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Title: Comparing the Effects of Various Types of Chronic Psychological Stress on Locomotor Activity, Learning, Memory, and Anxiety-Like Behaviors in Rats

Running Title: Chronic Psychological Stress and Brain Functions.

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Abstract

Background: Psychological stress impairs cognitive performance and affects mood states. This study has compared the effect of four types of psychological stress (i.e., crowding, relocation, isolation, and restraint) on locomotor activity, learning, and memory, as well as anxiety-like behaviors performed by the open field, elevated plus maze, and passive avoidance tests (OFT, EPM, and PA).

Methods: Wistar rats were randomly assigned to different groups of crowding, relocation, isolation, and restraint stress, as well as control. The stress induction was administered for 21 consecutive days (6 hrs/day). To evaluate various types of behaviors, OFT, EPM, and PA tests were used.

Results: According to the PA test results, the latency to enter the darkroom decreased significantly in all stress groups, especially in the crowding and isolation stress groups. However, it had an inverse relationship with serum corticosterone levels. The total dark stay time increased significantly in the restraint and crowding stress groups, and also particularly, in the isolation stress group. In the isolation stress group, the number of darkroom entries decreased significantly. All stress groups spent a significantly shorter time in the open arms of the EPM apparatus. Finally, the total distance traveled, in the OFT was significantly lower in all stress groups, particularly in the isolation stress group.

Conclusion: Crowding and social isolation were the two stress types that had the most adverse effect on cognitive performance, as they induced stress-driven anxiety-like behaviors, probably due to increased corticosterone secretion. A high or low population of social density may create a condition, in which the nervous system could not efficiently manage stress, particularly at chronic levels.

Keywords: Stress, Learning, Memory, Corticosterone, Anxiety-like behaviors, Rat.

Introduction

Psychological stress is a common term to describe the processes that are thought to contribute to the onset and maintenance of various mental and physical conditions. These stress types enable higher-order brain structures to provide additional interpretation of perceived danger (Fuchs & Flügge, 2003). Additionall, they need cortical processing and depend on former experiences or current activations. Moreover, the psychological information would be assembled within limbic circuits (e.g., hippocampus, amygdala, and prefrontal cortex) to induce neuroendocrine and behavioral responses (Fuchs & Flügge, 2003). Therefore, it is a common daily occurrence in today's society that people experience various types of stress (Alkadhi, 2013). Stress activates the hypothalamic–pituitary–adrenal (HPA) axis and the secretion of glucocorticoids or corticosteroids in rodents like corticosterone (CORT) (Radahmadi et al., 2015), which can affect cognitive functions, such as learning, memory, and mood states (Abou-Hany et al., 2018; Khani et al., 2018). Psychological stress could be categorized based on timing and type (Dastgerdi et al., 2020; Radahmadi et al., 2017). The effect of stress on physiological and psychological procedures is recognized by its stimulation aspects (Crestani, 2016). Some brain structures, including the limbic system and amygdala, are involved in causing various types of stress (Herman et al., 2005). Therefore, stress may result in a variety of behavioral issues (Hodgson et al., 2004; Watson et al., 2005). Nowadays, the most prevalent stress types in different societies are crowding, relocation, isolation, and restraint stress (as emotional stress). Previous studies have reported that crowding, relocation, isolation, and restraint stress have destructive effects on physiological systems and behaviors (Chotiwat & Harris, 2006; Dastgerdi et al., 2017; Davenport et al., 2008; Eid et al., 2010; Hodgson et al., 2004; Watson et al., 2005). Social isolation stress has commonly coincided with anxiety-like behaviors, cognitive impairments, reduced social interactions, and weight loss (Qin et al., 2011). While stress has been indicated as beneficial or harmful to the neural health, in some cases it has had no effect on the neural health (Radahmadi et al., 2013). Therefore, stress may exhibit paradoxical effects on cognitive functions and behaviors (Schwabe & Wolf, 2013) depending on the secretion of stress hormones at different levels. Compared to physical stress, psychological stress is known to affect the physiological system more adversely due to the interactions of the limbic system. However, the most prevalent psychological stress types in society that lead to such adverse effects are not indicated. Hence, understanding the effects of various types of prevalent social stress on cognitive performance and behavior is important. The current study has investigated the effects of four major types of psychological stress (crowding, relocation, isolation, and restraint) on locomotor activity, learning, memory, anxiety-like behaviors, body weight differences (BWDs), serum CORT levels, and their correlations in rats.

Materials and methods

Experimental animals

Forty male Wistar rats, aged approximately 3 months (250–300 g weight), were obtained from the Isfahan Royan Institute for the experiments. Rats were housed in similar cages with 42×27×15 cm^3 dimensions (Tajhiz Gostar Omid Iranian Co., Tehran, Iran) under controlled conditions (light on 07:00–19:00, 50±5% humidity, and 23±2 °C room temperature) and were given *ad libitum* water. The study was approved by the Ethics Committee of Animal Use at the Isfahan University of Medical Sciences (IR.MUI.Research.REC.1399.677). After a 1-week adaptation period, the animals were randomly assigned to five equal groups (n=8) of control (Co), crowding stress (Cro-St), relocation stress (Rel-St), isolation stress (Iso-St), and restraint stress (Res-St). During the test period, rats in the Co group were handled similarly to those in the other groups. All behavioral tests were accomplished between 14:00 and 16:00 on Day 21 (fig. 1).

In this study, different behavioral tests, including open field (OFT), elevated plus maze (EPM), and passive avoidance (PA) tests were performed respectively. To investigate the lasting effects of exposure to chronic stress on different behavioral variables, 30-min intervals were given between behavioral tests on Day 21, after the last stress session. As shown in Figure 1, the passive avoidance test was performed as the last behavioral test in all experimental groups. Therefore, the received shock in the shuttle box did not affect the animals' anxiety-like behaviors. All measured parameters in the OFT and EPM tests had changed definitely due to the lasting effects of chronic stress.

