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Physiological effects of environmental enrichment on BALB/c and C57BL/6 strain mice

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Abstract
Our aim was to assess the effects of enriched environment (EE) on some physiological variables, comparing BALB/c and C57BL/6 strain mice. Female BALB/c and C57BL/6 were individually housed under standard laboratory cages for 6 weeks and randomly separated into groups (n = 7 per strain per group): Standard environment (SE); Environmental enrichment (EE). The EE protocol consisted of toys and nesting material in home cages for each mouse. Weight gain, systolic blood pressure (SBP), heart rate, and gastrointestinal transit were assessed in SE and EE groups after 6 weeks. At sacrifice, corticosterone serum, thymus and splenic weight, and intestinal histology were also determined. There was no significant difference in body weight gain or heart rate; however, EE significantly reduced the plasma levels of corticosterone and SBP compared to SE groups in BALB/c and C57BL/6. Gastrointestinal transit was faster in BALB/c mice compared to C57BL/6 mice in SE. EE did not significantly change the transit for BALB/c mice, but was markedly accelerated in C57BL/6 mice. There was no difference in the thymus/body weight ratio for strain or housing condition; however, BALB/c mice displayed a higher spleen/body weight ratio compared to C57BL/6 regardless of housing conditions. Our data reinforce the importance of choosing the better experimental model due to the difference between the strains and importance of including EE, especially for the C57BL/6 strain, where there seems to be more physiological impact.

Keywords Enriched environment · Female mice · Physiology system · Gastrointestinal tract

1 Background

The enriched environment (EE) is an animal housing approach in which animals are given increased amounts of space, variety of toys, physical activity, and social interaction [1]. Such modifications are expected to increase sensory, cognitive, and motor stimulation, in contrast to the standard laboratory environment (SE) [1–3]. EE provides animals with opportunities to experience quantitatively more abundant and qualitatively more complex stimuli through different protocols [4]. The scientific evidence supports the use of EE to improve the welfare of mice used in research [5], resulting in stress response reduction [6], as well as improving neural, immune, and metabolic health [7, 8]. However, the resistance to its implementation seems to be related to possible mischaracterization of several well-described experimental models, so broader studies to clarify the physiological variables are necessary. Inbred mouse strains have specific characteristics

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since the genetic history of the mouse strains is strictly controlled, allowing the physical and pathophysiological response to be better predicted [9]. It is widely acknowledged that BALB/c and C57BL/6 mice differ in several aspects of gastrointestinal motility [10], immune [3], and autonomic response [11]. Several studies related to EEs verify their effects only in the context of neurological diseases [1, 8]. Additionally, BALB/c mice demonstrate less sociability and higher anxiety-like behaviors than C57BL/6 [12, 13], and seem to be more affected by EE [14]. Apart from the fact that the EEs effect has been demonstrated in several biological systems, little is known about how EE affect the cardiovascular variables and gastrointestinal morphofunctional aspects in different mouse strains. Thus, our goal was to assess the effects of EE on a variety of physiological variables, comparing BALB/c and C57BL/6 strain mice.

2 Methods

2.1 Animals

Twenty-eight female 5-week-old BALB/c and C57BL/6 inbred mice were used for experiments. BALB/c and C57BL/6 breeders were purchased from the animal breeding facility of the Institute of Biomedical Sciences, University of São Paulo (USP), Brazil, and were maintained in the animal facility of Universidade Federal de Mato Grosso (UFMT). Mice were kept in polyethylene cages (30 × 20 × 13 cm) in controlled conditions of temperature (22 ± 2 °C), humidity (60 ± 5%), and a 12-h light/dark cycle with free access to food (Purina®) and filtered water. All the experimental procedures were approved by and following the Ethics Committee on the Use of Animals at the UFMT, protocol number 23108.017861/2019–42. All animal experiments complied with Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria.

