Effect of Sex and Environmental Enrichment on Predator Odor Contextual Fear Conditioning and Extinction in Laboratory Mice

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Abstract

This study investigated the effects of sex and environmental enrichment (EE) on predator odor contextual fear conditioning and extinction in BALB/c mice. Forty adult mice (20 males, 20 females) were randomly assigned to four groups (n=10 each): male enrichment, female enrichment, male non-enrichment, and female non-enrichment. Enriched groups were housed in larger cages (60×40×20 cm) with tunnels and nesting materials, while non-enriched groups were housed in standard laboratory cages (30×20×15 cm). The experiment included a multi-day predator odor contextual fear conditioning paradigm. Days 1-3 consisted of a 10-minute familiarization session, a 10-minute predator odor exposure session (using cat feces), and a 10-minute conditioned context session. Extinction sessions (10 minutes each) were conducted on Days 4, 6, and 10. Behavioral responses (e.g., avoidance, freezing, locomotion, grooming) were assessed across all sessions. Results revealed that EE significantly reduced anxiety-related behaviors (e.g., hiding, freezing) and enhanced exploratory and grooming behaviors. Sex differences were also observed: male mice exhibited longer freezing and hiding durations, while females showed higher locomotor activity. Temporal patterns showed distinct dynamics in fear and exploratory behaviors, with EE facilitating adaptive coping mechanisms. These findings suggest that EE provides a protective effect against fear-related behaviors and that sex differences exist in the response to predator odor fear conditioning, highlighting the importance of considering both factors in preclinical anxiety research.

Keywords:

Enrichment; fear; conditioning; predator odor; sex difference; mice

1. Introduction

The ability to form, retain, and extinguish fear memories is essential for survival, enabling organisms to respond adaptively to threats. However, when fear responses are exaggerated or persist beyond the threatening context, they can contribute to debilitating anxiety disorders, including post-traumatic stress disorder (PTSD) ***(Correa, 2023; Lokshina et al., 2023)***. Animal models of fear conditioning have widely been used to understand neural mechanisms underlying fear acquisition and extinction, with implications for understanding human fear responses ***(Delgado et al., 2006)***. More recently, research has focused on predator odor contextual fear conditioning as a paradigm with unique ecological validity, mimicking natural predator-prey dynamics ***(Takahashi et al., 2008)***. This paradigm utilizes predator odors, which are rich in sulfur-containing compounds, as unconditioned stimuli. These odors elicit innate fear responses in rodents, such as freezing, avoidance, and risk assessment. These behavioral responses mirror core features of PTSD, providing a translational framework for investigating fear and anxiety-related pathologies ***(Blanchard et al., 2005; Wallace & Rosen, 2000)***.

Environmental enrichment (EE), which involves complex sensory, social, and cognitive stimulation, has emerged as a potential intervention for enhancing brain function and health. Studies have shown that EE improves cognitive functions, ameliorates depressive and anxiety-like behaviors, and may act as a neuroprotective strategy against neurodegenerative diseases ***(Liew et al., 2022)***. However, its effects on fear conditioning and extinction remain inconsistent. While some studies report that EE enhances fear memory and attentional processing of contextual cues, others suggest a reduction in overall anxiety-related behaviors ***(Barbelivien et al., 2006; Hegde et al., 2017; Mitra & Sapolsky, 2012)***. These contrasting findings underscore the need for a more nuanced understanding of the conditions under which EE influences fear responses.

In addition to environmental factors, biological variables such as sex significantly modulate fear learning and extinction. Hormonal differences, particularly the effects of estrogen and progesterone, are known to influence neural pathways associated with fear and anxiety ***(Milad et al., 2009)***. Females, for instance, often demonstrate superior extinction of conditioned fear compared to males, with estrogen enhancing extinction learning during certain phases of the estrous cycle ***(Chang et al., 2009; Milad et al., 2009)***. Despite these insights, the interaction between EE, sex, and predator odor fear conditioning has been underexplored, representing a critical gap in the literature.

This study investigates how environmental enrichment (EE) and sex differences influence predator odor fear conditioning and extinction in mice. The research hypothesizes that EE will reduce fear acquisition and enhance extinction, while female mice will show better fear extinction than males, particularly when enriched. The study aims to advance understanding of environmental and biological factors in fear learning, potentially informing individualized anxiety disorder treatments while emphasizing the importance of context-specific and sex-specific analyses in behavioral neuroscience.

2. Materials and Methods

2.1. Ethical considerations

All experimental procedures were conducted following the ethical standards approved by the Research Ethics Committee **(approval number: 2022055)** of the Faculty of Veterinary Medicine, Suez Canal University, Egypt. The study adhered to national and international laboratory animal care and welfare guidelines.

2.2. Animals and housing

Forty BALB/c albino mice (20 males, 20 females), aged 8-10 weeks (mean weight: 27 ± 3 g), were obtained from the Faculty of Pharmacy, Ain Shams University. The study was conducted at the animal laboratory facility of the Faculty of Veterinary Medicine, Suez Canal University, between October 22 and November 1, 2023.

Following a seven-day acclimation period, mice were randomly assigned to four experimental groups (n=10 per group): male enrichment (ME), female enrichment (FE), male non-enrichment (MN), and female non-enrichment (FN). Enrichment groups were housed in large cages (60 × 40 × 20 cm) equipped with environmental enrichment items (tunnels, shelters, and nesting materials), while non-enrichment groups occupied standard laboratory cages (30 × 20 × 15 cm) without enrichment.

Environmental conditions were maintained at 22 ± 2°C, 45-55% relative humidity, and 350 ± 30 Lux constant illumination with a 12-hour light/dark cycle. Food and water were provided ad libitum. Wood shavings served as bedding material and were replaced biweekly or as needed, with cage sanitization performed weekly.