[Figure 1]

Experimental procedures

Stress paradigm

Crowding stress was generated by increasing the population density in specified areas (Eid et al., 2010). In other words, inadequate space for the same number of subjects leads to crowding stress (Calhoun, 1973). Therefore, twice the original number of rats were placed in a normal cage (i.e., 8 instead of 4 rats) to increase the population density and generate crowding stress (Eid et al., 2010). During the relocation stress, the animals experienced environmental changes and displacement disturbances as the new condition was felt as a threat to their lives (Watson et al., 2005). Accordingly, the second group was relocated to a new cage with an unfamiliar conspecific to generate relocation stress. Also, to induce isolation stress, the animals were placed in different cages separately (individual housing) before being placed in their home cage (group housing). During the stress period, each rat was placed in a cage in isolated conditions without other mates (Khani et al., 2018; Song et al., 2021). Finally, in the last experimental stress group, the rats were placed in Plexiglas cylindrical restrainers to generate restraint stress (Adachi et al., 2021), commonly characterized by either physical or physiological types of stressful stimuli (Dastgerdi et al., 2017; Sunanda et al., 2000). All of these stress types were induced for 21 consecutive days (6 hrs/day, 08:00-14:00).

Behavioral paradigm

Passive avoidance test

The passive avoidance (PA) test was used to assess learning, memory, memory consolidation, and locomotor activity (Vohora et al., 2000). The PA apparatus (64×25×35 cm^3) contained two identical rooms (light and dark, 32×25×35 cm^3) with grid floors and a sliding door. A stimulator was used to administer electric shocks to the floor. The apparatus habituation impacts the performance of behavioral tasks negatively. The passive avoidance test includes three phases of habituation (Hab.PA), PA learning (PAL), and PA memory (PAM). Based on the common protocols in several studies, on Day 19, each rat was placed in the apparatus for 300 s (habituation) to diminish the novelty effects of the PA apparatus (Tatem et al., 2014). On Day 20, the rats were placed in the lightroom individually (PAL phase). The sliding door was raised after 10 s; as the rat fully entered the darkroom, this door was closed and a single electric shock (0.5 mA, 50 v, and 3 s; once) was delivered to the animal's foot (Huang et al., 2013). The initial latency (IL) to cross through the darkroom (the pre-shock latency) was recorded on Day 20. On the next day (Day 21), the PAM phase was performed and the latency of entrance to the darkroom was measured up to a maximum delay of 300 s. The memory experiment was terminated if the rat did not enter the darkroom within 300 s. If the rat avoided the darkroom entry and stayed in the lightroom, a positive response was recorded (Dastgerdi et al., 2018). The total dark stay (DS) time was attributed to memory consolidation or storage of new information (Dastgerdi et al., 2018). The number of entries to the darkroom was interpreted as locomotor activity (Vohora et al., 2000). Also, the difference between the IL and latency after a day was seen to be the occurrence of learning (Dastgerdi et al., 2018). The animal's ability to remember the foot shock was attributed to memory acquisition.

Elevated plus maze test

The elevated plus maze (EPM) test is commonly used to assess stress levels and anxiety-like behaviors (Walf & Frye, 2007). In this study, the EPM apparatus comprised a black opaque Plexiglas structure, elevated 70 cm above the ground. The apparatus consisted of two open arms (60 cm × 10 cm × 10 cm) and two closed arms (60 cm × 10 cm × 30 cm), extended from the central platform (10 cm × 10 cm). On Day 21, each animal was separately placed in the center of the EPM apparatus, facing the open arms. According to the EPM criteria for anxious behaviors, an expert recorded the number of open arm entries (OAE) and the total time spent in the open arms (OAT) within 300 s (Foldi et al., 2019). The following formulae were used to calculate the percentage of OAE (OAE%=[OAE/Total entries to the open and closed arms]×100) and OAT (OAT%=[OAT/300]×100) (Serafim et al., 2012).

Open field test (OFT)

Another experiment to assess mobility and anxiety-like behaviors is the open field test (OFT) (Hines & Minton, 2012). The OFT equipment consists of a box-shaped platform (90×90×60 cm^3), including painted grids that mark the floor with square crossings. In this experiment, the apparatus was placed in a silent room with no stressful stimulation. On Day 21, the rats were placed separately at the center of the device before the test. Their activities within 300 s were recorded by a mounted video camera that had tracking software (Ranjbar et al., 2017). Each animal was only tested once in this apparatus. The number of passages through the center of the platform and the total distance traveled on this platform were recorded as indices for anxiety-like behaviors and locomotor activity (Ranjbar et al., 2017). After each experiment, the rat was removed from the apparatus; then, the square was wiped with a cotton towel (soaked in 70% alcohol) to eliminate the odorant signals (Quillfeldt, 2016).

Determination of serum corticosterone levels

On Day 22, the rats were anesthetized with an intraperitoneal injection (i.p.) of urethane (1.5 g/kg; Sigma-Aldrich Chemical Co., USA), and then sacrificed between 16:00 to 17:00. The blood samples were taken from the animal's trunk. Subsequently, the serum was separated by centrifugation (6000 rpm, 20 min) to be stored at -80 °C until the analyses. The serum CORT levels were measured using a commercial enzyme-linked immunosorbent assay (ELISA) corticosterone kit (Zellbio Co., Germany). The detection limit for the rat CORT was set to 0.1–20 ng/mL and sensitivity at 0.05 ng/mL (coefficient of variation percentage [C.V.%] for the intra- and inter-assay was less than 10% and 12%, respectively).

Body weight differences

The body weight was measured on Day 1 and Day 21. The difference between the final and initial weight for each animal was calculated as body weight difference ($BWD=BW_{Final}-BW_{Initial}$).

Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple groups. In addition, the paired sample t-test was used to compare the IL and the latency after a day (within-groups). Using Pearson's correlation analysis (Coefficient of determination [R^2]), the correlation analyses of behavioral tests and BWDs with serum CORT levels were investigated. Furthermore, all data were estimated as means±SEM and a *p*-value less than 0.05 was considered statistically significant. The calculations were performed using the SPSS Statistics software v26.0 (IBM SPSS Inc., Chicago, USA).

Results

Effects of stress on the PA test

As shown in Figure 2A, IL had no significant difference in the experimental groups. The latency of entrance to the darkroom after a day was significantly lower in the Rel-St, Res-St, Cro-St, and Iso-St groups (respectively, $p<0.05$, $p<0.01$, $p<0.001$, and $p<0.001$) compared to the Co group (fig. 2B); thus, memory impairment occurred as a result of different stress types, particularly in the Cro-St and Iso-St groups. Also, the latency after a day in the Iso-St group was significantly lower ($p<0.01$) compared to the Rel-St group (fig. 2B).

[Figure 2]

IL and latency after a day were analyzed using a paired-sample t-test to evaluate the within-group latency changes. Significant differences were observed between IL and the latency after a day in all experimental groups (Co group: $p<0.001$; Rel-St, Res-St, and Cro-St groups: $p<0.01$; and Iso-St group: $p<0.05$). These results indicated the occurrence of learning in these groups (fig. 3). However, the lowest and highest degrees of learning occurred in the Iso-St and Co groups, respectively.

