BJPS

Brazilian Journal of Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902024e23474

Article

The gestational exposure to *Luffa operculata* and a late stressor in young Wistar rats induce sex-specific behavioral, inflammatory, and stress hormone responses

Ivana Barbosa Suffredini[1,5*], Cinthia dos Santos Alves[1], Humberto Vieira Frias[1], Leoni Villano Bonamin[1], Eduardo Fernandes Bondan[1], Cidéli de Paula Coelho[2], Maria de Fátima Monteiro Martins[3], Yasmin de Oliveira Santos[4], Maria Martha Bernardi[1]

[1]Graduate Program in Environmental and Experimental Pathology, Paulista University, São Paulo, SP, Brazil, [2]Santo Amaro University, São Paulo, SP, Brazil, [3]Cruzeiro do Sul University, São Paulo, SP, Brazil, [4]Health Sciences Institute, Paulista University, São Paulo, Brazil, [5]Center for Research in Biodiversity, Paulista University, São Paulo, Brazil

Luffa operculata (L.) Cogn. (Cucurbitaceae) aqueous extract (EBN) was gestationally administered to Wistar rats to evaluate sex-related behavioral changes in young individuals receiving a two-hit stimulus of 1 mg/kg EBN, followed by stress challenge (NYM) or lipopolysaccharide (LPS) exposure. EBN exposition occurred by gavage at gestational days (GDs) 17 to 21, and NYM or LPS was conducted at post-natal day 60 (PND60). Behavior was evaluated in the open field (OF) and light-dark box (LDB) apparatuses. Serum hormones, pro-inflammatory cytokines, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatinine were measured. The liver and kidneys were histologically evaluated. Male and female young rats responded differently in latency to the light side and locomotion frequency on the dark side, corticosterone (females, LPS), and ACTH (females, stress). Male rats showed improved TNF-α (LPS group) and melatonin (LPS group). No histological or biochemical differences were observed in the liver or kidneys. Female young rats were more resilient to the two-hit-stimulus than the male rats. The gestational administration of EBN to male and female Wistar rats and a second stimulus (stress challenge or LPS exposure) at a young age allowed for discriminating differences in behavior, stress hormones, and cytokines according to the sex of the rat. However, no histological alterations in the liver or kidneys were observed, nor in the AST, ALT, or creatinine levels.

Keywords: Animal behavior. *Buchinha-do-norte*. Cognitive performance. Interleukins. Reproduction. Gestational treatment. Reproductive pharmacology. Traditional medicine Meso- and Southern America.

HIGHLIGHTS

- *L. operculata* intake followed by stress-challenge/LPS exposure provokes behavior and stress alterations in male and female rats
- Young females are susceptible pro-inflammatory cytokine and grooming improvement
- Young males are susceptible to behavior, corticosterone, melatonin, and TNF-α alterations

INTRODUCTION

Buchinha-do-norte, or *Luffa operculata* (L.) Cogn. Cucurbitaceae (Flora do Brasil, 2023; theplantlist.org, 2023], is widely used in Brazil for human and animal

***Correspondence:** I.B. Suffredini. Av. Paulista, 900, 1 andar, Bela Vista. 01310-100, São Paulo, SP, Brasil. Phone: 55 11 3170-3776. FAX: 55 11 3170-3978. E-mails: ivana.suffredini@docente.unip.br; ibsuffredini@yahoo.com.br. https://orcid.org/0000-0002-5237-101X.

purposes. People use fruit tea as an abortifacient, and to treat sinusitis. The dried fruit, also known as loofah or *bucha*, is used to prepare a tea that is consumed to provoke abortion (Barilli, Santos, Montanari, 2007; Nunes, Silva, 2021; Revilla, 2002) or is instilled in the nostrils to remove the mucus obstructing breathing during sinusitis (Menon-Miyake *et al*., 2005a,b). The plant is widespread in Brazil and is available to treat sinusitis, although it is easily consumed to provoke abortion. The tea is also used to remove worms from goats (Boelter, 2003).

There are studies in the specialized literature reporting on the plant's toxicological, antimicrobial, cytotoxic, and antioxidant activities. Regarding plant toxicity, our group reported the effects of 1.0 mg/kg *L. operculata* aqueous fruit extract (EBN) administered orally to adult Wistar rats (Alves *et al*., 2018, 2021), and in gestationally exposed male and female Wistar rats (Alves *et al*., 2023; Frias *et al*., 2021). There were different levels of toxicity and behavioral disruption in the rats. Nonetheless, there is a lack of information regarding the sexual dimorphism between male and female pups exposed to EBN during the final period of gestation, which is crucial for the regular and healthy development of the central nervous system (CNS).

MATERIAL AND METHODS

Plant extract and dose used in the experiments

L. operculata fruits were acquired (lot SantosFloraBUCHO01/0914, collection on 09/24/2014, valid until 09/24/2017, Brazil). A decoction was made with the dried fruits with seeds, in the proportion of 7.0 g/L, for 10 minutes to emulate the method of preparation in popular use. After cooling, the decoction was frozen and lyophilized (EBN; 42.7% yield). The EBN was prepared at 1.0 mg/mL, a dose that did not provoke the reabsorption of the rat's embryos, as described elsewhere (Alves *et al*., 2021).

Animals

Ethics

The experiments were conducted under Ethics Committee for Animal Use (ECAU) protocol #043/2016, and the 3Rs concepts were adopted as guidance to reduce animal suffering and promote animal well-being during the experiments.