2.3. Experimental apparatus

The behavioral testing apparatus **(Figure 1)** comprised a two-compartment Plexiglas® enclosure (75 × 37 × 40 cm). The main testing arena (60 × 37 × 40 cm) featured nine demarcated sections with black gridlines. An adjacent black Plexiglas® hide box is connected to the testing arena through a 6 × 6 cm access portal. Four identical apparatuses were equipped with ceiling-mounted HD video cameras (Hikvision, China) connected to a digital video recording device

positioned outside the testing room for live viewing and recording.

2.4. Predator odor preparation

Fresh fecal matter from a healthy one-year-old male domestic cat (Felis catus) was collected and divided into one-gram portions. The samples were frozen to maintain their integrity until use in the behavioral trials.

2.5. Behavioral testing procedures

Before experimental procedures, all mice underwent identical handling for five days between 10:00 and 17:00. Handling included daily weighing, one-minute holding periods, and transport to the testing room. Protective rubber gloves and metal forceps were used for all animal handling.

2.5.1 The behavioral assessment consisted of four sequential phases:

2.5.1.1. Familiarization Phase: Each mouse underwent a 10-minute habituation session in the testing apparatus without predator odor to establish baseline behavior and reduce novelty stress.

2.5.1.2. Predator Odor Exposure: Twenty-four hours after familiarization, mice were exposed to one gram of cat feces placed on filter paper in the testing arena for 10 minutes to induce contextual fear conditioning.

2.5.1.3. Contextual Fear Assessment: On day three, mice were returned to the apparatus for 10 minutes without predator odor to evaluate short-term memory and context-odor association retrieval.

2.5.1.4. Extinction Sessions: Extinction trials were conducted on days 4, 6, and 10, with each session lasting 10 minutes in the apparatus without predator odor.

2.6. Behavioral Analysis

Behavioral responses were recorded and analyzed using the Behavioral Observation Research Interactive Software (BORIS, v. 2.95, University of Torino)***(Friard & Gamba, 2016)***. Nine distinct behavioral parameters were quantified including avoidance, where mice spend most of their time near the square in front of the hide box ***(Dielenberg & McGregor, 2001)***; jumping, characterized by standing on their hind legs, raising their forelimbs, and leaping; and Concealing was observed when mice retreated into the hide box ***(Yin et al., 2011)***; while freezing described a state of immobility, except for respiration, with the body supported by all limbs in a prone posture ***(Blanchard et al., 2003)***. Grooming comprised a cephalocaudal sequence of forelimb strokes and body licks during cohesive grooming episodes aimed at body care ***(Berridge, 1990)***. Locomotion was noted as movement between defined sections, initiated by crossing lines ***(Dielenberg & McGregor, 2001)***. The head-out behavior involved mice positioning their head or both head and shoulders outside the entrance of the hide box while keeping most of the body concealed inside ***(Dielenberg & McGregor, 2001)*;** Lastly, vigilant

rearing was defined as standing on hind legs with raised forelimbs unsupported ***(Dielenberg & McGregor, 2001)***.

3. Statistical Analysis

Statistical analyses were performed using SPSS 27.0 (IBM Corp., USA). A mixed-design ANOVA was conducted with environmental enrichment (enriched vs. non-enriched) and sex (male vs. female) as between-subjects factors, and time (Days 1, 2, 3, 4, 6, and 10) as the within-subjects factor for nine behavioral parameters. Significant effects were analyzed using Bonferroni-corrected pairwise comparisons ($p < 0.05$).

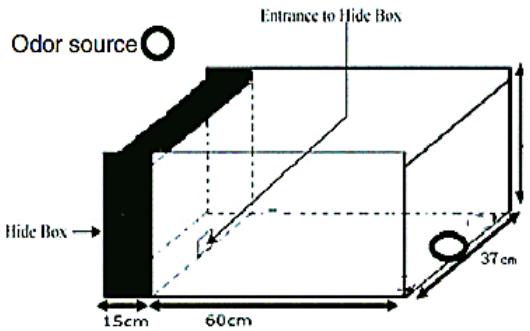

Figure (1): Testing apparatus

4. Results

A mixed-design ANOVA was conducted to explore the effects of sex (male vs. female) and treatment (enrichment vs. standard environment) on various behaviors (avoidance, freezing, grooming, hiding, head-out, jumping, locomotion, rearing, and vigilant rearing) measured over six days (Days 1, 2, 3, 4, 6, and 10).

4.1. Avoidance behavior(sec.)

Analysis of avoidance behavior revealed a significant main effect of the day ($F_{5,160} = 11.78$, $p < 0.001$, $\eta^2 = 0.27$), with the highest duration observed on day 2 (29.68 ± 2.78 sec) and the lowest on days 6 and 10 (12.97 ± 1.26 sec and 13.19 ± 1.40 sec, respectively)**(Figure 2)**. Treatment also significantly affected avoidance behavior ($F_{1,32} = 59.77$, $p < 0.001$, $\eta^2 = 0.65$), with mice in the standard environment showing significantly higher avoidance (25.57 ± 1.15 sec) compared to those in the enriched condition (12.98 ± 1.15 sec). A significant day × treatment interaction was observed ($F_{5,160} = 4.01$, $p = 0.006$, $\eta^2 = 0.11$). The highest avoidance in the standard environment group was recorded on day 2 (42.73 ± 3.93 sec), while the lowest in the enrichment group was observed on day 10 (10.31 ± 1.98 sec) **(Figure**

2).Neither the main effect of sex ($F_{1,32}$ = 4.05, p = 0.053, η^2 = 0.11) nor the day × sex interaction ($F_{5,160}$ = 1.10, p = 0.356, η^2 = 0.03) reached statistical significance. The three-way interaction (day × sex × treatment) was also non-significant ($F_{5,160}$ = 1.26, p = 0.293, η^2 = 0.04).

