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EFFECT OF RUNNING WHEEL AS ENVIRONMENTAL ENRICHMENT FOR BALB/C MICE ON ANXIOLYTIC-TYPE BEHAVIOR

EFEITO DA RODA DE CORRIDA COMO ENRIQUECIMENTO AMBIENTAL PARA CAMUNDONGOS BALB/C SOBRE COMPORTAMENTO TIPO-ANSIOLÍTICO

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ABSTRACT

Since ancient times, the animals have been used to advance scientific development. However, the management of these animals might trigger unwanted behavioral and physiological changes. Therefore, there has been a lot of discussion about the environmental enrichment. In this context, this study aims to evaluate the effects of the running wheel on the anxiolytic-type behavior in Balb/c mice. Thirty mice were used, distributed in 2 groups, Control and Enriched Group, with 15 animals each. In the beginning of the research, the animals were fasted for 12 hours and the glucose measurement and weighing were performed. After that, the animals were kept in mini-insulators for 30 days and the fasting, the measurement and weighing procedures were performed again. After 24 hours, the Open Field test and the Elevated Plus Maze test were performed. The statistic analysis Shapiro-Wilk, Mann-Whitney and Wilcoxon, Student-newman-keuls were performed. When evaluating glucose and body weight, a decrease in values was noted in the Enriched Group ($p<0.05$). In the Elevated Plus Maze test, a longer period of time spent in the open arm was also observed ($p<0.05$) in this group. There was no statistical difference in the number of entries ($p>0.05$). In the Open Field, a longer center time and a greater number of rearings ($p<0.05$) were found in the Enriched Group. Regarding the distance traveled, there was statistical difference ($p<0.0001$), however, defecations and groomings there was no difference ($p>0.05$). It is concluded that the running wheel showed positive results in blood glucose levels and body weight. In addition, the stress caused by housing was minimized through environmental enrichment.

Keywords: behavior; stress; glucose.

RESUMO

Desde a antiguidade os animais têm sido utilizados no avanço do desenvolvimento cientifico. Todavia, o manejo em cativeiro desses animais pode provocar alterações comportamentais e fisiológicas não desejáveis. Por isso, muito tem-se discutido a respeito do enriquecimento ambiental. Neste contexto, o presente estudo teve como objetivo avaliar os efeitos da roda de corrida sobre o comportamento tipo-ansiolítico em camundongos Balb/c. Foram utilizados 30 camundongos, distribuídos em 2 grupos, Grupo Controle e Enriquecido, com 15 animais cada. No momento inicial da pesquisa, os animais foram mantidos em jejum por 12 horas e foi realizado a mensuração de glicose e pesagem. Após isso os animais foram mantidos em mini-isoladores por 30 dias e o procedimento de jejum, mensuração e pesagem foi refeito. Após 24 horas foi realizado o teste de Campo Aberto e Labirinto em Cruz Elevado. Foi realizado análise estatística Shapiro-Wilk, Mann-Whitney e Wilcoxon, Sudent's-Newman-Keuls. Na avaliação da glicose e peso corporal, notou-se diminuição nos valores no Grupo Enriquecido ($p<0,05$). No Labirinto em Cruz Elevado, também foi observado um maior

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tempo de permanência no braço aberto (p<0,05) neste grupo. No número de entradas não houve diferença estatística (p>0,05). No Campo Aberto foi encontrado um maior tempo de centro e um maior número de rearings (p<0,05) no Grupo Enriquecido. Em relação a distância percorrida houve diferença estatística (p<0,0001), todavia, em defecações e groomings não houve (p>0,05). Conclui-se que a roda de corrida, mostrou resultados positivos nos níveis de glicose sanguínea e peso corporal. Além disso o estresse causado pela habitação, foram minimizados através do enriquecimento ambiental.

Keywords: *comportamento; estresse; glicose.*

INTRODUCTION

Since ancient times, several animals have been used in the pursuit of advancing scientific development, in order to benefit both medicine and veterinary medicine (GUIMARÃES, 2016). At first, in 450 BC, Hippocrates used animals only to observe anatomical differences between them and humans with diseases, but later, in 129-210 AD, Galen performed what is believed to be the first vivisection to analyze induced variables in animals (GREIF; TRÉZ, 2000).