2.2 Housing conditions and experimental design

Female BALB/c and C57BL/6 were evaluated after each strain was randomly separated into two groups according to the experimental protocols: (1) Standard environment (SE, n = 7 per strain): individually housed under standard laboratory cages for 6 weeks; (2) Environmental enrichment (EE, n = 7 per strain): individually housed in home cages with EE for 6 weeks. The EE protocol consisted of toys (shelters, tubes, paper balls) and nesting material in home cages for each mouse.. The objects in EE were randomly rearranged and cleaned weekly.

Body weight was measured at the beginning and end of 6 weeks and weight gain was calculated. The systolic blood pressure, heart rate, and gastrointestinal transit were assessed in SE and EE groups after exposure to 6 weeks of housing manipulation. At the end of the experimental period, mice were sacrificed, and blood and organs were collected to determine corticosterone levels, thymus and splenic weight, and intestinal histology for SE and EE groups in both strains (Fig. 1).

2.3 Systolic blood pressure and heart rate

The systolic blood pressure (SBP) and heart rate (HR) were assessed by non-invasive tail-cuff plethysmography after three days of adaptation to the device (PowerLab 4/S, ADInstruments Pty Ltd., Castle Hill, Australia). For recording, the mice were subjected to a box with ultraviolet (UV) light at 37 °C for 10 min and contained in an acrylic cylinder with an opening for the muzzle and tail for 5 min. An occluder was fitted to the proximal portion of the tail and coupled to a PE-399 electrical caudal pneumatic transducer connected to a transduction system. The final value of the caudal arterial

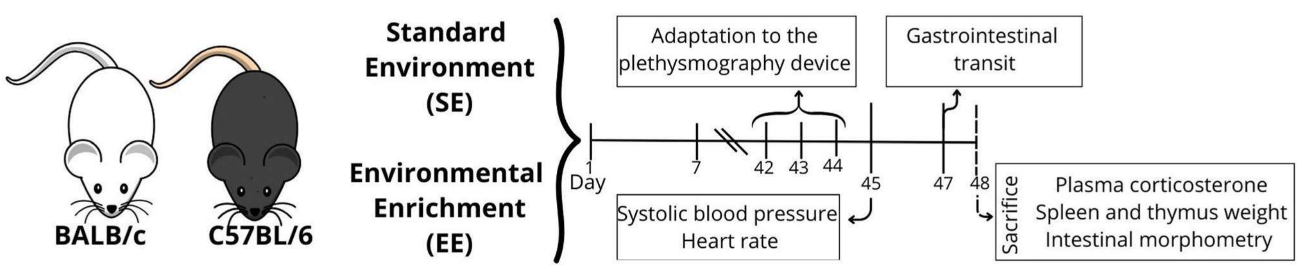


Fig. 1 Experimental setup for the study protocol in BALB/c and C57BL/6

pressure of each animal represents the arithmetic average of three readings. SBP levels measurements were recorded in awakening, immobilized mice, in the same conditions of adaptation sections.

2.4 Gastrointestinal transit

Recording of gastrointestinal transit was performed using the Alternating Current Biosusceptometry (ACB) technique (Br4-Science®, Brazil) [15, 16]. The ACB sensor consists of a set of induction coils for detecting the signals generated by magnetic materials in response to an externally applied magnetic field. Ferrite ($MgZnFe_2O_3$, $53 \le ø \le 75$ µm) is often used as a magnetic material because it is non-absorbable and inert at any pH. The signal intensity depends on the amount of magnetic material and the distance between the sensor and the magnetic material.

For gastrointestinal transit, a magnetically marked chow (200 mg standard laboratory chow homogenized with 50 mg ferrite powder) was used [10]. The magnetic intensity of each magnetically marked chow was determined through the ACB sensor, and, posteriorly, the signal intensity value was adopted to calculate the percentage of ferrite excretion in feces.