4.2. Freezing behavior(sec.)

Freezing behavior showed significant variation across days ($F_{5,160}$ = 17.56, p < 0.001, η^2 = 0.35), with the highest duration on day 4 (77.53 ± 7.00 sec) and the lowest on day 10 (19.34 ± 2.08 sec))**(Figure 3)**. The treatment had a significant effect on freezing ($F_{1,32}$ = 384.79, p < 0.001, η^2 = 0.92), with mice in the standard environment displaying significantly higher freezing durations (85.52 ± 2.63 sec) compared to those in the enriched condition (12.65 ± 2.63 sec.).A significant sex effect was also observed ($F_{1,32}$ = 10.88, p = 0.002, η^2 = 0.25), with males exhibiting longer freezing durations (55.21 ± 2.63 sec) than females (42.96 ± 2.63 sec).The day × treatment interaction was significant ($F_{5,160}$ = 21.45, p < 0.001, η^2 = 0.40). The highest freezing duration was recorded in the standard environment group on day 4 (148.50 ± 9.90 sec), while the lowest was observed in the enrichment group on the same day (6.56 ± 9.90 sec) **(Figure 3)**. Similarly, the sex × treatment interaction was significant ($F_{1,32}$ = 13.57, p = 0.001, η^2 = 0.30). Males in the standard environment exhibited more freezing (98.49 ± 3.72 sec) compared to those in the enrichment condition (11.93 ± 3.72 sec) **(Figure 4)**.Neither the day × sex interaction ($F_{5,160}$ = 1.50, p = 0.219, η^2 = 0.05) nor the day × sex × treatment interaction ($F_{5,160}$ = 1.55, p = 0.207, η^2 = 0.05) reached statistical significance.

4.3. Grooming behavior(sec.)

Grooming behavior varied significantly across days ($F_{5,160}$ = 6.37, p < 0.001, η^2 = 0.17), with the highest duration on day 4 (118.05 ± 7.06 sec) and the lowest on day 2 (74.01 ± 5.14 sec)**(Figure 5)**. Treatment significantly influenced grooming behavior ($F_{1,32}$ = 328.18, p < 0.001, η^2 = 0.91), with mice in the enriched environment grooming significantly more (157.85 ± 4.86 sec) than those in the standard condition (33.24 ± 4.86 sec). There was a significant difference between sexes ($F_{1,32}$ = 9.74, p = 0.004, η^2 = 0.23), with males showing higher grooming durations (106.28 ± 4.86 sec) compared to females (84.81 ± 4.86 sec).A significant day × treatment interaction was observed ($F_{5,160}$ = 8.11, p < 0.001, η^2 = 0.20). The highest grooming duration was recorded in the enrichment group on day 4 (207.36 ± 9.99 sec), while the lowest was observed in the standard cage group on day 6 (20.96 ± 14.63 sec) **(Figure 5)**. The sex × treatment interaction was also significant ($F_{1,32}$ = 5.28, p = 0.028, η^2 = 0.14). Males in the enrichment condition groomed significantly more (176.48 ± 6.88 sec) compared to males in the standard environment (36.07 ± 6.88

sec) **(Figure 6)**.Neither the day × sex interaction ($F_{5,160} = 1.12$, $p = 0.351$, $\eta^2 = 0.03$) nor the day × sex × treatment interaction ($F_{5,160} = 1.94$, $p = 0.108$, $\eta^2 = 0.06$) was statistically significant.

4.4. Head-out behavior(sec.)

Head-out behavior showed significant variation across days ($F_{5,160} = 7.34$, $p < 0.001$, $\eta^2 = 0.19$), with the highest duration on day 10 (45.74 ± 7.03 sec) and the lowest on day 3 (17.88 ± 1.48 sec)**(Figure 7).** Treatment significantly affected head-out behavior ($F_{1,32} = 7.16$, $p = 0.012$, $\eta^2 = 0.18$), with mice in the standard environment exhibiting more head-out activity (32.49 ± 2.44 sec) than those in the enriched condition (23.25 ± 2.44 sec).

A significant day × sex interaction was observed ($F_{5,160} = 3.98$, $p = 0.010$, $\eta^2 = 0.11$). The highest head-out behavior was recorded for females on day 10 (63.22 ± 9.94 sec) while the lowest was observed in males on day 3 (12.16 ± 2.09 sec) **(Figure 7).** The main effect of sex was not significant ($F_{1,32} = 3.76$, $p = 0.061$, $\eta^2 = 0.11$). Similarly, the day × treatment interaction ($F_{5,160} = 0.36$, $p = 0.788$, $\eta^2 = 0.01$), sex × treatment interaction ($F_{1,32} = 1.15$, $p = 0.293$, $\eta^2 = 0.04$), and day × sex × treatment interaction ($F_{5,160} = 1.39$, $p = 0.250$, $\eta^2 = 0.04$) did not reach statistical significance.