Among the animals used for experimentation, rodents stand out, especially rats and mice, because they are small animals, easy to handle, short reproductive cycle and with desired similarities in scientific experiments (FAGUNDES and TAHA,2004). The isogenic BALB/c mouse has become the choice in several researches, since its use guarantees genetic uniformity and standardization (LIRA, 2022).

Animals of the Balb/c lineage show high adaptation to new environments and individuals, however, among other animals, they are considered very aggressive and territorial animals, being common the existence of a dominant mouse in the environment (BATISTA *et al*., 2012). Due to this, male Balb/c mice are often found with lesions on the tail and regions without the presence of hair on the face and body, that happens as a result of the strong social hierarchy characteristic of the species. Among females, episodes of aggression are uncommon (KO *et al*., 2017).

In addition, the practice of captive management of these animals can cause undesirable behavioral and physiological changes in a scientific experiment, because they are created in small environments and without sensory stimuli, thus they're restricted from manifesting natural behaviors of the species (GOZZER *et al*., 2018).

Due to this, with the constant advance of technology, much has been discussed about environmental enrichment (KO *et al*., 2017). According to Gozzer *et al.* (2018), this practice has been widely used nowadays and aims to provide the animal with a variety of objects, such as a running wheel, tunnels, hiding places, in order to enhance motor, sensory and cognitive stimuli, awakening its interest in exploring the environment. As a consequence, animals living in enriched environments become less stressed, more docile and with a greater capacity for physiological and behavioral adaptation (GARBIN *et al*., 2012).

Selye (1976) describes stress as "nonspecific response of the body to any demand" and the stressor as "agent that produces stress at any time". Therefore, it represents the reaction of the animal organism to stimuli that disturb it's normal physiological balance or homeostasis.

Besides, Faccini (2020) describes that stress can be classified according to its release, and it can be defined as acute stress or chronic stress. Acute stress is released in transient traumatic situations, while chronic stress is released constantly and both can bring various negative effects on the body. These changes caused by stress can affect various systems such as the nervous, endocrine and immune systems of the animal.

One of the negative effects is the release of hormones that occurs after the animal experiences some type of stress. Among them there's cortisol, which after its release the increase of the glucose concentrations levels is observed (TEIXEIRA, 2002).

It is known that, during the practice of physical activity, glucose is the main source of energy expended, in addition, there is an increase in plasma concentrations of amino acid substrates, free fatty acids and glycerol, leaving the hepatocyte to promote hepatic gluconeogenesis (SOUZA *et al.*, 2019).

Another gain in performing physical activities, besides the glycemic control, is related to the release of endorphins and dopamine, since this release provides a relaxing and analgesic effect after exertion and it can bring euphoria and an improvement in mood (GOMES, 2014). As a consequence, it reduces the parameters of depression and anxiety and increases levels of well-being (BARBOSA, 2016).

In scientific experiments, spontaneous physical activities are used, through voluntary running by the provision of a wheel, so that such an act is possible (NAKAMA *et al.*, 2020). However, its main objective has been energy expenditure and not gains related to animal behavior (ALTHAUS, 2020).

Therefore, the aim of the present study was to evaluate the effects of environmental enrichment "running wheel" on type-anxiolytic behavior in Balb/c mice.

MATERIAL AND METHODS

The project was submitted and approved by the Ethics Committee on the use of animals under protocol 17/23, on March 22, 2023. This study was performed using mice from the Vivarium of University Center of Patos de Minas-UNIPAM. This one has a system of two hallways (clean and dirty) between the experimentation rooms with defined flow of people and inputs and they are protected with sanitary barriers (barrier autoclave, air filtration system, pressure differential, air-lock etc.). The temperature of the environment is controlled at 22ºC. It has a system of insufflation, exhaust and filtration of the air from the rooms, preventing the dispersion of ammonia in the environment, performing 15 to 20 air changes/h and the light cycle is set 12 hours of light and 12 hours of dark. The animals were placed in mini insulators, with three BalbC/c mice each, coupled to a ventilated rack. Food and water were provided ad libidum. The bed was made of pine wood shavings. The entire system was connected to a generator which ensures maintenance in the event of a power outage.