After the 12-h fast, each mouse ingested an entire magnetically marked chow, and the time was set as *t*0. All mice ingested the same amount of magnetically marked chow (250 mg) at $t = 0$. Then, the animals were placed individually in a polyethylene box with a grid floor to collect fecal pellets and to prevent coprophagy. During this period, a plastic tube (shelter) was maintained in each box for environmental enrichment. Each animal was manipulated at regular 20-min intervals for 10 h for fecal pellet collection, as detailed in Gama et al. [10]. Fecal pellets expelled at each time interval were allocated in a volume-controlled tube which was placed on the surface of the ACB sensor to record the magnetic signal intensity. The following gastrointestinal transit variables were also determined:

Oro-Anal Transit Time (OATT) is defined as the time *t* (minute) taken for the excretion of the first fecal pellet containing magnetic material starting from *t*0 [10];

Fecal Pellet Elimination Rate (FPER), in minutes, is defined as the time in which a mean amount of magnetic material was excreted in feces during 10 h of evaluation. FPER was calculated using the statistical moment in which the time-weighted average data were calculated by the area under the curve of magnetic signal intensity obtained in feces for 10-h measurements [10, 17].

2.5 Corticosterone levels

On the morning of the experimental endpoint, the mice were anesthetized with 100 mg/kg of ketamine (Cetamin®, Syntec) plus 10 mg/kg of xylazine (Xilazin®, Syntec), via intraperitoneal (i.p.) injection. Analgesia was tested by loss of reflexes after pinching the mice between the toes. The blood was always collected at 10 a.m. via heart puncture in a syringe prepared with ethylenediamine tetraacetic acid disodium salt hydrate (EDTA) anticoagulant 3% and plasma levels of corticosterone were determined by electrochemiluminescence immunoassays (Roche Diagnostics, Switzerland).

2.6 Assessment of spleen and thymus weight

The thymus and spleen were collected, and weighed, and the organ/body weight ratio was calculated individually by the following formula:

Organ/body weight ratio (%) = mouse's organ weight/body weight × 100%.

2.7 Intestinal morphometry

Specimens from the duodenum were removed and immersion-fixed for 24 h in 10% buffer formalin, dehydrated with serial alcohol, diaphonized in xylol, and embedded in paraffin. Tissues were cut into 4 µm thick sections and stained with hematoxylin and eosin (HE). HE staining was used for morphometric analysis of the thickness of the muscular layer, villus height, and crypt depth. Images were captured using an Eclipse E200-Nikon optical microscope (Nikon®), in objective × 10, equipped with 5.0 MP [10]. The morphometric measurements were carried out blindly using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.8 Statistical analysis

The Shapiro–Wilk test was performed to determine the distribution of the variables. Results were displayed for data distributed normally as mean ± standard deviation (SD) with a limit of statistical significance up to 5%. For comparisons of variables among groups, a two-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed. Variables that were non-normally distributed were analyzed by Kruskal–Wallis's test followed by Dunn's test and presented as median ± range.

3 Results

BALB/c mice presented larger body weight than C57BL/6 mice, independently of housing conditions ($F_{(1, 24)} = 44.64$, $p < 0.0001$, Fig. 2A). At 6 weeks of environmental enrichment, mice from BALB/c and C57BL/6 strains showed no significant difference in body weight gain compared to those from the standard environment (Fig. 2B). However, EE significantly reduced the plasma levels of corticosterone compared to SE groups in BALB/c and C57BL/6 ($F_{(1, 24)} = 18.86$, $p = 0.0002$, Fig. 2C).

Regarding cardiovascular variables, mice from the EE group showed a decrease in SBP compared to SE groups in both strains' mice ($F_{(1, 24)} = 845.9$, $p < 0.0001$, Fig. 3A). C57BL/6 also presented a very reduced variability on SBP recording independently from the environment. On the other hand, the heart rate was similar between BALB/c and C57BL/6, regardless of housing condition (Fig. 3B).