4.5. Hiding behavior (sec.)

Hiding behavior differed significantly across days ($F_{5,160} = 6.81$, $p < 0.001$, $\eta^2 = 0.18$), with the highest duration on day 4 (72.16 ± 6.42 sec) and the lowest on day 2 (33.85 ± 2.73 sec) **(Figure 8)**. Treatment significantly affected hiding behavior ($F_{1,32} = 66.68$, $p < 0.001$, $\eta^2 = 0.68$), with mice in the standard environment showing significantly more hiding (68.89 ± 3.04 sec) compared to those in the enrichment condition (33.77 ± 3.04 sec). A significant sex difference was observed ($F_{1,32} = 4.52$, $p = 0.041$, $\eta^2 = 0.12$), with males hiding more (55.90 ± 3.04 sec) than females (46.76 ± 3.04 sec).The day × treatment interaction was significant ($F_{5,160} = 4.84$, $p = 0.002$, $\eta^2 = 0.13$). The highest hiding duration was observed in the standard environment group on day 4 (107.35 ± 9.08 sec), while the lowest was recorded in the enrichment group on day 3 (24.18 ± 7.43 sec) **(Figure 8)**. The sex × treatment interaction was also significant ($F_{1,32} = 12.36$, $p = 0.001$, $\eta^2 = 0.28$). Males exhibited higher hiding behavior in the standard environment (81.02 ± 4.30 sec) compared to other conditions **(Figure 9)**. Neither the day × sex interaction ($F_{5,160} = 0.52$, $p = 0.704$, $\eta^2 = 0.02$) nor the day × sex × treatment interaction ($F_{5,160} = 1.45$, $p = 0.225$, $\eta^2 = 0.04$) reached statistical significance.

4.6. Jumping behavior

Jumping behavior varied significantly across days ($F_{5,160} = 14.27$, $p < 0.001$, $\eta^2 = 0.31$), with the highest duration on day 1 (21.78 ± 2.35 sec) and the lowest on day 4 (6.62 ± 0.72 sec) **(Figure 10)**. Treatment significantly affected

jumping behavior ($F_{1,32}$ = 4.39, p = 0.044, η^2 = 0.12), with mice in the standard environment showing significantly higher jumping durations (12.31 ± 0.69 sec) compared to those in the enrichment condition (10.26 ± 0.69 sec).The main effect of sex was not significant ($F_{1,32}$ = 3.10, p = 0.088, η^2 = 0.09). Similarly, the day × sex interaction ($F_{5,160}$ = 1.56, p = 0.206, η^2 = 0.05), day × treatment interaction ($F_{5,160}$ = 2.69, p = 0.053, η^2 = 0.08), sex × treatment interaction ($F_{1,32}$ = 0.00, p = 0.967, η^2 = 0.00), and day × sex × treatment interaction ($F_{5,160}$ = 0.38, p = 0.758, η^2 = 0.01) did not reach statistical significance.

4.7. Locomotion behavior(sec.)

Locomotion differed significantly across days ($F_{5,160}$ = 4.69, p = 0.004, η^2 = 0.13), with the highest duration on day 10 (221.79 ± 16.31 sec) and the lowest on day 4 (156.62 ± 11.04 sec) **(Figure 11)**.Treatment significantly affected locomotion behavior ($F_{1,32}$ = 77.65, p < 0.001, η^2 = 0.71), with mice in the enrichment condition exhibiting more locomotion (241.17 ± 8.52 sec) compared to those in the standard environment (135.02 ± 8.52 sec). A significant sex difference was observed ($F_{1,32}$ = 7.22, p = 0.011, η^2 = 0.18), with females showing higher locomotion (204.28 ± 8.52 sec) than males (171.91 ± 8.52 sec).The Sex × Treatment interaction was significant ($F_{1,32}$ = 5.40, p = 0.027, η^2 = 0.14). Males in the enrichment condition demonstrated higher locomotion (238.99 ± 12.05 sec) compared to males in the standard environment (104.83 ± 12.05 sec) **(Figure 12)**.Neither the day × treatment interaction ($F_{5,160}$ = 2.34, p = 0.079, η^2 = 0.07), day × sex interaction ($F_{5,160}$ = 0.75, p = 0.523, η^2 = 0.02), nor the day × sex × treatment interaction ($F_{5,160}$ = 0.36, p = 0.782, η^2 = 0.01) reached statistical significance.

4.8. Rearing behavior (sec.)

Rearing behavior displayed significant variations across days ($F_{5,160}$ = 7.95, p < 0.001, η^2 = 0.20), with the highest duration on day 3 (95.80 ± 11.16 sec) and the lowest on day 1 (50.10 ± 5.45 sec) **(Figure 13)**.Treatment significantly affected rearing behavior ($F_{1,32}$ = 36.08, p < 0.001, η^2 = 0.53), with mice in the standard environment exhibiting more rearing (90.89 ± 4.97 sec) compared to those in the enrichment condition (48.66 ± 4.97 sec).The main effect of sex was not significant ($F_{1,32}$ = 0.57, p = 0.455, η^2 = 0.02). Similarly, the day × sex interaction ($F_{5,160}$ = 2.03, p = 0.098, η^2 = 0.06), day × treatment interaction ($F_{5,160}$ = 2.43, p = 0.054, η^2 = 0.07), sex × treatment interaction ($F_{1,32}$ = 2.15, p = 0.152, η^2 = 0.06), and day × sex × treatment interaction ($F_{5,160}$ = 0.67, p = 0.605, η^2 = 0.02) did not reach statistical significance.

