement test: One of the familiar objects was retained in its original position (stationary object, S), while the second object was relocated to the diagonally opposite corner (displaced object, D). The mouse was returned to the arena for a 5-min test session.

Behavioral Scoring and Definition

Video recordings were scored by trained observers. Measured parameters were as follows:

a) Active Exploration (T_{expl}): Directing the nose toward the object at a distance ≤2 cm or touching it with the snout/vibrissae. Chewing, rearing without directing attention toward the object, or looking over it from afar were excluded [1, 11].

b) Passive Sitting (T_{sit}): Placement of at least three paws on top of the object, resting the body trunk on the flat surface, or grooming while perched, accompanied by absence of vibrissal palpation or active orienting toward the object surface.

c) Raw Exploration (T_{raw}): Total contact duration including sitting time ($T_{expl} = T_{active} + T_{expl}$).

d) Corrected Exploration (T_{corr}): Active exploration strictly subtracting sitting episodes ($T_{corr} = T_{raw} – T_{sit}$).

e) The Discrimination Index (DI) was calculated for both raw and corrected durations:

$$DI = \frac{TD - TS}{TD + TS}$$

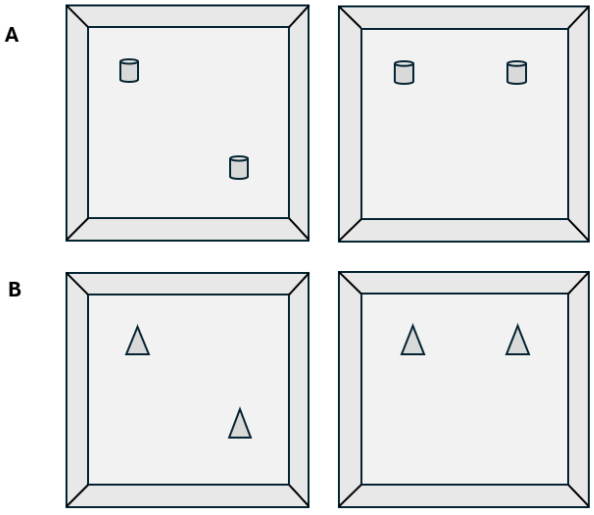


Figure 1: Comparison of Discrimination Index (DI) across Familiarization and Test phase for conical versus cylindrical objects. A: Cylinder Group, B: cone group

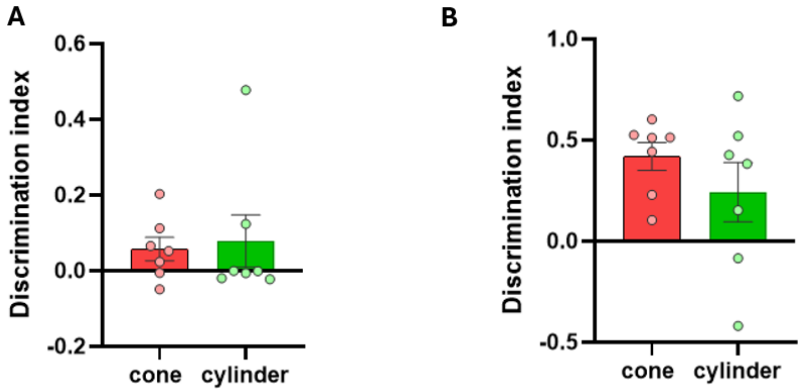


Figure 2: Comparison of Raw cone and cylinder Group. There was no significant difference between two groups.