Regarding gastrointestinal motility variables, OATT was faster in BALB/c mice compared to C57BL/6 mice in SE housing conditions ($F_{(1, 24)} = 15.10$, $p = 0.0007$, Fig. 4A). Environmental enrichment did not significantly change the OATT for BALB/c mice, but markedly reduced the OATT, both concerning the average value and the variability, in C57BL/6 mice (Fig. 4A). Similarly, the fecal pellet elimination rate (FPER) was also faster in BALB/c mice compared to C57BL/6 mice in the standard environment ($F_{(1, 24)} = 15.27$, $p = 0.0007$, Fig. 4B). However, the housing condition does not significantly influence the FPER in BALB/c mice. Conversely, the FPER was accelerated in C57BL/6 mice of the EE group compared to its control.

Regarding the histomorphometry of intestines, BALB/c mice presented larger muscle layer thickness ($F_{(1, 24)} = 47.63$, $p < 0.0001$, Fig. 5A) and crypt depth ($F_{(1, 24)} = 149.4$, $p < 0.0001$, Fig. 5B) than C57BL/6 mice, independently of housing conditions. In contrast, no significant effects of strain or housing conditions were observed in the villus height (Fig. 5C).

Regarding the relative weight of the lymphoid organs, there was no significant difference in the thymus/body weight ratio for strain or housing condition (Fig. 6A). However, BALB/c mice displayed a higher spleen/body weight ratio compared to C57BL/6 and regardless of housing condition ($F_{(1, 24)} = 370.4$, $p < 0.0001$, Fig. 6B).

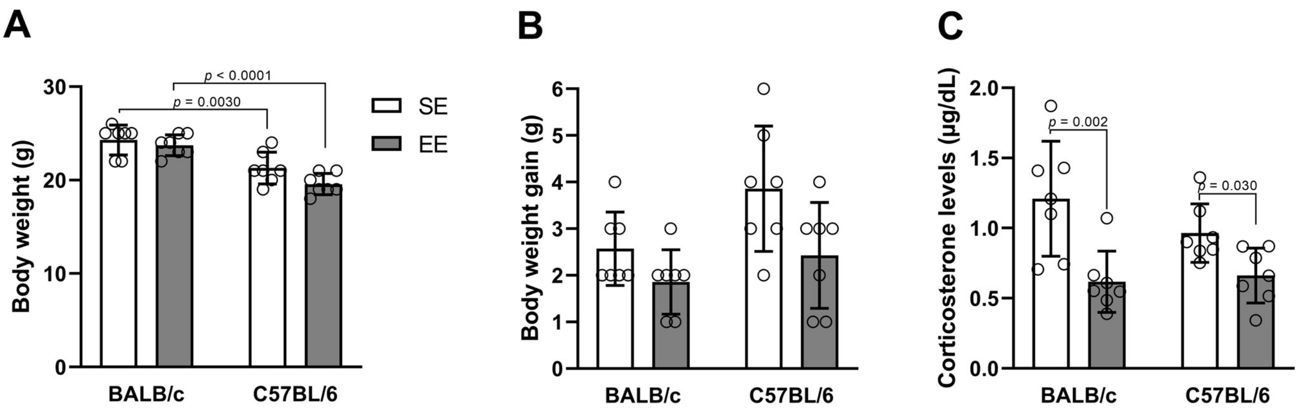


Fig. 2 Body weight (**A**), Body weight gain (**B**) and corticosterone plasma levels (**C**) in Standard Environmental (SE, white bars) and Environmental Enrichment (EE, gray bars) groups of BALB/c and C57BL/6 mice strains. Blood was always collected at 10 a.m. Lines over bars indicate significant differences between groups of mice. Statistical significance was determined by two-way ANOVA followed by Tukey's post hoc test. The results are expressed as mean ± SD (n = 7)