[Figure 3]

The total DS time was significantly higher in the Res-St, Cro-St, and Iso-St groups ($p<0.05$, $p<0.01$, and $p<0.001$, respectively) In comparison with the Co group; however, compared to the Rel-St group, it was significantly higher only in the Iso-St group ($p<0.05$) (fig. 4A).

The number of entries to the darkroom had no significant differences in the Rel-St, Res-St, and Cro-St groups compared to the Co group. However, it was significantly lower in the Iso-St group ($p<0.05$) compared to the Co group. These results suggested locomotor activity reduction in the PA apparatus due to isolation stress (fig. 4B).

[Figure 4]

Effects of stress on the EPM test

The OAE% decreased significantly in all stress groups (Rel-St group: $p<0.05$; other stress groups: $p<0.001$) compared to the Co group. Furthermore, OAE% had significant decreases in the Cro-St and Iso-St groups (both $p<0.001$) compared to the Rel-St group. Similarly, OAE% was significantly lower in the Cro-St and Iso-St groups (both $p<0.05$) compared to the Res-St group (fig. 5A).

A significant reduction of OAT% was observed in all stress groups (Rel-St group: $p<0.05$; other stress groups: $p<0.001$) compared to the Co group. Furthermore, OAE% in the Cro-St and Iso-St groups showed significant decreases (both $p<0.01$) compared to the Rel-St group (fig. 5B).

[Figure 5]

Effects of stress on the OFT

The number of entries to the platform's center was significantly lower in the Cro-St and Iso-St groups (both $p<0.01$) compared to the Co group (fig. 6A). Also, this value had a significant decrease in the Cro-St and Iso-St groups (both $p<0.01$) in comparison with the Rel-St group (fig. 6A), although it was significantly lower in the Cro-St and Iso-St groups (both $p<0.05$) compared to the Res-St group.

The time spent in the central area of the OFT platform showed a significant decrease only in the Iso-St ($p<0.05$) compared to the Co group (fig. 6B).

A significant decrease in the total distance traveled was observed in all stress groups (Res-St, Rel-St, and Cro-St groups: $p<0.01$; Iso-St group: $p<0.001$) compared to the Co group (fig. 6C). However, the highest decrease in exploration activities was observed in the Iso-St group in comparison with other stress groups.

[Figure 6]

Effects of stress on serum CORT levels

Serum CORT levels increased in all stress groups (Rel-St group: $p<0.05$; Res-St group: $p<0.01$; Cro-St and Iso-St groups: $p<0.001$) compared to the Co group. Furthermore, the serum CORT levels showed significant enhancement in the Iso-St group ($p<0.05$) compared to the Rel-St group (fig. 7).

[Figure 7]

Effects of stress on BWDs

Compared to the Co group, the BWDs significantly declined in all stress groups (Rel-St group: $p<0.05$; other stress groups: $p<0.01$) in comparison with the Co group (fig. 8).

[Figure 8]

Correlation analyses of behavioral tests and BWDs with serum CORT levels

In the correlation analysis of the PA test data, the latency after a day exhibited no significant correlation with serum CORT levels in the Co group (R^2=0.2327). However, they had significant negative correlations in the Rel-St, Res-St, Cro-St, and Iso-St groups (respectively, R^2=0.5934, 0.2619, 0.5945, and 0.6616; p<0.05) (fig. 9A). These findings supported the proposition that the serum CORT levels should be involved in the memory impairment in the Cro-St and Iso-St groups, as per the PA test.

In the correlation analysis of the EPM test data, the OAE% presented no significant correlation with serum corticosterone levels in the Co group (R^2=0.983). However, they had significant negative correlations in the Rel-St, Res-St, and Cro-St groups (respectively, R^2=0.5556, 0.2220, 0.5945, and 0.5199; p<0.05) and Iso-St group (R^2=0.775; p<0.01) (fig. 9B). These findings supported the proposition that serum CORT levels should be involved in anxiety-like behaviors in all stress groups, as per the EPM test.

In the correlation analysis of the OFT data, the total distance traveled revealed no significant correlation with serum CORT levels in the Co group (R^2=0.424). As seen in Figure 9C, there were significant negative correlations in the Rel-St, Res-St, and Cro-St groups (R^2=0.6278, and R^2=0.7398 and R^2=0.6848 respectively; $p<0.05$ in all) and Iso-St group (R^2=0.8129; $p<0.01$). These findings supported the proposition that serum CORT levels should be involved in locomotor activity in all stress groups, as per the OFT.

Another correlation analysis between the BWD data and serum CORT levels exhibited no significant correlation in the Co group (R^2=0.3242). However, significant negative correlations were observed in the Rel-St, Res-St, Cro-St, and Iso-St groups (respectively, R^2=0.6815, 0.383, 0.5544, and 0.6614; $p<0.05$) (fig. 9D). These findings supported the proposition of serum CORT levels should be involved in the body weight loss under stress.

[Figure 9]

Discussion

This study investigated the effects of four major types of psychological stress, crowding, relocation, isolation, and restraint (emotional stress), on learning, memory, memory consolidation, locomotor activity, anxiety-like behaviors, and BWDs in rats, as well as the correlations between their results.

According to the present PA data, learning occurred at different levels in all experimental groups. However, the lowest degree of learning occurred under the isolation stress. In line with current findings, learning was reported to have occurred under stress (Dastgerdi et al., 2018); that is because stress influences the onset and intensity of learning as a cognitive brain function (Rafah

Sami, 2009). Although stress did not impede learning, it impaired brain performance by blocking the changes regarding habit memory formation (Schwabe et al., 2010). The impact of stress on learning has nevertheless been controversial. Based on different studies, stress either adversely or positively affected learning, or did not affect it at all depending on various high and low ranges of stress curve (i.e., distress and eustress, respectively) (Joëls et al., 2006; Rudland et al., 2020; Salehi et al., 2010). Moreover, stress altered the equilibrium between multiple underlying systems involved in learning and memory (Vogel & Schwabe, 2016).