Experimental design

Wistar rat male and female pups (F1 generation) were submitted to a two-hit treatment composed of a five-day-multiple-dose gestational exposure to EBN on gestational days 17 to 21 (GD17-GD21), and a posterior stress challenge or LPS exposure at post-natal day 60 (PND60). The experiments were carried out according to the design described in Figure 1. Starting with the reproduction protocol, the female dam rats were divided into two groups: one that received the EBN (the experimental group, EG), and the other that received water as the vehicle (the control group, CG).

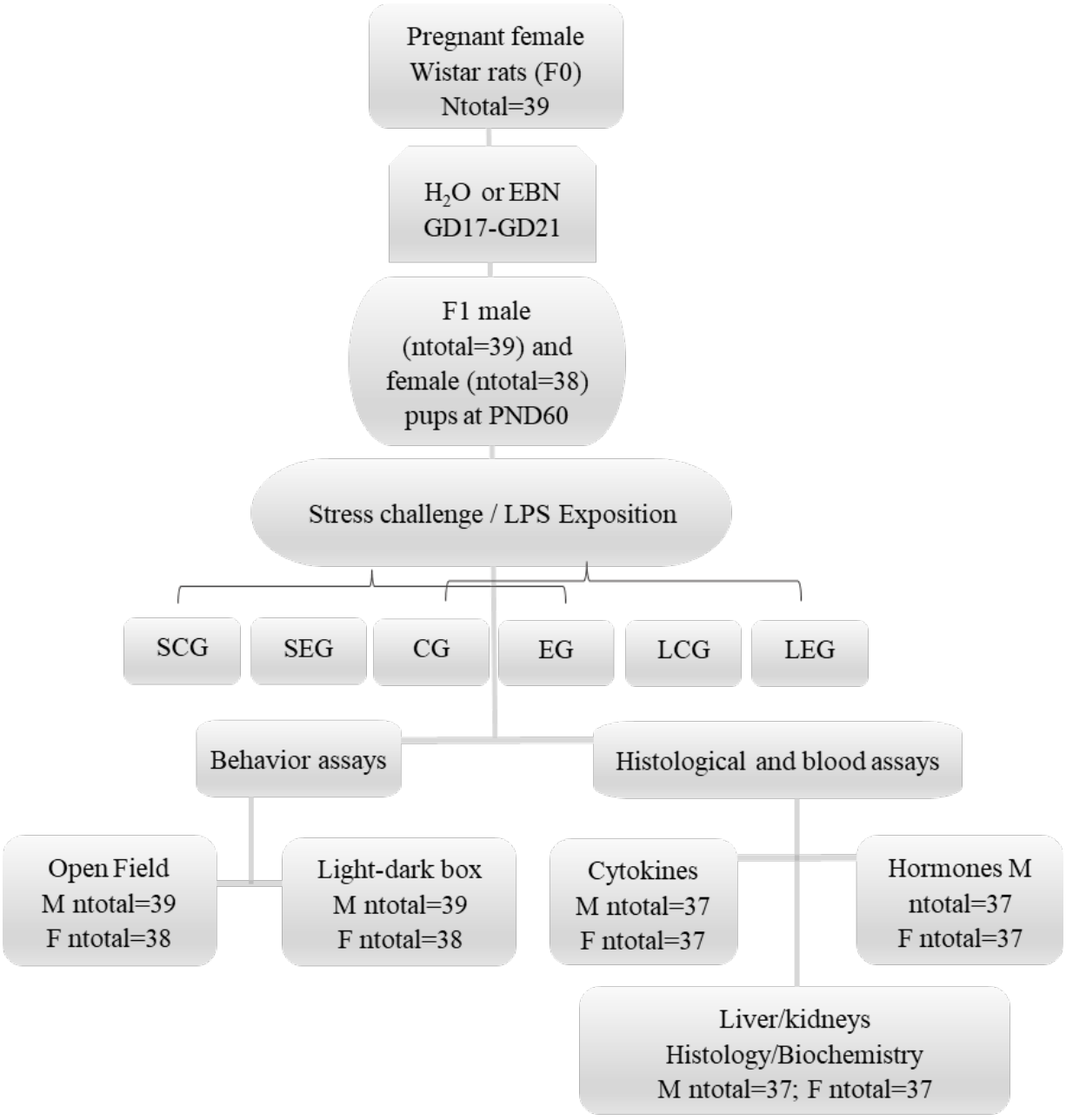

FIGURE 1 – The experimental design of the study performed with male and female Wistar rat pups after gestational treatment with *Luffa operculata* aqueous extract and posterior stress challenge or LPS exposure at PND60. EBN=*buchinha-do-norte* aqueous extract; GD=gestation day; PND=postnatal day; M=F1 male rats; F=F1 female rats.

Mating and birth protocol

Sex-inexperienced female Wistar rats and sex-experienced male Wistar rats were donated by the *Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo*, Brazil. The animals were allowed a ten-day habituation period in the laboratory before beginning the experiments. The mating protocol was carried out as previously described (Alves *et al*., 2021; Frias *et al*., 2021). The animals had free access to filtered water and irradiated food (BioBase®, Águas Frias, Brazil).

Treatment of pregnant rat dams to promote offspring gestational exposure

The pregnant rats from the experimental group (EG, n=19) received 1.0 mg/kg of EBN, which corresponds to one quarter of the abortive dose (Barilli, Santos, Montanari, 2007), administered by gavage for five consecutive days, from GD17 to GD21 (Alves *et al*., 2018). The pregnant rats from the control group (CG, n=20) received water as the vehicle control, corresponding to 100 mL/kg, but not exceeding 1 mL to ensure animal well-being.