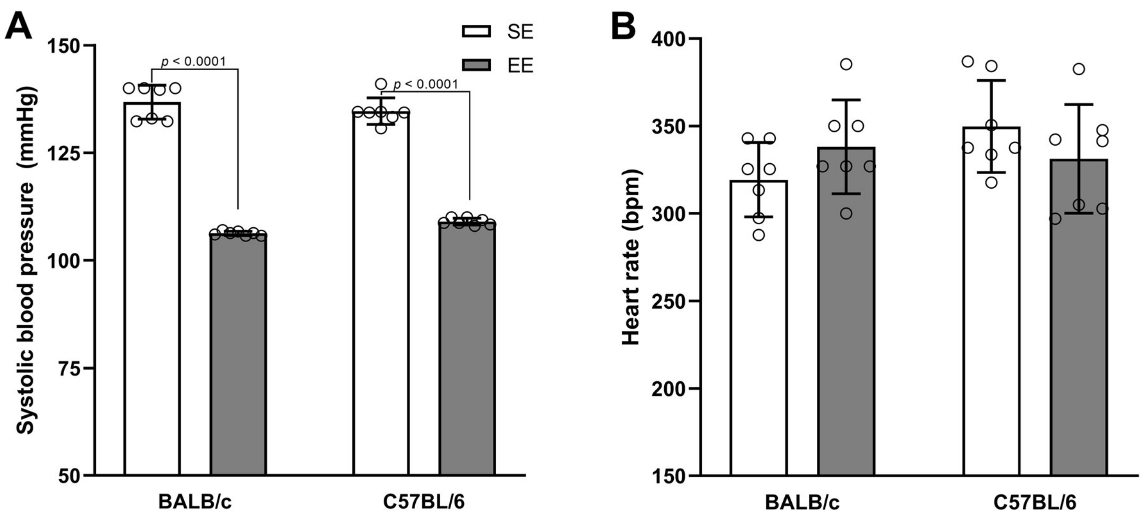


Fig. 3 Systolic blood pressure (**A**) and heart rate (**B**) in Standard Environmental (SE, white bars) and Environmental Enrichment (EE, gray bars) groups of BALB/c and C57BL/6 mice strains. Lines over bars indicate significant differences between groups of mice. Statistical significance was determined by two-way ANOVA followed by Tukey's post hoc test. The results are expressed as mean ± SD (n = 7)

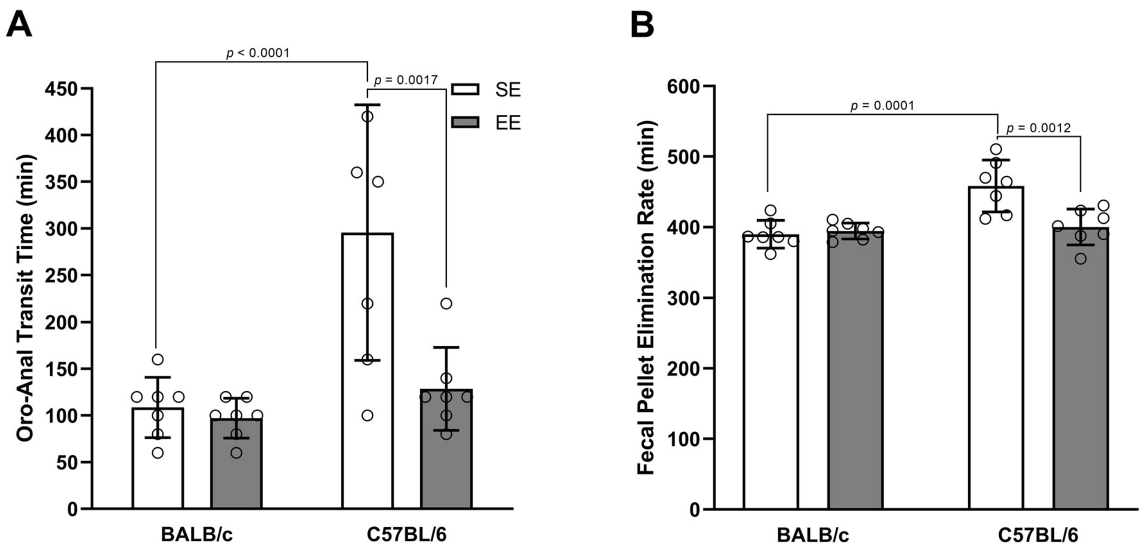


Fig. 4 Oro-anal transit time (**A**) and Fecal pellet elimination rate (**B**) in Standard Environmental (SE, white bars) and Environmental Enrichment (EE, gray bars) groups of BALB/c and C57BL/6 mice strains. Lines over bars indicate significant differences between groups of mice. Statistical significance was determined by two-way ANOVA followed by Tukey's post hoc test. The results are expressed as mean ± SD (n = 7)