Thirty male mice of the BALB/c strain were used and they were divided into two groups with 15 animals each. For the control group, no environmental enrichment was provided, and for the enriched Group, a running wheel was provided to each box. The 15 mice from each group were allocated to five mini-isolators, with three animals in each.

All race wheels used for environmental enrichment have been packaged and autoclaved so that they are sterile at the time of use. The boxes were changed twice a week, ensuring that the environment was always kept clean.

At the beginning of the research, all animals were weighed for control, through a precision scale model S3201, (Bel Photonics, Brazil, São Paulo). After, the animals were fasted for 12 hours, and then the first glucose measurement of all animals in the two groups was performed, through the capillary glucose test (NAKAMA, 2020).

The measurement was performed through the blood sample obtained by puncturing the caudal end of the animals. To perform the collection, the mice were manipulated by the tail with tweezers and then contained in acrylic containers suitable for the species (Bonther, Brazil, São Paulo). After the puncture, the drop of blood was placed on test strips for glucose quantification (Accu-Chek Performa Brasil test strips, São Paulo). The strips were placed in the glucose meter (Accu-Chek Performa Nano, Brazil, São Paulo) to obtain the glucose values of these mice (Figure 1A).

After this procedure, the animals were kept in mini-insulators and observed for 30 days. During this period, the Control Group did not receive any special treatment, while in the Enriched Group, the running wheel was provided (Figure 1B). The management of these animals was done only by the same researcher, quickly and cautiously, always changing the procedure glove to each box, so as not to transfer the smell of the animals from one box to the other, in order not to compromise the final result of the experiment.

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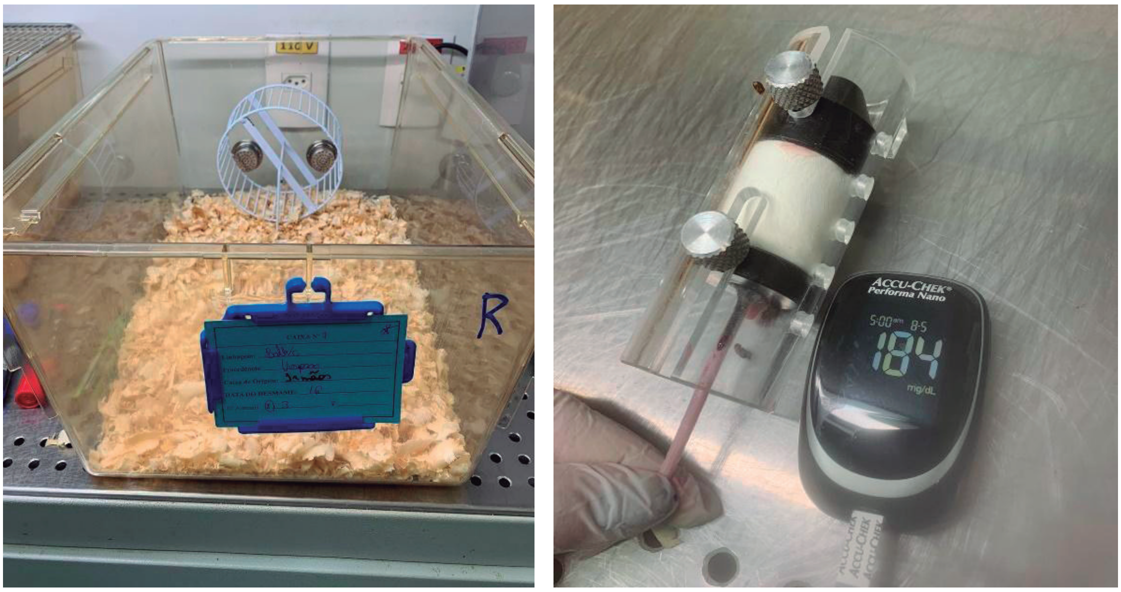
Figure 1 (A) - Measurement of glucose by the glucose meter.
(B) Mini-insulator containing the racing wheel for the enriched Group.