According to other PA findings, not only memory was impaired in all chronic stress conditions, but also memory consolidation was impaired by restraint, crowding, and especially isolation stress. Further findings related to the hormonal levels indicate that stress-driven memory deficits mainly occurred because of the changes in the CORT levels. Notably, the correlations between our findings in the PA test with the serum CORT levels verify this proposition. Previous studies have stated chronic stress as an inevitable phenomenon that has impaired memory through the secretion of stress hormones (e.g., corticosterone) and other neurochemical factors (Jeong et al., 2006; Sandi & Pinelo-Nava, 2007; Sunanda et al., 2000). In addition, the comparison between previous studies demonstrated that isolation stress was more destructive to memory processing compared to restraint stress (Hosseini Dastgerdi et al., 2021; Khani et al., 2022). Other findings in this study suggested that cognitive performance strongly corresponds with social density (the average conspecific encounter rate in an animal population). A research study indicated that social density was influenced by the physical area and availability of resources in the habitat (Love & Zelikowsky, 2020). In humans and rodents, social stress could be triggered by interpersonal encounters, arguments, and fights (Love & Zelikowsky, 2020). In comparison to moderate levels of social density, extremely low or high levels of social density create a situation, in which the nervous system may not efficiently handle stress, especially chronic stress. With the increase in population, the behavior of individuals changes. Crowding increases the stress level as the competition for limited resources exacerbates and leads to increased aggression (Agrell et al., 1995).

According to the result of the PA test, locomotor activity was decreased in the subjects enduring isolation stress. However, the effect of stress on locomotor activity remains paradoxical as reduced (Sestakova et al., 2013) or increased locomotor activity (Weiss et al., 2000) is discussed in various studies. In these studies, besides behavioral assessment methods, stress duration and type seem to have influenced the results concerning locomotor activity as well (Ranjbar et al., 2016). Along with altered secretion of hormones like glucocorticoids (Miranda & Oliveira, 2015), different mechanisms might be involved in stress-related memory impairment and behavioral changes. This includes the secretion of neurotransmitters (serotonin, dopamine, and norepinephrine) (Brenes et al., 2008; Dalesman & Lukowiak, 2011) and brain morphological changes (reduced expression of new neurons, synaptic proteins, dendritic density and length of neurons) (Bianchi et al., 2006).

The findings of the EPM test showed a significant reduction in the time spent in the EPM open arm and the number of entries to the open arm in all stress groups. The crowding and isolation stress increased anxiety-like behaviors more than other types of stress did. Another study demonstrated that animals' behaviors on the EPM platform were influenced by the stress type (Nazeri et al., 2017). In the present study, the increased serum CORT levels seemed to elevate anxiety-like behaviors. In line with other studies, the correlations between EPM findings and serum CORT levels confirmed that corticosterone has an influential role in causing anxiety-like behaviors. As such, longer and continuous periods of social isolation induce a cascade of negative behaviors in animal models, humans, and neural mechanisms, facilitating this shift (Love & Zelikowsky, 2020). The network organization of structural connectomes will begin to differ in stress conditions related to social isolation. For instance, some measures of the network structure, such as modularity (i.e., the strength of network division into modules) and small-worldness (the degree a network could be cluster-organized) decrease, indicating greater homogeneous connections (Liu et al., 2016). These changes depend on the outcome of the disrupted inter-hemispheric and inter-modular connections in the dorsolateral orbitofrontal cortex (Liu et al., 2016). Other studies have confirmed the association of social isolation with decreased myelination, altered dendritic development, decreased plasticity in the prefrontal cortex (Makinodan et al., 2012; Medendorp et al., 2018), and changes in the prefrontal cortex connectivity (Hermes et al., 2011), in particular concerning the prefrontal cortex-amygdala circuit (Castillo-Gómez et al., 2017). Also, the serotonergic fiber density in the inferior colliculus is a factor that is reduced by developmental isolation (Keesom et al., 2017). Moreover, chronic isolation stress induces a phenotype with similar aspects to anxiety, depression, and social withdrawal in adult rodents (Ieraci et al., 2016; Liu et al., 2012; Scaccianoce et al., 2006).

Based on the present OFT findings, the exploration activity was decreased in the subjects of the Iso-St and Cro-St groups that exhibited anxiety-like behaviors. Therefore, isolation and crowding stress types were more destructive than the other stress groups in this study. The locomotor activity decreased in all stress groups, especially in the Iso-St group. The changes in locomotor activity on the OFT platform were related to the serum CORT levels and were accordingly confirmed by the correlation analysis of the OFT findings with serum CORT. Another study reported that crowded housing (for mice) reduced exploration, locomotor activity, and anxiety-like behaviors in the OFT and EPM tests (Reiss et al., 2007). Furthermore, environmental factors seem to affect the expression of behavioral phenotypes. Therefore, social housing, as a stress factor, could affect psychological reactivity significantly. Also, there were locomotor activity differences in response to various stress types in the OFT and PA tests. Some behaviors, like locomotor activity, originate from certain brain areas; thus, their evaluation requires specific behavioral methods. In other words, specific behavioral tests should be considered for different behavioral assessments. Moreover, the open field is more specific test for evaluation of passive avoidance test.

Based on current findings regarding the hormonal changes, different stress types, particularly crowding and isolation stress, increased serum corticosterone levels more significantly than other stress types. According to previous studies, adrenal gland weight increased with the greater population density, indicating a probable increase in adrenal function (Love & Zelikowsky, 2020). In addition, isolation, restraint, relocation, and crowding stress increased CORT levels in rats (Djordjevic et al., 2003; Khani et al., 2018; Radahmadi et al., 2020). In another study, isolation stress strongly affected behavior but did not enhance plasma corticosterone levels, which were induced by other stressors (Scaccianoce et al., 2006). These differences might be related to the methodology, age, gender, physical area, as well as stress duration and type (Radahmadi et al., 2017; Ranjbar et al., 2016). It is critical to determine the time when social isolation level and duration begin to have detrimental effects on the subject (Love & Zelikowsky, 2020). The role of other variables, such as the group size and housing duration, should be considered as well (Van Loo et al., 2001).