Litter standardization

The births occurred at GD21±1. At PND1, no manipulations were made in the pups (F1 generation) to prevent maternal rejection or cannibalism (DeSantis, Schmaltz, 1984). At PND2, the pups were manipulated over a heat blanket adjusted to a physiological temperature. The manipulator's gloves were first rubbed in the cage shaving bed. The pups' sexual standardization was determined by measuring the distance between the sex organ, not yet wholly developed and distinguishable, and the anus (JHU, 2020; Witschi, 1962), where the smallest distance between the orifices corresponded to a female pup. At PND2, each litter was gauged into four males and four females (Udo *et al*., 2014) to avoid differences in the development of the pups due to maternal care, including milk supply and pup leak frequency. The pups were weaned at PND21, and animals of the same sex were housed together in groups of four in each cage. Identifications were made according to their mothers, such as CG and EG (Figure 1).

Stress or lipopolysaccharide (LPS) challenge at PND60

At PND60, all pups were subjected to a stress challenge or LPS exposure before being introduced to the open field (OF) and light-dark box (LDB) behavior apparatuses. The stress challenge was induced according to the New York Metro (NYM) protocol (Dhabhar, McEwen, 1997), as described previously (Alves *et al*., 2018, 2021; Frias *et al*., 2021), resulting in a stress control group (SCG) and a stress experimental group (SEG) from both sexes. LPS exposure (Kirsten *et al*., 2013) was achieved by the intraperitoneal administration of a 100 µg/kg dose to yield the LPS control group (LCG) and LPS experimental group (LEG).

Behavior evaluation
Open field (OF)

The rats' locomotion and anxiety-like behaviors were assessed in a circle OF apparatus (Belovicova *et al*., 2017). Briefly, the animal was placed in the central area of the arena, and remained under evaluation for 3 minutes. Parameters such as the locomotion and rearing frequencies, immobility and grooming times, time spent in the center and the periphery of the apparatus (in seconds), and the number of fecal *boli* produced were analyzed (Alves *et al*., 2018; Estork *et al*., 2016, 2014; Frias *et al*., 2021; Gusmão *et al*., 2013a,b; Suffredini *et al*., 2017). After each evaluation, the apparatus was cleaned with a 5% ethanol solution to avoid animal odor interference.

Light-dark box (LDB)

The animals were also submitted to evaluation in the LDB apparatus to assess anxiety immediately following completion of the OF evaluation. The LDB is a box divided into a dark side (1/3 of the total area) and a light side (2/3 of the total area), separated by a hole that allows the animals to cross sides as they wish.

The experiment followed the format previously described in other studies (Alves *et al*., 2021; Frias *et al*., 2021) to assess behavioral changes. Briefly, the rat was placed on the dark side of the apparatus (Takao, Miyakawa, 2006) and observed for 5 minutes (Shimada *et al*., 1995). Behavioral parameters related to locomotion and anxiety were assessed (Alves *et al*., 2018, 2021; Crawley, Goodwin, 1980; Frias *et al*., 2021).

Pro-inflammatory cytokines, hormone, biochemical, and histological studies

Immediately following the behavioral testing, the pups were euthanized and blood was collected. The blood was centrifuged at 2,000 rpm for 15 minutes to

remove serum, which was used to quantify the cytokines interleukin-1α (IL-1α), IL-1β, IL-6, and tumor necrosis factor-α (TNF-α) using the Magpix RECYTMAG 65K kit (Corrêa *et al*., 2018), and the hormones involved in stress (corticosterone, adrenocorticotropic hormone (ACTH), and melatonin) using the Magpix RESHMAG 59K kit (Faria *et al*., 2013).

Liver and kidney histological and biochemistry studies

Liver and kidney tissues were prepared according to the previously described conventional paraffin embedding and hematoxylin-eosin (HE) staining method (Alves *et al*., 2021; Frias *et al*., 2021). The evaluation was based on scores for parameters linked to liver degeneration, such as pyknosis, karyorrhexis, karyolysis (related to the nucleus morphology), and cytoplasm vacuolization. The glomerular hypercellularity and the tubular vacuolization related to kidney degeneration were scored. The scores were between 0 and 2, where (0) means absence, (1) means moderate, and (2) means severe expressions of the parameter being analyzed.

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatinine (CRE) were quantified in the serum by the biochemical analyzer Cobas C111® (Roche® Diagnostics, Basel, Switzerland) and commercial reagents AST IFCC code 04657543190, ALTL code 04718569190, and CREJ2 code 04718569190, as previously described (Borzouie *et al*., 2020).

Statistical analysis

Statistical premises such as independence, randomness, and normality assays were considered to perform the analysis. Data were tested for normality using the Shapiro-Wilk test. Two-way ANOVA/Tukey's, or Kruskal-Wallis/Dunn's post-tests were then chosen accordingly to be applied for the comparison of the means or medians of the groups. The sample size was determined using the equation $\{n=1+[2C*(s/d)^2], C=(z\alpha+z\beta)^2\}$, where the confidence interval was 0.95/2 (0.475); z=1.96; the test power was 90% (zβ=1.282); the maximum deviation 0.2; and the difference between groups 0.5, resulting in n=4.4, or five animals per group (Eng, 2003). Significant statistical findings were analyzed by principal component analysis (PCA), considering 18 variables composed of the parameters used in the analysis and 64 cases consisting of the total number of rats participating in the assays. Statistical significance was defined as α=0.05 (Zar, 1999). The Excel statistics package Realstats®, GraphPad Prism 7.0 and 9.0, and MVSP Multivariate Statistical Package (Kovach Comp.) were used for the calculations.