4 Discussion

Environmental enrichment is a strategy focusing on enhancing animal well-being by providing animals with sensory and motor stimulation, without negatively impacting the health and safety of the animal [5]. Our results showed that EE during six weeks altered physiological variables by reducing plasma corticosterone levels and systolic blood pressure in both BALB/c and C57BL/6 strains, as well as reduced gastrointestinal transit time in C57BL/6 mice. Furthermore, EE showed no changes in weight gain, heart rate, thymic and splenic body weight ratios, and intestinal histomorphometry.

Stressful stimuli induce a cascade of events in the hypothalamic–pituitary–adrenal (HPA) axis, which culminate in the secretion of glucocorticoids from the adrenal cortex [18]. The HPA axis is an autoregulatory network that employs corticosterone in rats and mice, and cortisol in humans, as its end products to control its activity through a multistep

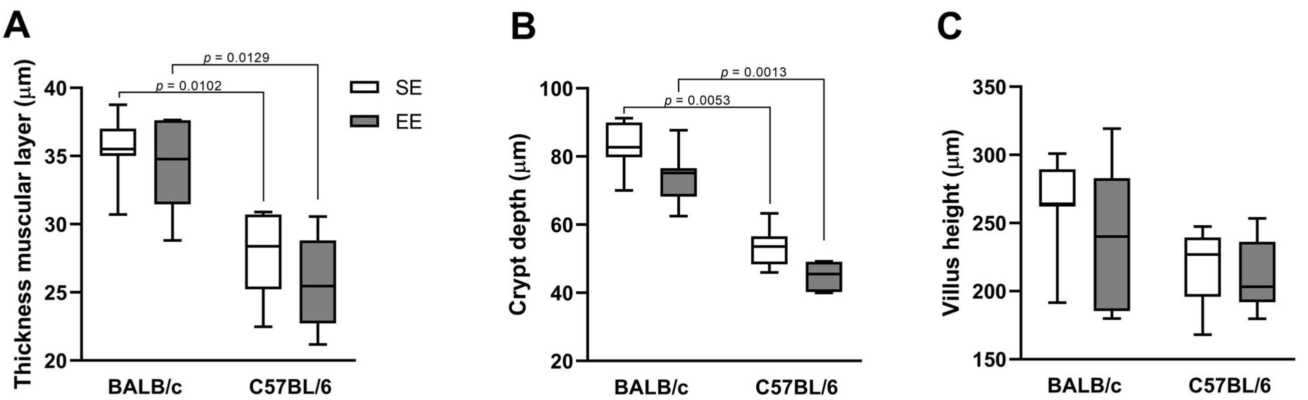
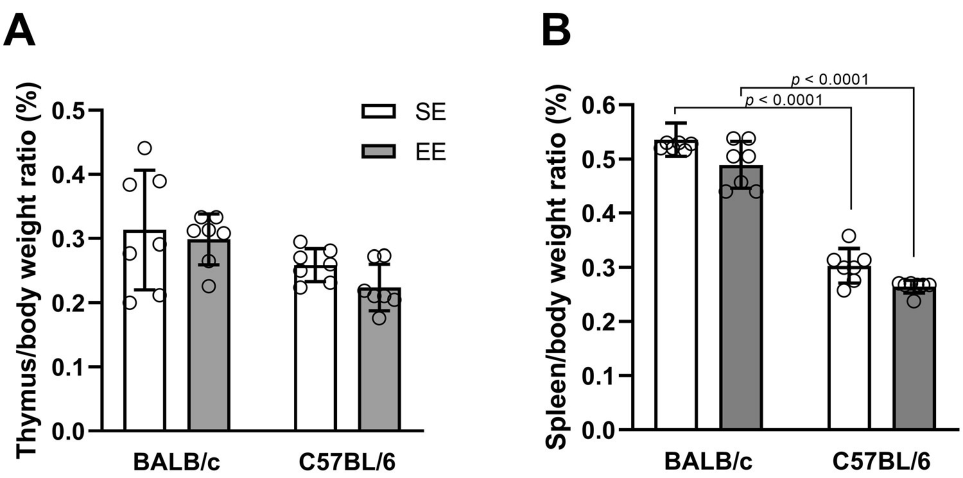