4.9. Vigilant rearing behavior (sec.)

Finally, vigilant rearing significantly differs between the

days ($F_{5,160}$ = 7.48, p < 0.001, η^2 = 0.19). The longest duration of vigilant rearing occurred on day 3, with an average of 41.57 ± 6.53 sec., while the shortest was recorded on day 6 (16.98 ± 1.71 sec.) (**Figure 14**). Additionally, the housing conditions significantly impacted vigilant rearing ($F_{1,32}$= 46.24, p < 0.001, η^2 = 0.59). Mice exposed to a standard environment displayed considerably more vigilant rearing (34.42 ± 1.68 sec.) than those in an enriched environment (18.31 ± 1.68 sec.). The results also showed that the main effect of sex was not significant ($F_{1,32}$= 2.94, p = 0.096, η^2 = 0.08) (24.336 ± 1.675 sec. for males, 28.397 ± 1.675 sec. for females). Furthermore, all the interaction combinations [day ×sex $F_{5, 160}$ = 1.43, p = 0.247, η^2 = 0.04), day ×treatment ($F_{(5,160)}$ = 1.50, p = 0.232, η^2 = 0.05) sex × treatment ($F_{1,32}$= 0.32, p = 0.577, η^2 = 0.01), and day ×sex × treatment ($F_{(5,160)}$ = 0.69, p = 0.504, η^2 = 0.02)] showed no significant influence of on vigilant rearing behavior (sec.)in mice.

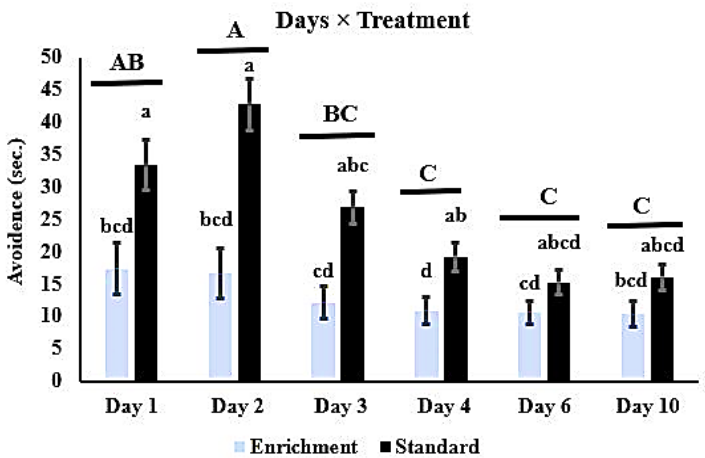

Figure (2): Mean of avoidance duration (sec.) in treatment groups over six days. Capital letters over the bars indicate significant differences between days ($p \leq 0.05$), while small letters indicate significant differences between treatments within the same day ($p \leq 0.05$).

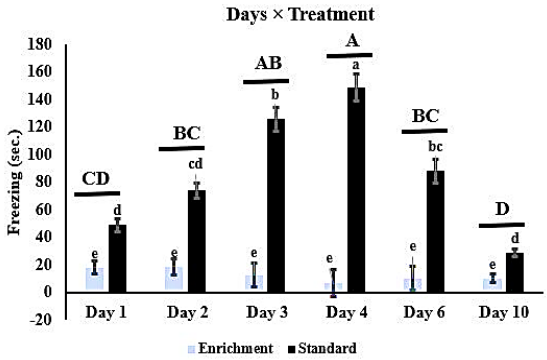

Figure (3): Mean of avoidance and freezing duration (sec.) in treatment groups over six days. Capital letters over the bars indicate significant differences between days ($p \leq 0.05$), while small letters indicate significant differences between treatments within the same day ($p \leq 0.05$).

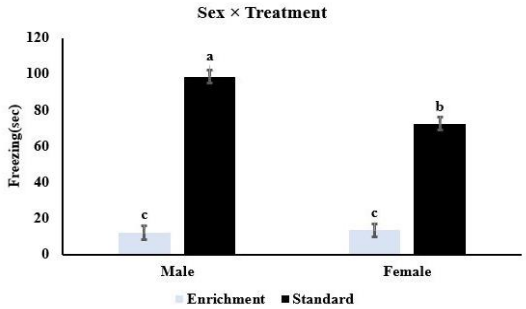

Figure (4): Mean of freezing duration (sec.) for males and females in treatment groups for freezing responses in mice. Different letters over the columns are significant at $p \leq 0.05$

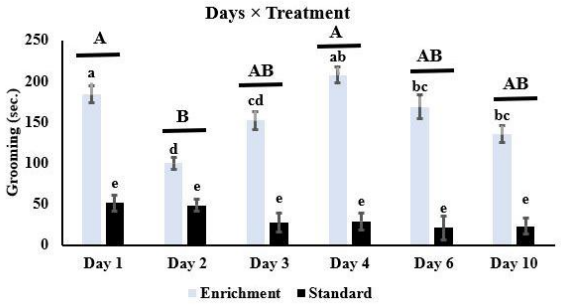

Figure (5): Mean of grooming duration (sec.) in treatment groups over six days. Capital letters over the bars indicate significant differences between days ($p \leq 0.05$), while small letters indicate significant differences between treatments within the same day ($p \leq 0.05$).

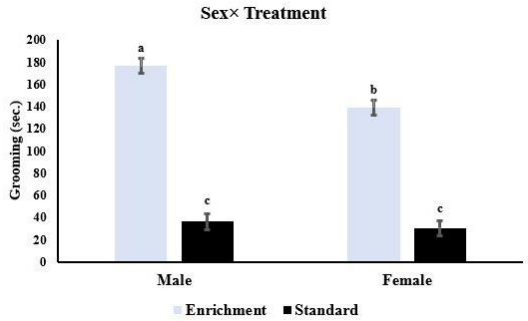

Figure (6): Mean of grooming duration (sec.) for males and females in treatment groups for grooming behavior in mice. Different letters over the columns are significant at $p \leq 0.05$.