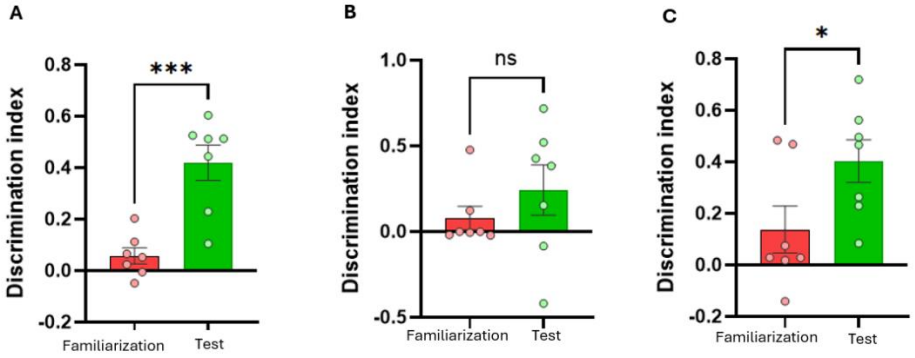


Figure 3: Spatial Discrimination Across Familiarization and Test Phases (A, B, C). C: after subtraction of sitting time.

In the test phase, TD represents the displaced object and TS the stationary object.

For the cylinder group, two sitting metrics were extracted:1) Total Sitting Percentage: ($T_{sit\text{-}total}$ / T_{raw_total}) × 100; 2) Differential Sitting Time (Δ Sitting): $T_{sit_displaced} - T_{sit_stationary}$

Statistical Analysis

Statistical evaluations were conducted in GraphPad Prism (version 10.4.1). Two-tailed unpaired Student's t-tests compared cone vs. cylinder groups across four primary outcomes: (1) raw familiarization DI, (2) raw test DI, (3) corrected familiarization DI, and (4) corrected test DI. Equality of variances was formally tested using the F-test. Relationships between sitting metrics and DI in the cylinder group were assessed using two-tailed Pearson correlation coefficients (r). Data was reported as mean ± SEM. Significance difference was set at $\alpha = 0.05$.

Results

During the familiarization phase, animals exhibited similar exploration among the two identical objects. Raw familiarization DI did not differ between the cone and the cylinder groups ($p = 0.7833$). Homogeneity of variance was preserved ($F(6,6) = 4.938$, $p = 0.0730$). Mean raw familiarization DI was 0.057 for the cone group and 0.08 for the cylinder group. The variance-ratio test was also non-significant, $F(6,6) = 4.938$, $p = 0.0730$.

In the test phase, raw discrimination scores showed a non-significant trend toward higher displacement discrimination in the cone group relative to the cylinder group. The group-mean comparison was non-significant ($p = 0.2997$) (Fig. 2).

To evaluate whether animals demonstrated intact spatial learning within their respective geometry conditions, paired analyses between familiarization and test phases were evaluated. In cone group, mice demonstrated marked spatial discrimination, with DI increasing significantly during the test phase ($p = 0.0010$). In cylinder group, raw metrics showed no significant difference between familiarization and test phases ($p = 0.2192$) (Fig. 3, A-B).

When passive sitting atop the cylinders were subtracted, mean corrected test DI remained non-significantly different between cone and cylinder

($p = 0.1911$). However, the variance-ratio F-test revealed significant variance heterogeneity between groups ($F(6,6) = 7.793$, $p = 0.0246$).

In the cylinder group, correcting for passive sitting resulted in a statistically significant increase in DI in test compared to familiarization phase ($p = 0.0245$). Individual trajectory inspection confirmed that passive sitting had masked the performance of multiple animals, whose corrected scores aligned with positive spatial preference (Fig. 3C).

To elucidate the mechanical driver of sitting-induced scoring distortion during the displacement-test phase, Pearson correlations were evaluated against raw DI. Total sitting duration did not correlate significantly with raw DI ($r^2 = 0.07243$, $p = 0.5595$). A strong, statistically significant positive correlation was observed between Δ 2-1 sitting time and raw DI ($r^2 = 0.6834$, $p = 0.0218$). Following correction for sitting, the correlation between sitting metrics and corrected DI was abolished ($p = 0.5458$).