Fig. 5 Histomorphometry of intestinal of Thickness muscular layer (**A**), Crypt depth (**B**), and Villus height (**C**) in Standard Environmental (SE, white bars) and Environmental Enrichment (EE, gray bars) groups of BALB/c and C57BL/6 mice strains. Lines over bars indicate significant differences between groups of mice. Statistical significance was determined by Kruskal–Wallis's test followed by Dunn's test. The results are expressed as median ± range (n = 7)

Fig. 6 Thymus/body weight ratio (**A**) and Spleen/body weight ratio (**B**) in Standard Environmental (SE, white bars) and Environmental Enrichment (EE, gray bars) groups of BALB/c and C57BL/6 mice strains. Lines over bars indicate significant differences between groups of mice. Statistical significance was determined by two-way ANOVA followed by Tukey's post hoc test. The results are expressed as mean ± SD (n = 7)



negative feedback mechanism of the HPA axis [19]. Thus, corticosterone levels depend on production in the adrenal cortex in response to the activation of the HPA axis [20]. The present study was performed in females, since they are generally considered to be more sensitive to environmental enrichment, compared to male. Studies have shown that female rodents often exhibit greater behavioral and physiological responses to enriched environments, such as improved cognitive function, reduced anxiety-like behaviors, and enhanced neuroplasticity [21, 22]. It is worth noting that the mechanisms controlling the stress response are complex and each parameter should not be interpreted on its own but in the context of other measures [23].

In our study, BALB/c and C57BL/6 female mice housed in an environmental enrichment showed a significant reduction in plasma corticosterone levels. This corroborates evidence that EE can reduce the systemic response to stress via reduced corticosterone levels in mice [24, 25]. Clipperton-Allen et al. [26], in turn, observed no differences in the levels of fecal corticoid metabolites in BALB/c and C57BL/6 housed in environmental enrichment for 6 months. In the present study, there were no significant changes in the weight gain of mice from both strains after 6 weeks independently of housing conditions. These data were corroborated by other studies carried out with BALB/c and C57BL/6 females kept in environmental enrichment [26, 27].

BALB/c mice are well known to be Th2-polarized immune responses, characterized by elevated antibody production and higher cytokine levels (IL-4, IL-5, IL-13) in response to allergic challenges [28]. In models of allergic rhinitis, for example, BALB/c mice developed robust systemic immune activation, while C57BL/6 mice show milder systemic responses [29]. The ability of organisms to trigger immune responses can be monitored by the conditions and characteristics of peripheral lymphoid organs such as the spleen and thymus [30]. The effects of EE on the indicative of immunological status from mice were assessed by evaluating thymus and spleen body weight ratios. The EE for

6 weeks did not significantly alter these parameters, demonstrating that the EE adopted did not interfere with this aspect of the immunological integrity of the animals. Tsai et al. [27] also did not observe a significant difference in spleen weight in both strains. However, the spleen/body weight ratio was higher in BALB/c compared to C57BL/6, regardless of environmental conditions, which must be related to differences in body weight characteristic of each mouse strain [31].