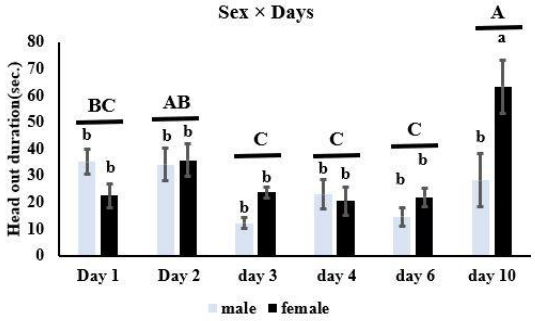

Figure (7): Mean of head-out duration (sec.) for males and females over six days for head-out responses in mice. Capital letters over the bars indicate significant differences between days ($p \leq 0.05$), while small letters indicate significant differences between treatments within days ($p \leq 0.05$).

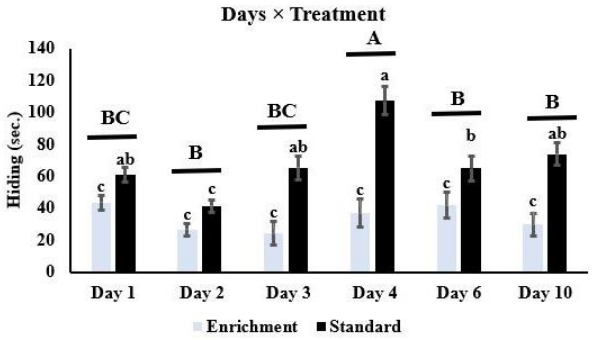

Figure (8): Mean of hiding duration (sec.) in treatment groups over six days. Capital letters over the bars indicate significant differences between days ($p \leq 0.05$), while small letters indicate significant differences between treatments within the same day ($p \leq 0.05$).

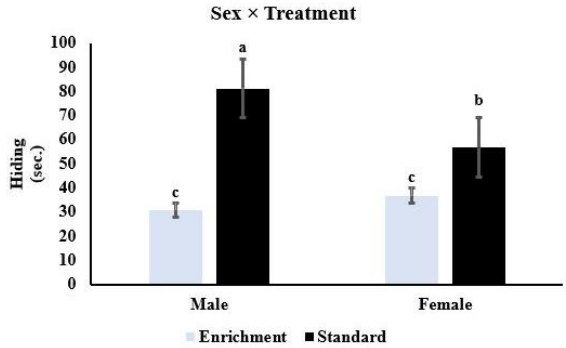

Figure (9): Mean of hiding duration (sec.) for sex treatment interaction for hiding responses in mice. Different letters over the columns are significant at $p \leq 0.05$

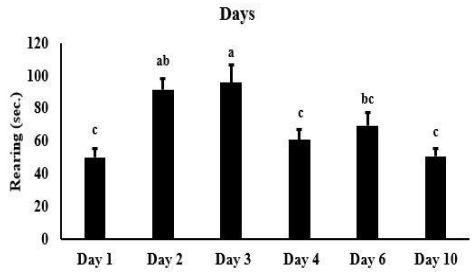

Figure (10): Mean of rearing duration (sec.) for sex treatment interaction for rearing responses in mice. Different letters over the columns are significant at $p \leq 0.05$.

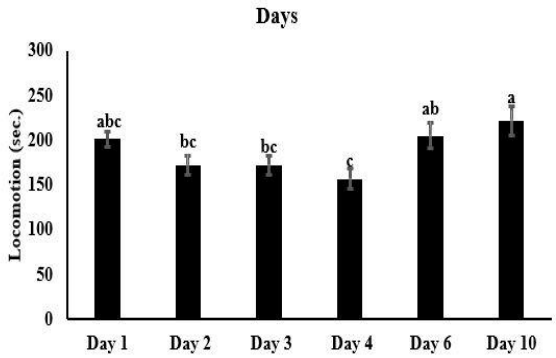

Figure (11): Mean of locomotion duration (sec.) over six days for locomotor behavior in mice. Different letters over the columns are significant at $p \leq 0.05$.

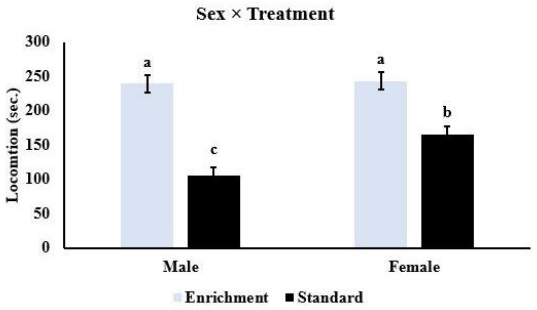

Figure (12): Mean of locomotion duration (sec.) for sex treatment interaction for locomotor behavior in mice. Different letters over the columns are significant at $p \leq 0.05$.

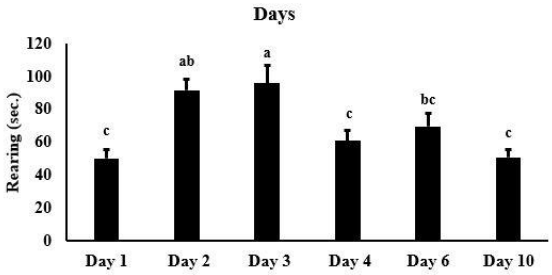

Figure (13): Mean of rearing duration (sec.) over six days for rearing responses in mice. Different letters over the columns are significant at $p \leq 0.05$

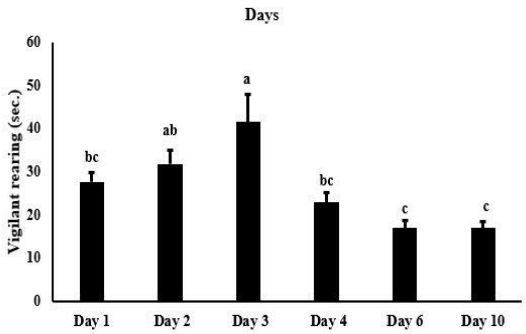

Figure (14): Mean of vigilant rearing duration (sec.) over six days for vigilant rearing responses in mice. Different letters over the columns are significant at $p \leq 0.05$.