According to BWD findings, all types of chronic stress decreased body weight gain significantly. A relationship between weight changes and corticosterone levels should be well noted as the BWD findings and serum CORT correlation confirmed it. It is noteworthy that some metabolic processes are mediated by glucocorticoids; thus, psychological stress could lead to body weight loss (Qin et al., 2011; van der Kooij et al., 2018). However, it was previously indicated that crowding increased adiposity without weight gain (Lin et al., 2015). Concerning stress-related body weight loss, a study has reported that epinephrine and norepinephrine stimulated hormone-sensitive lipase, whereas cortisol increased lipid cell sensitivity to epinephrine and norepinephrine (Lafontan & Langin, 2009). The secretion of corticotrophin-releasing hormones due to stress could decrease food ingestion and body weight (Heinrichs et al., 2001). The BWDs were related to stress exposure (Ranjbar et al., 2016). In another research study, various types of chronic stress, except chronic restraint stress, induced body weight loss because of the stress exposure (Marin et al., 2007). Finally, the potential effects of stress caused by social density could be highlighted as a neuroendocrine stress response, regulated or deregulated by the HPA-axis, as well as behavioral and neural alterations that are either primary or secondary responses to psychogenic stress (Love & Zelikowsky, 2020). However, understanding those brain mechanisms concerning chronic social stress that have such subserving adaptive functions should be of primary concern. This is because social stress is the major cause of stress stimuli in humans that lead to psychopathology. However, further cellular, biochemical, and structural research is needed to explain its underlying physiological mechanisms.

Conclusion

Overall, learning occurred at different levels in all experimental groups although the lowest level of learning occurred under isolation stress conditions. The crowding and isolation stress, as two models of social density stress, had further destructive effects on the impairment of cognitive functions in comparison with the relocation and emotional stress. As such, these stress models seem to severely impair learning, memory, memory consolidation, locomotor activity, and body weight. The crowding and isolation stress increased anxiety-like behaviors and serum corticosterone levels more than other types of stress, (i.e., relocation and restraint stress). Thus, stress, which was caused by social density (housing density: crowding and spatial isolation), led to the most negative effects on memory and mood, probably due to different corticosterone levels, as the main stress hormone. Finally, high or low populations of social density may create a condition, in which the nervous system could not efficiently handle stress, at chronic levels in particular.

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Conflict of interest
The authors declare no conflict of interest.

Data availability
The authors confirm that all data supporting the findings in this study are included in the graphs. Other analyzed data during the study are available from the corresponding author upon reasonable request.

Ethical Statement
All experiments were approved by the Research and Ethics Committee of Isfahan University of Medical Sciences (IR.MUI.Research.REC.1399.677).

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Contributors/Credit author statement
MR designed the study, conducted research, and supplied research materials. In addition to performing the experiments, *HA* collected and organized the data. Subsequently, *MR* analyzed and interpreted the data. *HA, MR, RK* and *HA* wrote the first and final drafts. *MR* and *RK* provided logistic support. All authors critically reviewed the final version, and remain responsible for the content and similarity index.

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Figures and legends

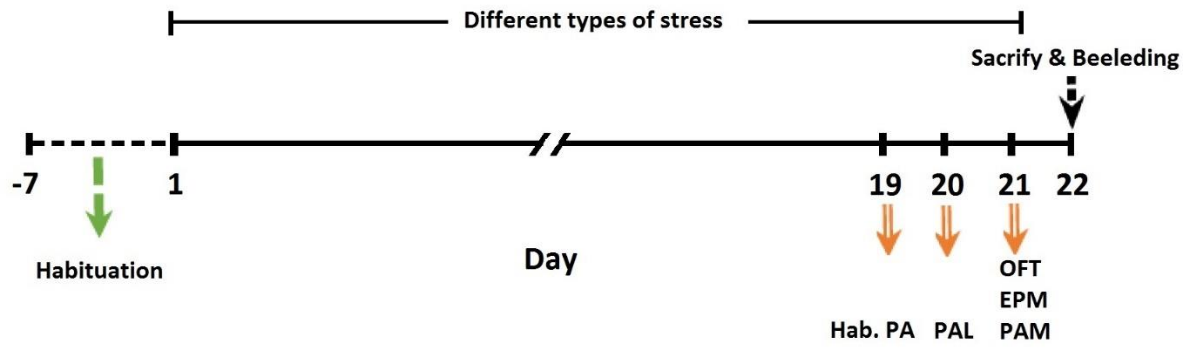

Figure 1: Timeline of all experimental groups. Animals were exposed to different stress types (relocation, restraint, crowding, and isolation stress). Different behavioral tests were respectively open field (OFT), elevated plus maze (EPM), and passive avoidance (PA), with an interval of 30 mins between each on Day 21 (after the last stress session).

Hab.PA: Habituation phase in passive avoidance test; **PAL:** Passive avoidance learning phase; **PAM:** Passive avoidance memory phase.

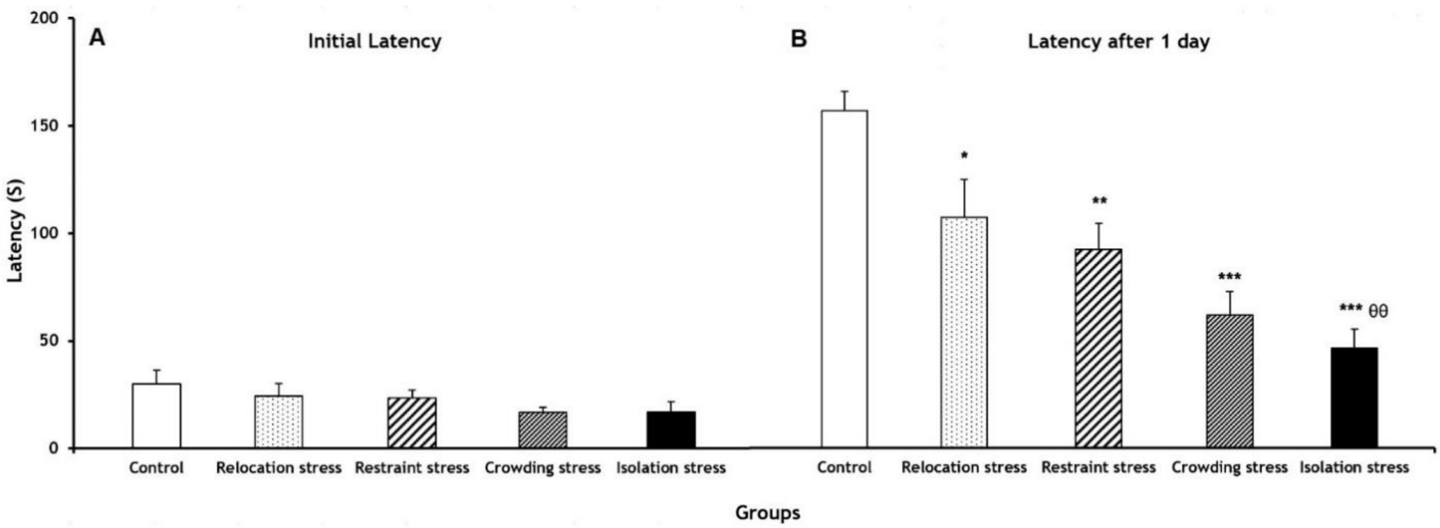

Figure 2: Darkroom entries **A)** initial latency (IL) and **B)** latency after a day in the PA apparatus for all groups (n=8) before and after receiving the foot shock. Results are expressed as means±SEM (One-way ANOVA, followed by Tukey's post-hoc test).