Changes in the HPA axis may affect many physiological systems simultaneously. The effects of EE on cardiovascular parameters were assessed using systolic blood pressure (SBP) and heart rate (HR) measurements. Mice kept in EE showed no significant changes in heart rate; however, they showed a significant decrease in SBP compared to animals kept in a SE in both strains of mice. SBP is the product of heart rate and peripheral vascular resistance and is therefore a variable modulated by several factors. In rodents, as in humans, threatening stimuli also elicit increased autonomic activity, including increased heart rate, body temperature, respiration, and blood pressure [11]. Also, several investigators have used autonomic measures to assess fear and anxiety in animal models [11, 32, 33]. C57BL/6 female mice housed in EE did not show significant variations in HR measured after implantation of the radiotelemetry transmitters [25]. A long-term study demonstrated that the autonomic nervous functions, especially parasympathetic activity, were significantly greater in Nagoya-Shibata-Yasuda (NSY) mice, and the HR was significantly lower in the EE group [6].

Stress affects gastrointestinal motility, visceral pain sensitivity, and intestinal epithelial cell permeability [34]. The factors stressors have been proposed to disturb gut-brain bi-directional communication, lead to gastrointestinal alterations, and may contribute to the development of functional gastrointestinal disorders [35–37]. Chronic psychological stress increases HPA axis and autonomic nervous system activity which promotes modulation in monoaminergic systems signaling involved in the regulation of gut physiology and brain-gut axis function [35, 37, 38]. Environmental enrichment seems to alleviate the deleterious effects of stress [39]; however, studies evaluating the role of EE on gastrointestinal motility are scarce.

Our data show a reduction in gastrointestinal transit time in C57BL/6 mice, but not in BALB/c. At baseline levels, there are important phenotypic differences between BALB/c and C57BL/6 strains for motility, which may influence the choice of model for that line of study. The same difference regarding gastrointestinal motility was also observed in a previous study in male mice using both strains BALB/c and C57BL/6 [10]. Our data demonstrates that EE has an interesting role in reducing the transit time by reducing the observed intraspecies variability. It seems that enriching the environment in which these animals were inserted reduces stress and contributes to modulating gastrointestinal motility in C57BL/6. The mechanisms regarding EE effects on the gastrointestinal tract still need to be better studied. Differences between strains were also observed in intestinal histomorphometry. BALB/c mice presented larger muscle layer thickness than C57BL/6 mice, independently of housing conditions, which partially explains faster transit in this strain. Previous study showed that BALB/c strains had higher duodenal frequencies and a shorter time to eliminate the ingested marker [10]. BALB/c mice presented larger crypt depth than C57BL/6, regardless of the environmental conditions to which they were exposed. In a previous study, our group observed greater crypt depth and smaller villus height in male BALB/c mice [10], demonstrating that sex was a factor that establishes structural differences in strains. The EE, in turn, does not cause significant changes in histomorphometry, which was understood positively to maintain the anatomic and functional characteristics of the animals.

It is important to study the influence of EE on different strains since genotype variations produce different experimental phenotypes that must be considered when choosing the best model. The methodology adopted in our study has the advantage of being non-invasive, which may be the differential factor for measuring these parameters. This means that most of these measurements can be repeated and monitored against other variables in the same animal, as recommended in the 3R guidelines [40]. It is important to point out that no studies were found comparing the influence of EE on BALB/c and C57BL/6 regarding blood pressure or gastrointestinal transit, reiterating the importance of our study.

5 Conclusions

In summary, the EE during six weeks altered physiological variables by reducing plasma corticosterone levels, and systolic blood pressure in both BALB/c and C57BL/6 strains, as well as reduced gastrointestinal transit time in C57BL/6 mice. Our results show that it is necessary to know the rodent strain differences to choose the better experimental model and the importance of including EE, especially for the C57BL/6 strain, where there seems to be more physiological impact.

Author contributions L.A.G. designed and performed experiments, analyzed, and interpreted the data, and wrote the paper; M.A.S.O.A. performed experiments, analyzed and interpret analyses; P.C.S.S. interpretated of data and revising it critically for important intellectual content; A.F.M.B. performed experiments, analyzed and interpret analyses; F.R.C.G. interpretated of data and revising it critically for important intellectual content; M.F.A. conceived the study, analyzed and interpreted the data, wrote and revised the paper. All authors read and approved the final manuscript.

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Data availability The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Competing interests The authors declare no competing interests.

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