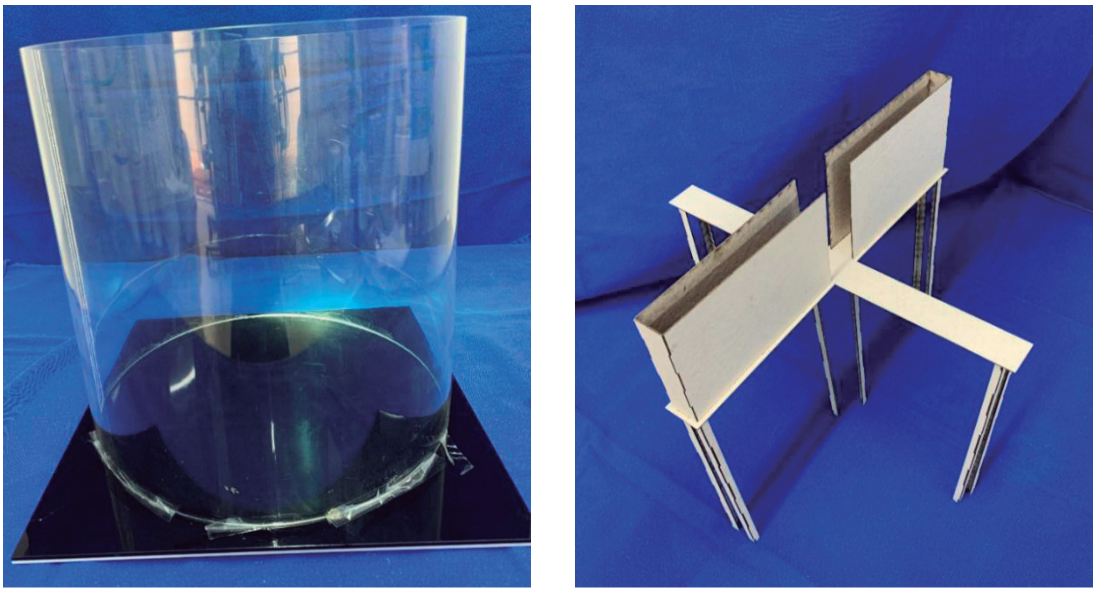


A B

Source: own authorship, 2023.

After 30 days, the weighing procedure was repeated. In addition, the animals were again subjected to 12-hour fasting and glucose measurement. After 24 hours of these procedures, behavioral tests Open Field (Figure 2A) and Elevated Plus Maze (Figure 2B) were performed.

Figure 2 - (A) Apparatus used for Open Field testing.
(B) Prototype of the apparatus for testing in Maze in Elevated Plus.



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Source: own authorship, 2023.

The open field test was carried out in order to evaluate the behavior (anxiety, fear) of the mice that participated in the two groups. The test was performed in the morning and the animals were placed individually inside the circular apparatus measuring 30 cm in diameter and 30 cm in height (Figure 2A) and were placed in a separate room. All behaviors seen during five minutes were computed, them being: number of times the animal stood up (rearings), frequency of defecation, self-cleaning (groomings), center time and distance traveled. These variables were recorded using a video camera. After testing each animal, the apparatus was sanitized with 70% alcohol, so that the smell of the previous animal was removed.

The distance traveled and the time spent in the central area by the animals were counted using the software Anymaze. Whereas, the number of rearings, frequency of defecation and groomings were evaluated manually, analyzing the footage taken.

Regarding the Elevated Plus Maze test, it was performed in order to evaluate the anxiety and fear of mice. The apparatus was formed by four arms of identical sizes, 40cm long x 8cm wide x 25cm high, two arms were closed laterally and two were open. The Labyrinth was 60cm suspended from the floor. (Figure 2B).