5. Discussion

The current experiment revealed that environmental enrichment and sex may significantly influenced fear-related behaviors such as hiding, avoidance, jumping, and freezing in laboratory mice tested in the contextual fear conditioning paradigm. The standard-housed mice exhibited

higher levels of hiding, avoidance, jumping, and freezing than the enriched-housed (EH) counterparts. This enrichment-related anxiolytic effect suggests that enrichment creates a robust stress-buffering capacity that persists throughout the testing period ***(Fox et al., 2006)***. This supports the notion that the standard-housed mice showed elevated anxiety-like behaviors, such as increased concealing and avoidance, as they lack the stimulating landmarks present in EH environments ***(Benaroya-Milshtein et al., 2004; Sherwin & Olsson, 2004)***. Moreover, ***(Klein et al., 1994)*** proposed that enriched environments may enhance stress resilience by increasing familiarity with novel stimuli, thereby reducing anxiety during predator encounters. These findings emphasize the protective role of environmental enrichment against predator-induced stress. Moreover, our findings align with ***Novaes et al. (2021)*** who reported that environmental enrichment over two weeks increased coping mechanisms and facilitated the extinction of conditioned fear memory, with reduced freezing behavior.

The current findings highlighted the sex-based variations that male mice exhibited longer freezing and hiding durations than females. These sex-specific findings align with those who demonstrated that male rats exhibited significantly longer freezing and hiding durations than female rats in a contextual fear conditioning paradigm ***(Alexandrov et al., 2023; Russo & Parsons, 2021; Shanazz et al., 2022; Sturman et al., 2018)***. Similarly, ***Daviu et al. (2014)*** found that females show less freezing responses, but greater HPA axis activation than males in contextual fear conditioning**.** Moreover, other studies showed that males even when tested in a novel context are more-risk aversive and less exploratory than females ***(Johnston and File, 1991; Kokras and Dalla, 2014)*.**

However, our findings contrast with those who found females exhibit higher freezing behavior compared to males in the Pavlovian auditory fear conditioning paradigm ***(Borkar et al., 2019)***. The differences are because our study focused on innate fear (predator odor) and measured contextual defensive behaviors over days, while Borkar et al examined learned fear (an auditory cue) and emphasized freezing and flight responses within sessions. Regarding the lack of significant sex differences in avoidance and jumping, it aligns with those who found no significant differences between males and females in active avoidance conditioning in the performance of rats ***(Ribeiro et al., 2010; Rubio et al., 1999)*.**

The differing temporal dynamics for each fear-related behavior on day 2, avoidance is driven by the acute introduction of predator odor, prompting the mice to

escape the immediate threat ***(Hwa et al., 2018; Takahashi et al., 2005).*** By Day 4, in the absence of odor, the context has become associated with the predator threat through fear memory consolidation, this aligns with those who found that rats exposed to cat odor displayed increased freezing behavior upon re-exposure to the same context 48 hours later ***(Hubbard et al., 2004; Rodríguez et al., 2021)***.

Regarding jumping behavior, the results indicate that it varied significantly throughout the experiment, with the highest duration observed on Day 1. This pattern suggests an initial escape-driven response to the novel context on Day 1, reflecting heightened arousal and exploratory attempts to escape potential confinement.

Our study found clear differences in grooming behavior between males and females, with males exhibiting consistently longer grooming durations compared to females. This enhanced grooming response in males suggests sex-specific differences in stress-coping strategies and self-maintenance behaviors. These findings align with ***Reis-Silva et al. (2019)*** who demonstrated that male mice show increased grooming during both low and high-stress levels. Moreover, our findings align with those who reported enhanced rearing and grooming in male *mice* ***(Borkar et al., 2019)*** .

Environmental enrichment demonstrated a unique effect on grooming behavior compared to other behavioral categories, significantly enhancing grooming duration across all experimental days. This consistent increase suggests that enrichment promotes adaptive self-maintenance behaviors rather than suppressing them as seen with defensive responses. This aligns with ***Rojas-Carvajal et al. (2018)***, who reported that enriched environments enhance and increase grooming behavior, particularly body licking, in rats.

Grooming exhibited a unique temporal pattern distinct from that observed for fear-related behaviors. It reached its lowest point on Day 2, coinciding with the introduction of the predator odor, and peaked on Day 4, before gradually declining. This pattern suggests that grooming is not simply a reflexive response to an immediate threat but is rather modulated by a more complex interplay of factors, including stress levels, adaptation, and coping mechanisms. The initial decrease on Day 2 likely reflects predator odors have significant effects on mammalian prey species, including suppressed non-defensive behaviors such as grooming ***(Apfelbach et al., 2005).***

Other Studies on laboratory and wild rats exposed to predator odors reveal increased avoidance, freezing, and grooming behaviors ***(Storsberg et al., 2018).*** The subsequent increase in grooming behavior and peak on Day 4 might indicate the onset of a coping

response, where grooming serves as a self-maintaining behavior, helping to reduce anxiety and restore emotional homeostasis as animals begin to adapt to stressors. The gradual decline thereafter suggests habituation and a return to baseline grooming levels as the perceived threat diminishes and the environment becomes more familiar.

Our findings found that environmental enrichment increases locomotor activity, aligning with ***Nag et al. (2009)***. Moreover, ***Singhal et al. (2019)*** found that Short-term environmental enrichment (EE) (4 weeks) enhanced home cage locomotion, while long-term EE (6 months) decreased locomotion in novel environments. Furthermore, females consistently displayed higher locomotion than males, which aligns with the findings by ***Shanazz et al. (2022)*** who found that females display higher locomotion than males in the contextual fear conditioning paradigm due to an "anxioescapic" behavioral strategy.