Evaluation of total cucurbitacins

Total cucurbitacins were isolated by two methods: partition and solid-liquid extraction. The partition was performed with 1 g EBN diluted in 50 mL Milli-Q grade water. After solubilization, the solute was transferred to a 125 mL separatory funnel. Three dichloromethane (DCM) portions of 34 mL were subsequently added to the separatory funnel, which was shaken to ensure intimate contact between the phases. The three DCM partitions were reunited to obtain Fraction DCM.LL. A similar partition was then made with three subsequent portions of 34 mL ethyl acetate, which were reunited to obtain Fraction AcOEt. LL. The residue was named Fraction H_2O.LL.

The solid-liquid extraction was performed by adding 100 mL DCM to a 250 mL beaker containing 1 mL EBN, and then vortexing them in a magnetic stirrer for 30 minutes. The supernatant was then filtered to give Fraction DCM.SL. Following this, 100 mL ethyl acetate was added to the beaker containing the cake, and the set was allowed to stir for another 30 minutes. The supernatant was filtered to obtain Fraction AcOEt.SL, and the remaining cake was named Fraction H_2O.SL.

Thin-layer chromatography analysis was performed to observe the cucurbitacin separation of both techniques. The system used a 7 × 15 cm pre-coated TLC sheet ALUGRAM® Xxtra SIL G/UV_{254} plates (Macherey-Nagel, lot 802046) and two mobile phases composed of chloroform:methanol (19:2) and ethyl acetate:methanol:water (38.5:7.5:4). As the revealing system, a vanillin phosphate VP41 solution was prepared according to previous descriptions (Wagner, Bladt, 1996). After VP41 nebulization, the plates were incubated at 100°C for 10 minutes and were visualized under natural light. Ten microliters of each sample were spotted on the plates.

RESULTS

Behavioral studies of male and female pups

Open field (OF)

In the groups that received the NYM stress challenge, treatment accounted for 31.89% of the total variance in locomotion frequency ($F_{3,43}$=8.173, p=0.0002), regardless of sex ($F_{1,43}$=4.014, p=0.0514), and the interaction between both factors ($F_{3,43}$=1.239, p=0.3072) accounted for less of the total variance (5.22% and 4.84%, respectively). Although differences in treatment were observed, there were no significant differences in locomotion frequency between the sexes (Figure 2A).

A similar observation was made in the rearing frequency (Figure 2B), in which treatment accounted for 28.11% of the total variance ($F_{3,44}$=6.25, p=0.0013), despite sex ($F_{1,44}$=0.03, p=0.8602), and the interaction between both factors ($F_{3,44}$=1.06, p=0.3764) accounted for less of the total variance (<0.1% and 4.76%, respectively). Although differences in treatment were observed, there were no significant differences in rearing frequency between the sexes (Figure 2B).

Females from the SCG group spent a significantly greater time performing grooming compared to the F1 male and female EG groups, and F1 females from the SEG group performed more grooming (Figure 2C) than F1 females from the EG group. There were significant differences in the grooming response between sexes (K-$W_{8,47}$=33.82, p<0.0001). No statistical differences in immobility (K-$W_{8,45}$=10.29; p=0.1729), time spent in the center (K-$W_{8,51}$=5.692; p=0.5762), or time spent in the periphery (K-$W_{8,51}$=5.692; p=0.5762) were observed.

F1 female rats from the LCG group locomoted more frequently than the males from the CG and EG groups (Figure 2D). Regardless of sex, there was significant variance ($F_{1,40}$=15.24, p=0.0004; accounting for 23.86% of the total variancep<0.05 for F1 males and female LEG groups). Treatment and interaction accounted for 8.86% and 6.07% of the total variance, respectively. Interaction accounted for 17.57% of the total variance in the rearing frequency (Figure 2E), while sex and treatment accounted for 2.80% and 10.66%, respectively. The interaction showed statistical significance ($F_{3,42}$=3.57, p=0.0218), while both variables, treatment and sex, did not show significance ($F_{3,42}$=2.16, p=0.1066, and $F_{1,42}$=1.70 p=0.1989, respectively). Differences were observed in the CG and LCG groups, and there was no significance between F1 male and female LCG groups (p<0.05).