The animals were placed individually in the center of the apparatus with their heads facing one of the closed arms. The number of times the animal entered the open arms was evaluated for five minutes as well as the number of times the animal entered the closed arms. In addition, the time of stay of the animal in each of them was counted. As in the open field test, this was also performed in the morning, and after each animal finished, the apparatus was sanitized with 70% alcohol. The behaviors were recorded using a Logitech C270 Webcam video camera (Logitech, Brazil, São Paulo).

The obtained results were subjected to statistical analysis. The data collected regarding glucose and body weight measurement were analyzed using the SPSS (Statistical Package for the Social Sciences) 20.0 software, adopting a 95% confidence level ($p<0.05$), and the Shapiro-Wilk tests were performed to verify normality and the Mann-Whitney and Wilcoxon test for inferential analysis.

For statistical analysis of behavioral tests, the GraphPad Prism software, version 5.0 for Windows (GraphPad Software, San Diego, California, USA) was used, and they were analyzed through one-way ANOVA, followed by the student's-Newman-Keuls test. Significant results were considered $p<0.05$.

RESULTS AND DISCUSSION

Regarding the blood glucose values, there was a significant increase in their values in the Control Group and a decrease in the Enriched Group, presenting statistical significance ($p<0.0018$). These data can be seen in Table 01.

Table 01 - Blood glucose values (mean±SD) in the Control and Enriched groups before and after the 30 days of experiment, Patos de Minas, MG, 2023

Variable	Control Group (n=15)		Enriched Group (n=15)		Significance
	Day 1	Day 30	Day 1	Day 30	
Glucose (mg / dL)	127.33	142.33	143.13	116.2	P< 0.0018

Source: own authorship, 2023.

These values were similar to those obtained by Strapazzon (2019), in which they evaluated mice fed a standard and high-fat diet and using the running wheel. The author also observed lower blood glucose results in mice that practiced physical exercise through the running wheel.

Another study that presented similar results was described by Nakama (2020), who used running wheels to evaluate the chronic effect of physical activity in an enriched environment on blood glucose levels in Wistar rats. The author reported that the practice of physical exercises through the use of the wheel caused better glycemic control by an increase in insulin sensitivity.

Thus, the implementation of the running wheel as environmental enrichment generated positive results, since it is known that exercise stimulates increased insulin sensitivity with reduced blood glucose (Marine *et al.*, 2014). In addition, according to Gomes (2014), exercise is able to reduce stress, since the release of endorphins and dopamine occurs.

Regarding the mean body weight between the two groups, an increase in the values of the Control Group was found when compared to the Enriched Group, presenting statistical significance ($p= <0.05$). These results are presented in Table 2.

This result found in the present study was similar to the one found by Junior (2019) when evaluating whether physical exercise increases dopamine and brain Irisin levels and produces antidepressant-like behaviors in mice. In it, it was observed that the animals that underwent endurance physical exercise, by the end of the experiment, gained less weight when compared to sedentary animals.

According to Machado *et al.* (2014), through the practice of physical exercises it is possible to find a higher energy expenditure, with a consequent reduction in fat mass. Unlike sedentary lifestyle, which can lead to low energy expenditure and an increase in adipose tissue.

Table 02 - Blood glucose values (mean±SD) in the Control and Enriched groups before and after the 30 days of experiment, Patos de Minas, MG, 2023

Variable	Control Group (n=15)		Enriched Group (n=15)		Significance
	Day 1	Day 30	Day 1	Day 30	
Body weight (g)	25.88	28.93	25.71	26.27	P <0.05

Source: own authorship, 2023.

When evaluating the effect of environmental enrichment on the percentage of time spent in open arms in the Elevated Plus Maze Test (Figure 3A), it was observed that the Enriched Group remained for a longer period of time ($t_{(28)} = 6.354$, $p<0.0001$) in the open arms compared to the Control Group, there was a statistical difference between them.