Temporal effects were highly divergent across exploratory behaviors. Locomotion peaked by Day 10 when the mice underwent multiple extinction trials (Days 6 and 10). The absence of predator odor during these sessions likely reduced the fear association with the context. Exploratory behaviors like locomotion increase, reflecting reduced fear and greater confidence in the environment. Fearful animals often tend to reduce activity when placed in environments they are unfamiliar with. This behavior likely reflects a survival strategy shaped over time, where minimizing movement reduces the chances of detection by predators or other threats. Such adaptive responses highlight the tactical ways animals have evolved to navigate risk in their habitats ***(Baron, 1963)***. On the other hand***, Naert et al. (2011)*** observed that socially isolated mice displayed nocturnal hyperactivity and delayed extinction of fear responses, indicating a relationship between increased locomotor activity and heightened anxiety.

Our results showed that environmental enrichment uniformly reduced both rearing, vigilant rearing, and head-out behaviors, with mice in enriched environments contributing to reducing fear and anxiety by creating a sense of safety. This safety diminishes the need for heightened risk assessment and leads to a lower perception of threats, which helps minimize excessive vigilance ***(Rossi & Neubert, 2008)***. In standard conditions, mice exhibit longer durations of the previous behaviors because they lack the cognitive and emotional-buffering effects of enrichment, leading to heightened vigilance in assessing potential threats. Enrichment likely enhances resilience and reduces the perception of threat through increased neuroplasticity, better stress regulation, and enhanced coping

strategies, thereby diminishing the need for these defensive *behaviors* ***(McCreary & Metz, 2016).*** In contrast, sex differences were absent for all the previous behaviors, indicating a lack of sex-specific influence. The absence of sex differences in rearing, head-out, and vigilant rearing behaviors aligns with ***Augustsson et al. (2005)*** who found no general sex differences in behavioral strategies for risk assessment under the same testing conditions in mice. However, other studies found that male rats exhibit more risk-prone behaviors in novel environments and predator-odor contexts compared to females, who are more risk-averse and respond more adaptively to environmental changes ***(Jolles et al., 2015).***

Temporal analysis showed differing trajectories for risk assessment behaviors. Vigilant rearing peaked on Day 3, which indicates heightened fear memory retention and active environmental scanning during the memory consolidation phase. Head-out behavior subsequently elevated on Day 10, which represents exploratory re-engagement during the extinction and recovery phase as the fear response diminished as animals assess their environment for potential threats ***(Schiller et al., 2008; Malik et al., 2023)***.

In conclusion, this study demonstrates the significant impact of environmental enrichment and sex differences on fear-related behaviors in laboratory mice exposed to predator odor contextual fear conditioning. Environmental enrichment emerged as a crucial factor in reducing defensive behaviors like hiding, avoidance, jumping, and freezing while promoting adaptive responses such as grooming and increased locomotion - suggesting its role in developing stress resilience. Sex differences were evident in specific behaviors, with males showing greater freezing, hiding, and grooming durations, while females exhibited higher locomotor activity. The temporal analysis revealed distinct patterns across behaviors, with fear responses peaking during predator odor exposure and context re-exposure, while adaptive behaviors showed varying trajectories during the extinction phase. These findings underscore the complex interaction between environmental conditions and sex in shaping fear responses and stress adaptation in mice, highlighting the importance of considering both factors in behavioral research and animal welfare practices.

In conclusion, this study explored the influence of sex and environmental enrichment on predator odor contextual fear conditioning and extinction in laboratory mice. Employing an experimental design, 40 mice were divided into enriched or standard housing conditions, with their behavioral responses to predator odor exposure monitored across multiple sessions. The results

revealed that environmental enrichment significantly decreased fear-related behaviors, accelerated extinction, and fostered adaptive actions like grooming and locomotion. Sex differences emerged, with males showing longer freezing durations and females demonstrating increased locomotion, indicative of distinct stress-coping mechanisms.

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Conflict of interests

The authors declare they have no competing interests.

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تأثير الجنس والإثراء البيئي على التكيف والانطفاء للخوف المشروط برائحة المفترس في فئران التجارب

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الملخص

تبحث هذه الدراسة في تأثير الجنس والإثراء البيئي على استجابات الخوف المشروط برائحة المفترس ومدى انطفائه لدى فئران التجارب. شملت التجربة 40 فأرًا بالغًا (20 ذكرًا و20 أنثى) وُزّعوا على أربع مجموعات: مجموعتان تعيشان في بيئات مثرية تحتوي على مساحات أوسع وعناصر تحفيزية مثل الأنفاق والأجسام التفاعلية، وأخريان في بيئات قياسية بدون محفزات.

اعتمدت التجربة بروتوكولًا محكمًا لتكييف الخوف السياقي باستخدام رائحة المفترس، تلاه تقييم الاستجابات السلوكية خلال جلسات انطفاء متكررة. أظهرت النتائج أن الإثراء البيئي خفف من مظاهر القلق مثل التجمد والاختباء، بينما عزز سلوكيات التكيف كالحركة والتنظيف. كما لوحظت فروق واضحة بين الجنسين؛ إذ أظهرت الذكور معدلات تجمد واختباء أعلى، بينما سجلت الإناث مستويات أعلى من النشاط الحركي والاستكشاف.

تشير هذه النتائج إلى أن الإثراء البيئي قد يسهم في تحسين القدرة على التكيف مع الخوف وتقليل الاستجابات الدفاعية المفرطة، مما يسلط الضوء على أهمية العوامل البيئية والجنسية في فهم اضطرابات القلق قبل السريرية.