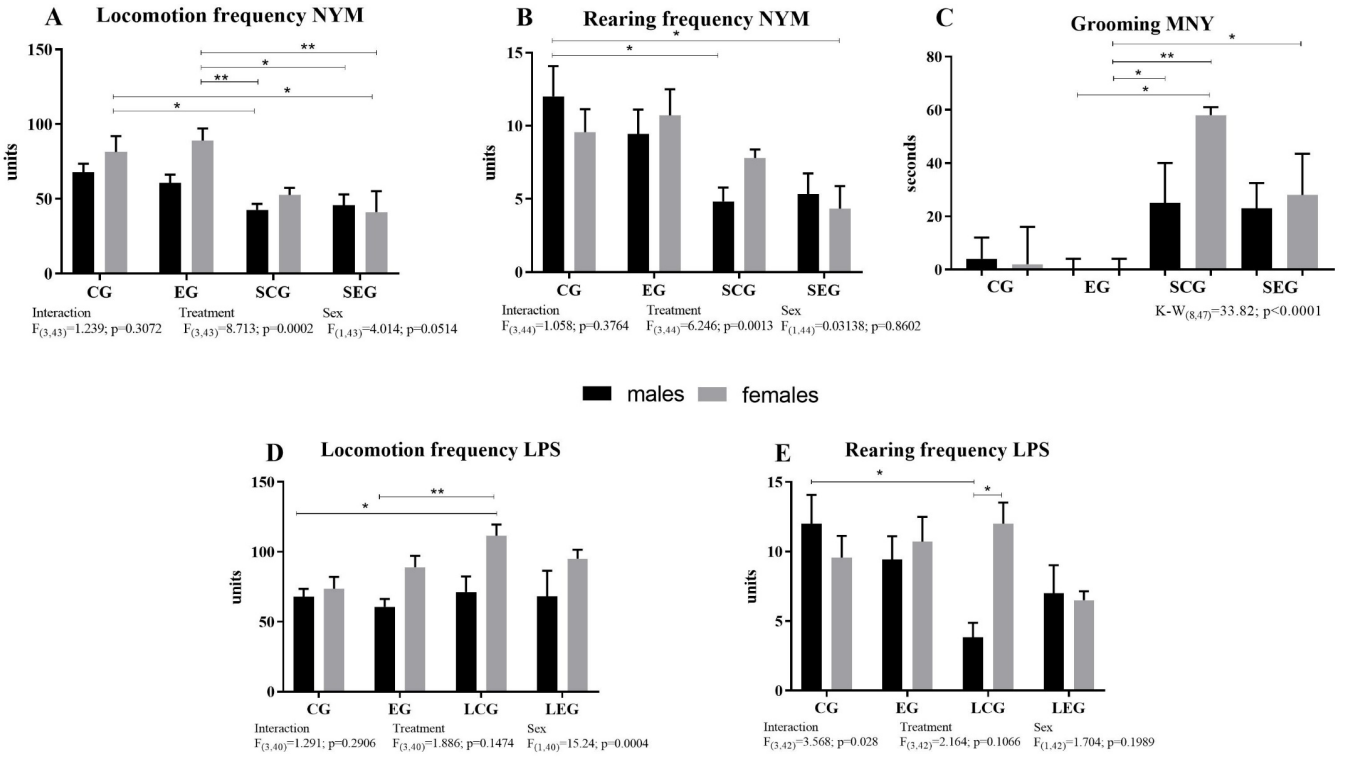

FIGURE 2 - Open field apparatus data comparing the behavior of young male and female F1 generation Wistar rats after being gestationally treated with *Luffa operculata* fruit aqueous extract and then challenged with New York Metro (NYM) stress or exposed to lipopolysaccharide (LPS). **A.** Locomotion frequency after NYM challenge; **B.** Rearing frequency after NYM challenge; **C.** Grooming after NYM challenge; **D.** Locomotion frequency after LPS exposure; **E.** Rearing frequency after LPS exposure. Two-way ANOVA/Tukey's post-test or Kruskal-Wallis/Dunn's post-test. Significance $\alpha<0.05$.

Light-dark box (LDB)

Figure 3 shows the male and female behaviors under LDB evaluation after gestational exposure to EBN and being submitted to the NYM stress challenge at PND60. F1 male and female rats took longer to enter the light side ($K\text{-}W_{8,47}=27.75$; p=0.0002; Figure 3A) compared to the CG groups (male CG X male SEG p=0.0135; male CG X female SEG p=0.0297; female CG X male SEG p=0.0157; female CG X female SEG p=0.0330), but no differences were related to sex in the F1 male and female SEG groups. The F1 female SEG group made more attempts ($K\text{-}W_{8,49}=24.44$; p=0.0010; Figure 3B) to enter the light side than the male CG (p=0.0081) and female CG (p=0.0180) groups. The F1 female SEG group crossed between the sides ($K\text{-}W_{8,46}=26.95$; p=0.0003; Figure 3C) less frequently than the F1 female CG (p=0.0368) and EG (p=0.0254) groups. In terms of locomotion (Figures 3D and 3E) and rearing (Figure 3F) frequencies, the F1 female SEG group showed a significant impairment of exploration, both horizontally ($K\text{-}W_{8,50}=31.21$; p<0.0001) and spatially ($K\text{-}W_{8,49}=26.08$; p=0.0005), relative to the CG and EG groups. There were no significant differences in exploration factors between the F1 male and female SCG and SEG groups (p>0.05). A subtle augmentation in grooming ($K\text{-}W_{8,43}=20.59$; p=0.0044; Figure 3G) was observed in the F1 female SEG group in relation to the F1 female CG group (p=0.0217). Males and females from the SEG group remained in the light side less than the female EG group ($K\text{-}W_{8,50}=25.16$; p=0.0007; Figure 3H). F1 male and female rats from the SEG group remained on the dark side for longer (Figure 3I) compared to the CG group (males p<0.0490, females p=0.0369), although males and females remained on the dark side for the same length of time (p>0.05).