According to Almeida (2016), in a study conducted in mice, in which they were subjected to classical music as a form of environmental enrichment, longer periods of time in open arms can be observed when anxiolytic stimuli occur that manage to prevent sensations of fear and anxiety generated by innate responses associated with open spaces.

Almeida (2012) found in his experiment similar results to the present study when evaluating mice subjected to environmental enrichment, through PVC pipe and paper towels for nesting.

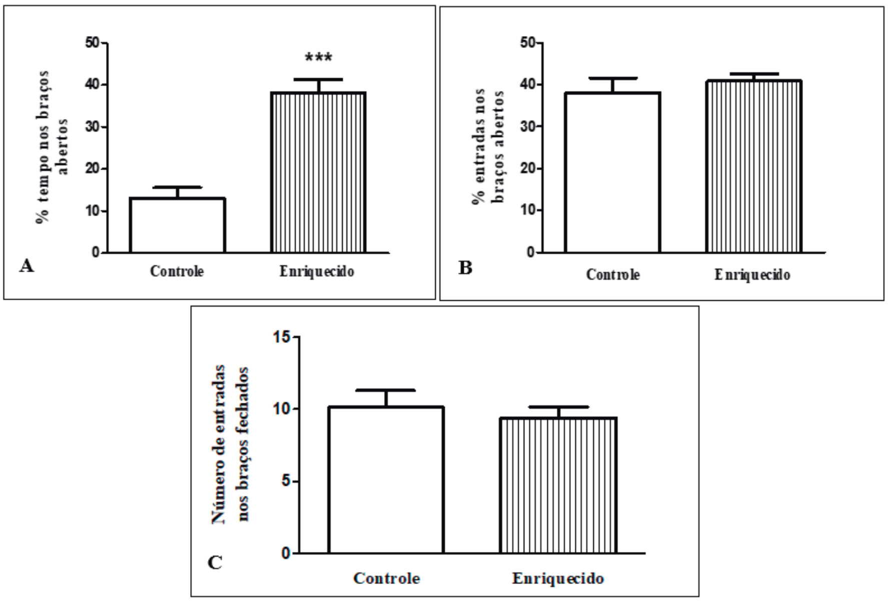
These animals remained longer in open arms in relation to the total time of the test, indicating a reduction in the animals ‘ anxiety levels.

However, when evaluating the percentage of entries in the open arms of the same test, no statistical difference was observed between the Control Group and the Enriched Group ($t_{(28)} = 0.7006$, $p>0.05$), as shown in Figure 3B.

The same happened with Ravache (2023) in his experiment in which the role of environmental enrichment in the recovery of memory loss in mice was evaluated. Similar results were also found in the research developed by Novaes (2017), in which the protection conferred by environmental enrichment in stress-induced anxiety was evaluated. In both studies, no statistical difference was found in the number of entries in open arms, finding only in the length of stay.

Although the results showed no statistical differences in the number of entries in the open arm between groups, as evidenced by Figure 3B, the high frequency of input may mean that animals are adapted and with a lower level of stress and anxiety (MCDERMOTT and KELLY, 2008).

Figure 3 - Effect of environmental enrichment on the percentage of:
(A) time spent in open arms in the elevated plus maze test.
(B) entries in the open arms in the elevated plus maze test.
(C) Number of entries in the closed arms in the elevated plus maze test.



The bars represent the mean ± E.P. M of the percentage of time and entry in the open arms, and the number of entries in the closed arms (Student’s t-test). Source: own authorship, 2023.

In view of this, it is evidenced that although the use of the running wheel as environmental enrichment did not cause changes in exploration in the labyrinth, it reduced anxiety due to the fact that the animals of the enriched group remained longer in an environment without protection against falls (open arm) and exposed to altitude (feeling of exposure to danger).

In the present study, there was no significant difference when analyzing the number of entries in the closed arms between the two groups, as shown in Figure 3C ($t_{(28)} = 0.5202$, $p>0.05$).

This result contradicts the data obtained by Almeida (2016). In his experiment, 24 mice were subjected to an environment with perinatal environmental enrichment. After that, their behaviors were analyzed using the elevated plus maze test and it was possible to notice a significant difference in relation to entering the closed arms. Since, in the animals that used environmental enrichment, a greater number of inputs were found.