$^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ compared to the Control (Co) group; $^{\theta\theta}p<0.01$ compared to the Relocation Stress (Rel-St) group.

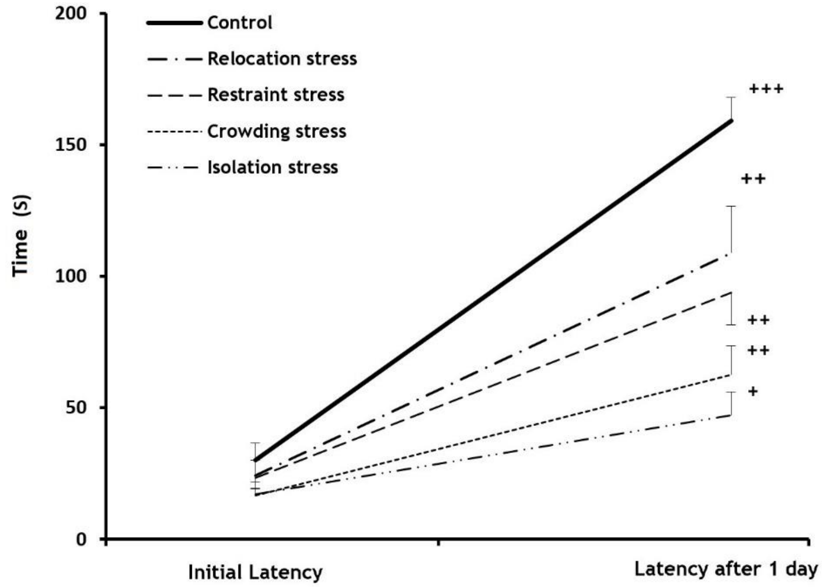

Figure 3: Initial latency (IL) and latency after a day in the PA apparatus before and after the foot shock (within-groups; n=8). Results are expressed as means±SEM (paired sample t-test).

$^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$ initial latency relative to latency after a day.

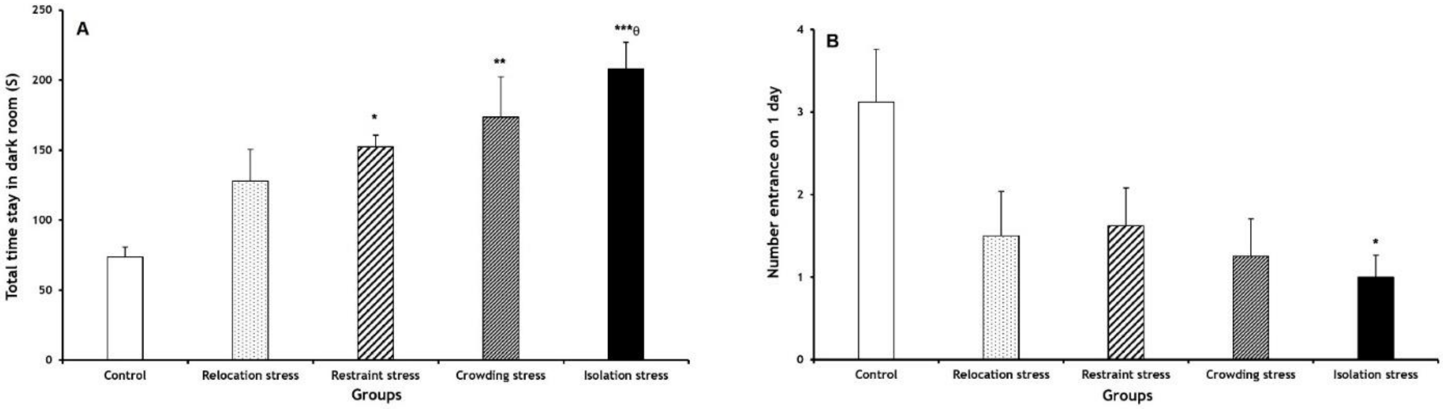

Figure 4: A) The total dark stay (DS) time and **B)** number of entries to the darkroom after a day in the PA apparatus for all groups (n=8). Results are expressed as means±SEM (One-way ANOVA, followed by Tukey's post-hoc test).

[*]p<0.05, [**]p<0.01, and [***]p<0.001 compared to the Control (Co) group; [Θ]p<0.05 compared to the Relocation Stress (Rel-St) group.

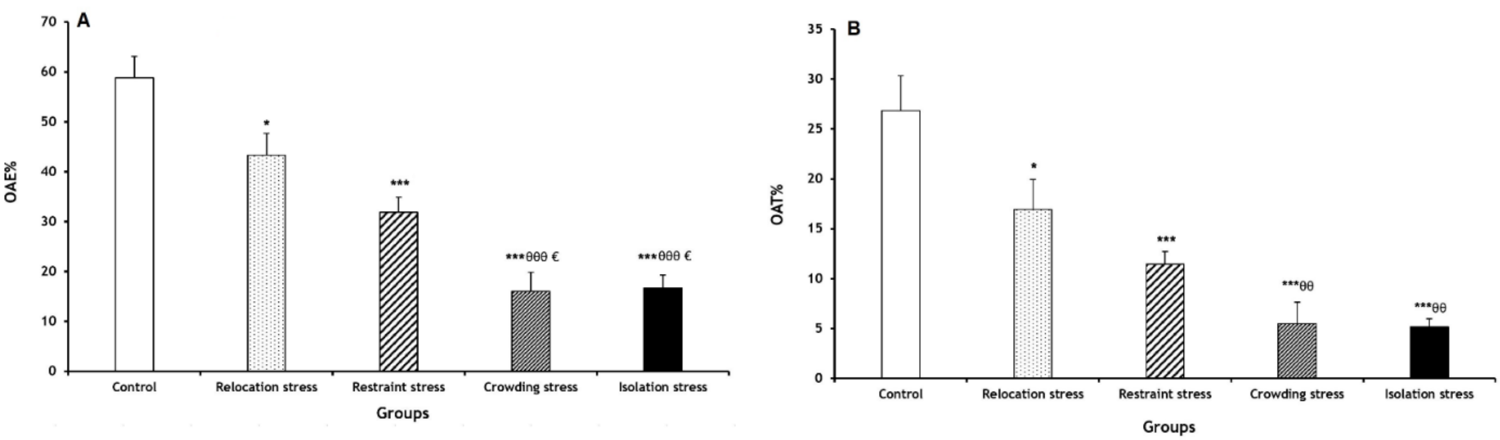

Figure 5: The percentage of **A)** open arms entries (OAE%) and **B)** time spent in the open arms (OAT%) in the EPM test in different groups (n=8). Results are expressed as means±SEM (One-way ANOVA, followed by Tukey's post-hoc test).