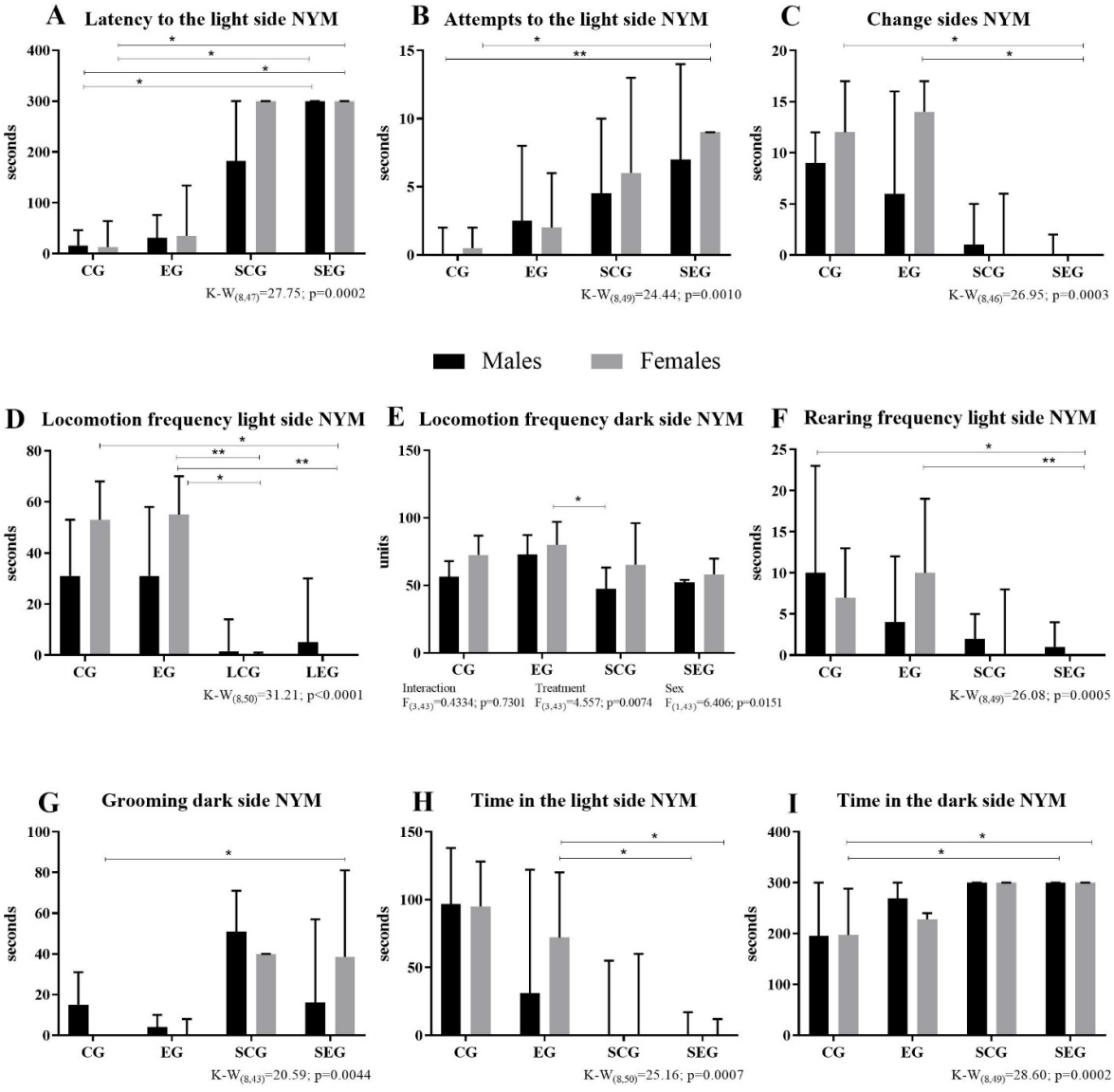

FIGURE 3 - Light-dark box apparatus data comparing the behavior of young F1 generation male and female Wistar rats after being gestationally treated with *Luffa operculata* fruit aqueous extract and then challenged with New York Metro (NYM) stress. **A.** Latency to the light side; **B.** Attempts to the light side; **C.** Changes between sides; **D.** Locomotion frequency in the light side; **E.** Locomotion frequency in the dark side; **F.** Rearing frequency in the light side; **G.** Grooming in the dark side; **H.** Time spent in the light side; **I.** Time spent in the dark side. Two-way ANOVA/Tukey's post-test or Kruskal-Wallis/Dunn's post-test. Significance $\alpha<0.05$.

Figure 4 shows the results of the LDB apparatus behavioral parameters assessed for the F1 male and female rats exposed to LPS at PND60. F1 male and female rats took longer to enter the light side (Figure 4A), and there were significant differences in interaction ($F_{3,38}$=3.576; p=0.0226; 11.97% of total variance), treatment ($F_{3,38}$=10.96; p<0.0001; 36.71% of total variance), and sex ($F_{1,38}$=8.473; p=0.0060; 9.46% of total variance) between the male LEG and male CG (p=0.0040); male LEG and female CG (p=0.0069); male LEG and male EG (p=0.0265); and male LEG and female EG (p=0.0288) groups. Figure 4D shows the locomotion

frequency results on the dark side (female CG and male LCG p=0.0348; male EG and male LCG p=0.0259; female EG and male LCG p=0.0038; male LCG and female LCG p=0.0101; LCG male and LEG female p=0.0059). Figures 4B, 4C, 4E, and 4F, which represent time on the dark side, locomotion frequency on the dark side, and rearing frequency on the light and dark sides, show that the statistical differences were not meaningful to the present analysis because no differences between LEG groups or between sexes were found.