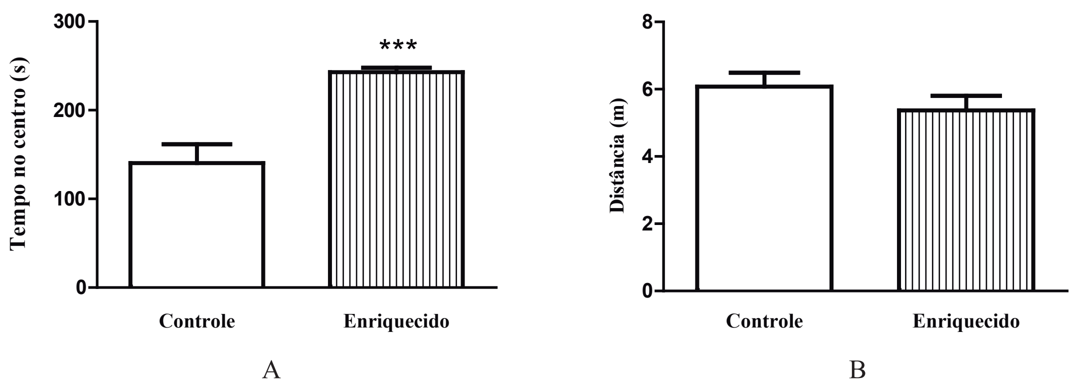
According to Almeida (2012), the percentage of entry into the closed arms is not directly linked to anxiety, but to the locomotor activity of the animal. However, this dressing has an important role to confirm the results as a whole, since it is related to the results found in the number of entries in the open arms.

As for the second test, the open field, as can be seen in Figure 4A, there was a statistical difference ($t_{(28)} = 4.680$, $p<0.0001$) between groups, it was noticed that the Enriched Group remained for a longer period of time in the center of the field than the CG.

According to Antioro *et al.* (2022), more anxious animals tend to stay on the periphery of the open field, as they have a greater survival instinct. This is because the peripheral regions have a sense of security in divergence from the center, where they feel exposed to possible dangers.

The results of the present study were similar to those found by Gozzer (2018) in which the effect of environmental enrichment on the behavior of Wistar rats, and observed in the Control Group a greater permanence in the periphery indicating fear and anxiety. While in the Enrichment Group it was possible to find 50% lower values, indicating anxiolytic effect.

Figure 4 - Effect of environmental enrichment evaluated in the open field test:
(A) time spent in the center. **(B)** distance traveled.



The bars represent mean distance ± E. P. M in meters and time in seconds (Student's t-test).
Source: own authorship, 2023.

When comparing the distance traveled between the two groups in the open field, no significant results were obtained, as shown in Figure 4B ($t_{(28)} = 1.189$, $p>0.05$).