$^{*}p<0.05$ and $^{***}p<0.001$ compared to the Control (Co) group; $^{\theta\theta}p<0.01$ and $^{\theta\theta\theta}p<0.001$ compared to the Relocation Stress (Rel-St); $^{€}p<0.05$ compared to the Restraint Stress (Res-St) group.

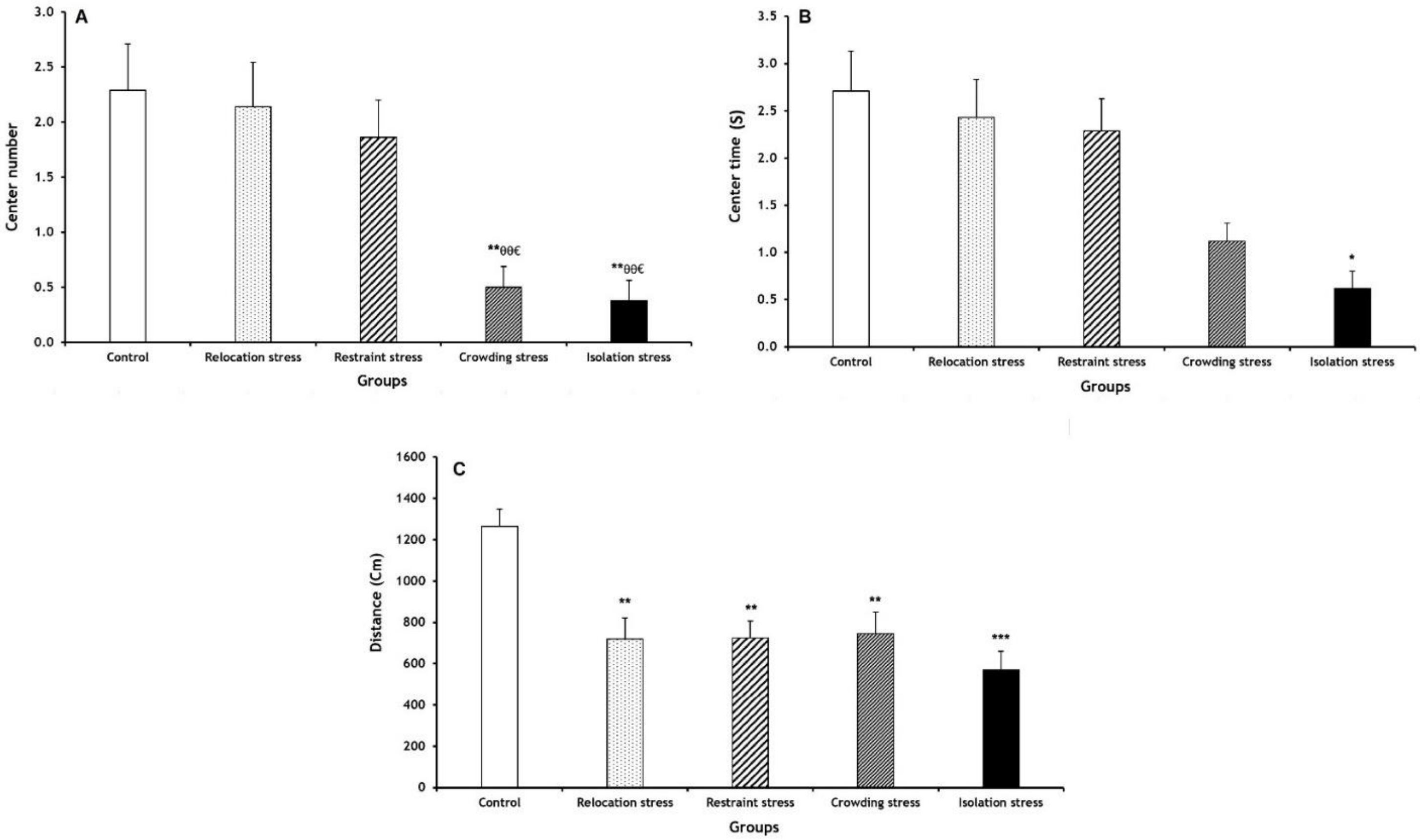

Figure 6: A) Number of entries to the center of the platform, **B)** time (s) spent in its center, and **C)** total distance (cm) traveled in the OFT in different groups (n=8). Results are expressed as means±SEM (One-way ANOVA, followed by Tukey's post-hoc test).

$^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ compared to the Control (Co) group; $^{\Theta\Theta}p<0.01$ compared to the Relocation Stress (Rel-St) group; $^{€}p<0.05$ compared to the Restraint Stress (Res-St) group.

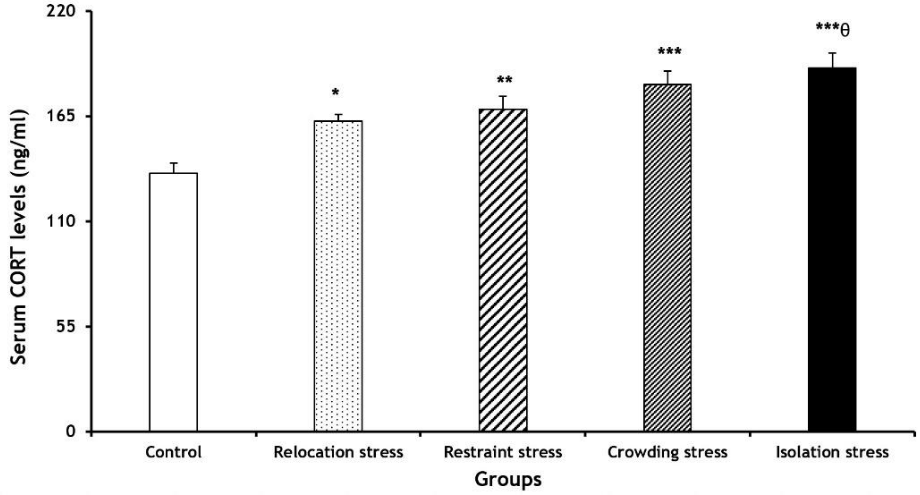

Figure 7: Comparisons of the serum corticosterone levels (ng/mL) in all experimental groups (n=8). Results are expressed as means±SEM (One-way ANOVA, followed by Tukey's post-hoc test).