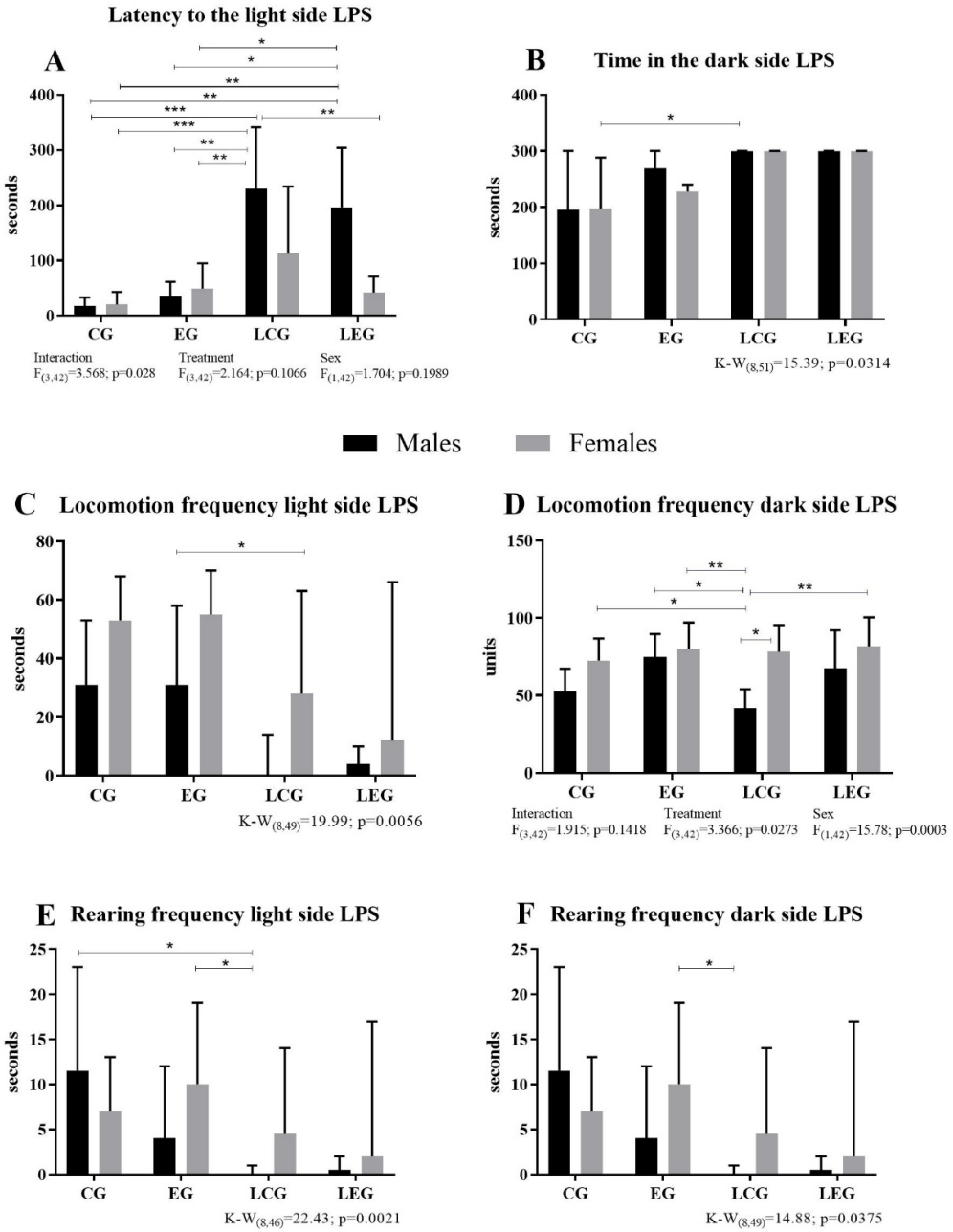

FIGURE 4 - Light-dark box apparatus data comparing the behavior of young F1 generation male and female Wistar rats after being gestationally treated with *Luffa operculata* fruit aqueous extract and exposed to lipopolysaccharide (LPS). **A.** Latency to the light side; **B.** Time spent in the dark side; **C.** Locomotion frequency in the light side; **D.** Locomotion frequency in the dark side; **E.** Rearing frequency in the light side; **F.** Rearing frequency in the dark side. Two-way ANOVA/Tukey's post-test or Kruskal-Wallis/Dunn's post-test. Significance $\alpha<0.05$.

Serological studies on the dams

Hormones

Figure 5 shows the levels of serum hormones related to behavior changes, such as corticosterone, ACTH, and melatonin. After the NYM stress challenge, corticosterone levels (Figure 5A) were not significantly different between the groups, considering the interaction between factors ($F_{3,42}$=0.01007; p=0.9986), treatment ($F_{3,42}$=2.646; p=0.614), and sex ($F_{1,42}$=0.03551; p=0.8514). ACTH (Figure 5B) was higher in the F1 female SEG group compared to the female EG group (p=0.0214 between groups; K-$W_{8,47}$=22.73; p=0.0019). Melatonin (K-$W_{8,42}$=26.08; p=0.0005; Figure 5C) was augmented in the male EG group in relation to the female EG group (p=0.0046) and the female CG group (p=0.0188).

In the animals exposed to LPS, corticosterone levels (Figure 5D) were greater in the male LEG group compared to the female LCG group (p=0.0390 between groups; K-$W_{8,48}$=12.99; p=0.0722), although results showed a type I error. No differences were observed among the means for ACTH (K-$W_{8,45}$=13.69; p=0.0571; Figure 5E). Melatonin (Figure 5F) was elevated in the male EG group that received LPS compared to female CG (p=0.0305) and female EG (0.0110) groups, while levels were lower in the male EG group relative to the female EG group (p=0.0208).