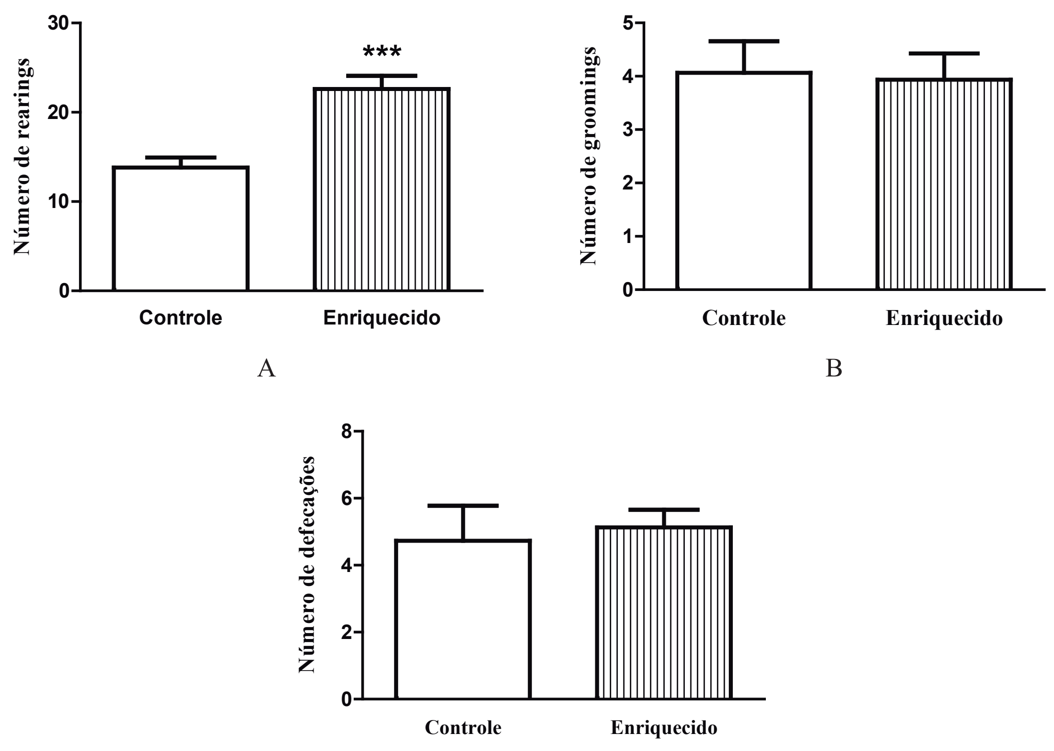
The same was also reported by Carmo (2022), whose study evaluated the effects of environmental enrichment on behavioral and memory changes in Wistar rats. No statistical difference was found between the total distance traveled between the control group and the group that was implemented environmental enrichment.

According to Mcdermott and Kelly (2008), increased locomotor and exploratory activity may be indicative of good adaptation. On the other hand, decreased activity may be indicative of an anxious temperament. According to the results obtained, it is possible to note that environmental enrichment did not cause changes in the locomotion of animals in the enriched group compared to the Control Group.

The number of rearings, groomings and defecations performed by the animals during the open field test were also analysed, as shown in Figure 5. Regarding the number of rearings, significant results were found, and the enriched group obtained superior results ($t_{(28)} = 4.694$, $p<0.0001$), that is, it performed this behavior more frequently. Regarding the number of groomings ($t_{(28)} = 0.1736$, $p>0.05$) and defecations ($t_{(28)} = 0.3424$, $p>0.05$) there was no statistical difference in these behaviors between the groups.

In the study conducted by Testa (2020), where the physiological and behavioral responses of rats subjected to voluntary physical activity and social isolation were evaluated, there were no changes between the groups, regarding the number of rearings, groomings defecation, as in the present study.

Figure 5 - Effect of environmental enrichment on open field testing:
(A) In the number of rearings. **(B)** In the number of groomings. **(C)** frequency (event) of defecation.



C

The bars represent the mean ± E. P. M of the number of rearings, groomings and defecation (Student's t-test).
Source: own authorship, 2023.

According to Blum (2017), the number of groomigs, that is, the number of self-cleaning, when decreased, can also identify depressive-like behavior. This is because animals with higher levels of stress tend to reduce their self-care and thus end up decreasing the habit of self-cleaning.

Regarding the increase in the number of defecations, according to Ramos (1997), this behavior may be an indicative factor of anxiety, since it occurs due to the activation of the sympathomimetic system.

Finally, regarding the number of rearings, that is, the number of times the animal raises its hind legs, is directly related to its exploratory activity, which when increased can mean a lower rate of anxiety of the same (ALMEIDA, 2012; ALMEIDA, 2016). These findings corroborate that environmental enrichment by the use of the running wheel promoted an increase in the number of rearings, suggesting a lower anxiety index of the animals.

CONCLUSION

It is concluded that the effects of the enriched environment through the running wheel show positive results with reduction in blood glucose levels and body weight, when compared to the control group.

Besides, the stress caused by housing is minimized by environmental enrichment, a result observed through behaviors related to decreased anxiety of these animals.

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