Discussion

Spontaneous recognition tasks depend on the assumption that exploratory contact reflects active cognitive processing [1, 3, 10]. Our findings demonstrated that flat-topped object geometries violated this premise by affording passive perching and resting postures. In the cylinder group during the displacement test, raw discrimination was heavily biased by asymmetrical resting behavior: the difference in sitting duration between objects (Δ Sitting) accounted for over 68% of the variance in raw DI, whereas total cumulative sitting time showed no meaningful correlation. This indicated that the confounding artifact was not passive immobility per se, but rather an unprompted positional bias where animals preferentially perched atop one object over another.

A conventional, superficial analysis relying solely on uncorrected between-group comparisons would yield non-significant outcomes and erroneously conclude that object geometry exerts negligible influence on behavioral readouts. Such an interpretation is methodologically misleading. Within-subject evaluations reveal that while conical exploration yielded robust spatial discrimination, uncorrected cylinder exploration failed to reach significance. Crucially, once passive sitting bouts were subtracted, discrimination in the cylinder group emerged as statistically significant, accompanied by a notable expansion in effect size. Furthermore, significant variance heterogeneity confirmed that individual rodents adopted divergent behavioral repertoire when confronted with flat-topped stimuli. Several animals spent disproportionate durations atop the displaced object, artificially distorting raw indices and masking genuine underlying memory retention.

These findings present profound implications for neuropharmacological screening and lesion models targeting spatial memory circuitry, including hippocampal LTP modulation, scopolamine-induced amnesia, and transgenic neurodegenerative strains [2, 7, 8]. If an experimental intervention induces sedation, altered thigmotaxis, or stereotypic perching, flat-topped stimuli risk misattributing posture artifacts to cognitive enhancement or impairment. Automated tracking systems that rely strictly on centroid or head-snout coordinates within standard proximity zones (e.g., <2cm) are extraordinarily prone to registering passive sitting as high-fidelity exploration [4, 11]. Rigorous manual behavioral scoring or adopting geometrically inaccessible objects (such as cones) is imperative to preserve construct validity in spontaneous spatial recognition paradigms.

This study has several limitations. First, the sample size ($n = 7$ per group) was designed to evaluate acute methodological differences but possesses limited statistical power for detecting subtle cognitive effects. Second, while male C57BL/6 mice are the standard model in cognitive neuroscience [11], testing across both sexes and diverse strains (e.g., BALB/c) is required to establish generalizability. Finally, while sharp-apex cones eliminated sitting, other behavioral affordances (e.g., object gnawing or olfactory tracing) should be evaluated systematically.

Conclusion

Passive perching on flat-topped objects introduces substantial artifactual variance into the displaced object recognition task. Standard group-mean null-hypothesis testing fails to detect these artifacts, which can only be identified through individual animal tracking, correlation analysis, and variance tests. Researchers are strongly advised to select geometries that

physically preclude mounting—such as steep cones or domes—or to implement strict video-scoring criteria that explicitly deduct passive sitting from exploration scores.

Author Contributions Statement

R.F. performed experiments and writing manuscript, N.H. performed parts of experiments, MR.R. and A.Sh. and J.M-Z. designed the experiments; J. M-Z. Supervised, directed and managed the study; R.F. and J.M-Z. discussed the results and final approved of the manuscript.

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Data availability statement

All data supporting the results and analysis in this research will be available on request.

Ethical Approval

All animal experiments were approved by the ethical committee of Tarbiat Modares University (Ethical code: IR.MODARES.AEC.1402.052).

Consent for publication

All authors have read and approved the final of the manuscript. We are interested in publication of the present manuscript in Pathobiology Research.

Conflicts of Interest

The authors declare no conflicts of interest directly relevant to the content of this article.

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Artificial intelligence (AI)-assisted technologies

During the preparation of this work, the authors used ChatGPT to improve language, grammar, and readability. After using this tool, the authors reviewed, edited, and took full responsibility for the final content of the article.

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