$^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ compared to the Control (Co) group; $^{\Theta}p<0.05$ compared to the Relocation Stress (Rel-St) group.

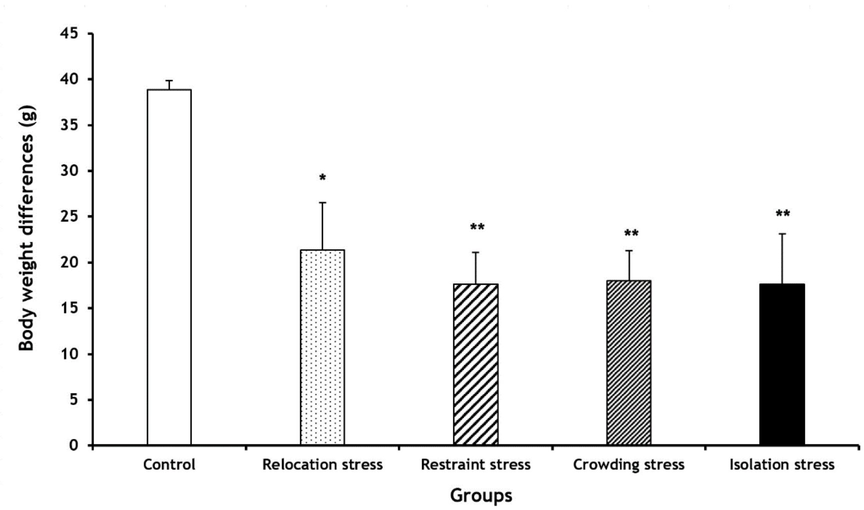

Figure 8: Comparison of the body weight differences (BWDs) in all experimental groups (n=8). Results are expressed as means±SEM (One-way ANOVA, followed by Tukey's post-hoc test). *$^{*}p<0.05$ and $^{**}p<0.01$ compared to the Control (Co) group.*

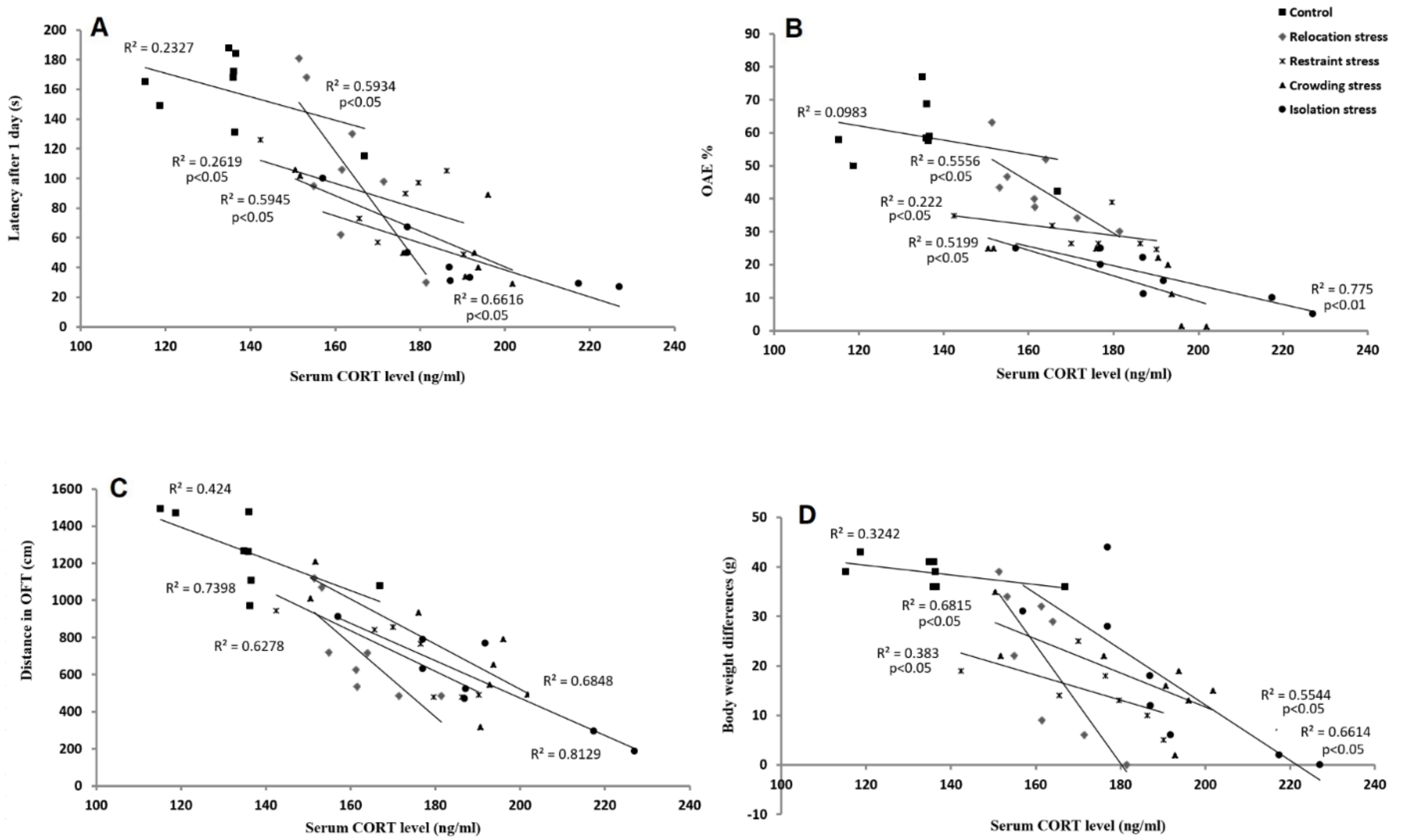

Figure 9: Correlation analysis between the latency after a day in the PA test, OAE% in the EPM, distance in the OFT, and body weight differences (BWDs) with corticosterone (CORT) levels in all experimental groups (n=8). Results are expressed as mean±SEM (Pearson's correlation test).