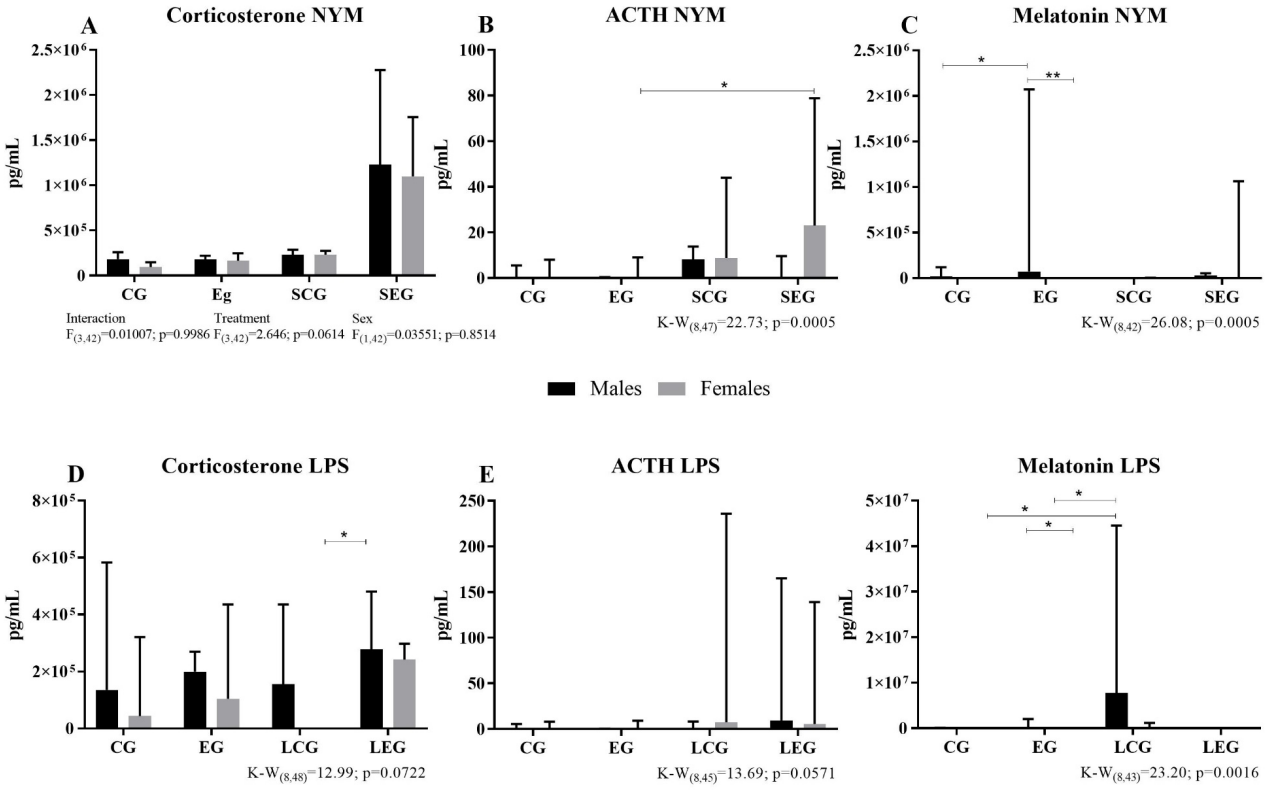

FIGURE 5 - Hormone quantification in the serum of young male and female rats exposed to *Luffa operculata* dried aqueous fruit extract and the New York Metro (NYM) stress challenge or exposed to lipopolysaccharide (LPS) at postnatal day 60. **A.** Corticosterone after NYM stress challenge; **B.** ACTH after NYM stress challenge; **C.** Melatonin after NYM stress challenge; **D.** Corticosterone after LPS exposure; **E.** ACTH after LPS exposure; **F.** Melatonin after LPS exposure. ACTH=adrenocorticotropic hormone. Two-way ANOVA/Tukey's post-test or Kruskal-Wallis/Dunn's post-test. Significance $\alpha<0.05$.

Pro-inflammatory cytokines

Figure 6 shows the serum pro-inflammatory cytokine quantification of F1 male and female Wistar rats gestationally treated with EBN and challenged with the NYM stress challenge or exposed to LPS at PND60. There was no difference in the cytokine levels of animals challenged with NYM stress. For IL-1α levels (Figure 6A), the interactions between factors ($F_{3,41}$=1.786; p=0.1649), treatment ($F_{3,41}$=0.7408; p=0.614), and sex ($F_{1,41}$=5.149; p=0.0286, probable error type II) were not significant. In addition, neither IL-1β (K-$W_{8,48}$=9.886; p=0.1951; Figure 6B), IL-6 (K-$W_{8,46}$=14.29; p=0.0462, possible error type II; Figure 6C), nor TNF-α (K-$W_{8,48}$=4.604; p=0.7081; Figure 6D) showed differences between the groups.

In the rats exposed to LPS, interaction between factors ($F_{3,42}$=0.5312; p=0.6633), treatment ($F_{3,42}$=0.6941; p=0.5608), and sex ($F_{1,42}$=2.707; p=0.1074) did not significantly affect the IL-1α levels among the groups (Figure 6E). IL-1β levels were significantly different (K-$W_{8,45}$=28.92; p<0.0001; Figure 6F) and were augmented in the female LCG group relative to the male EG (p=0.0009), male CG (p=0.0159), and female EG (p=0.0038) groups. IL-6 levels (K-$W_{8,43}$=26.79; p=0.0004; Figure 6G) were significantly higher in the female LCG group relative to the male CG (p=0.0119), female CG (p=0.0263), and male EG (p=0.0301) groups. TNF-α levels (K-$W_{8,44}$=29.29; p=0.0001; Figure 6H) were significantly higher in the male LEG group relative to the male EG (p=0.0134) and female EG (p=0.0425) groups, while the female LCG group had higher levels than the male EG group (p=0.0340).

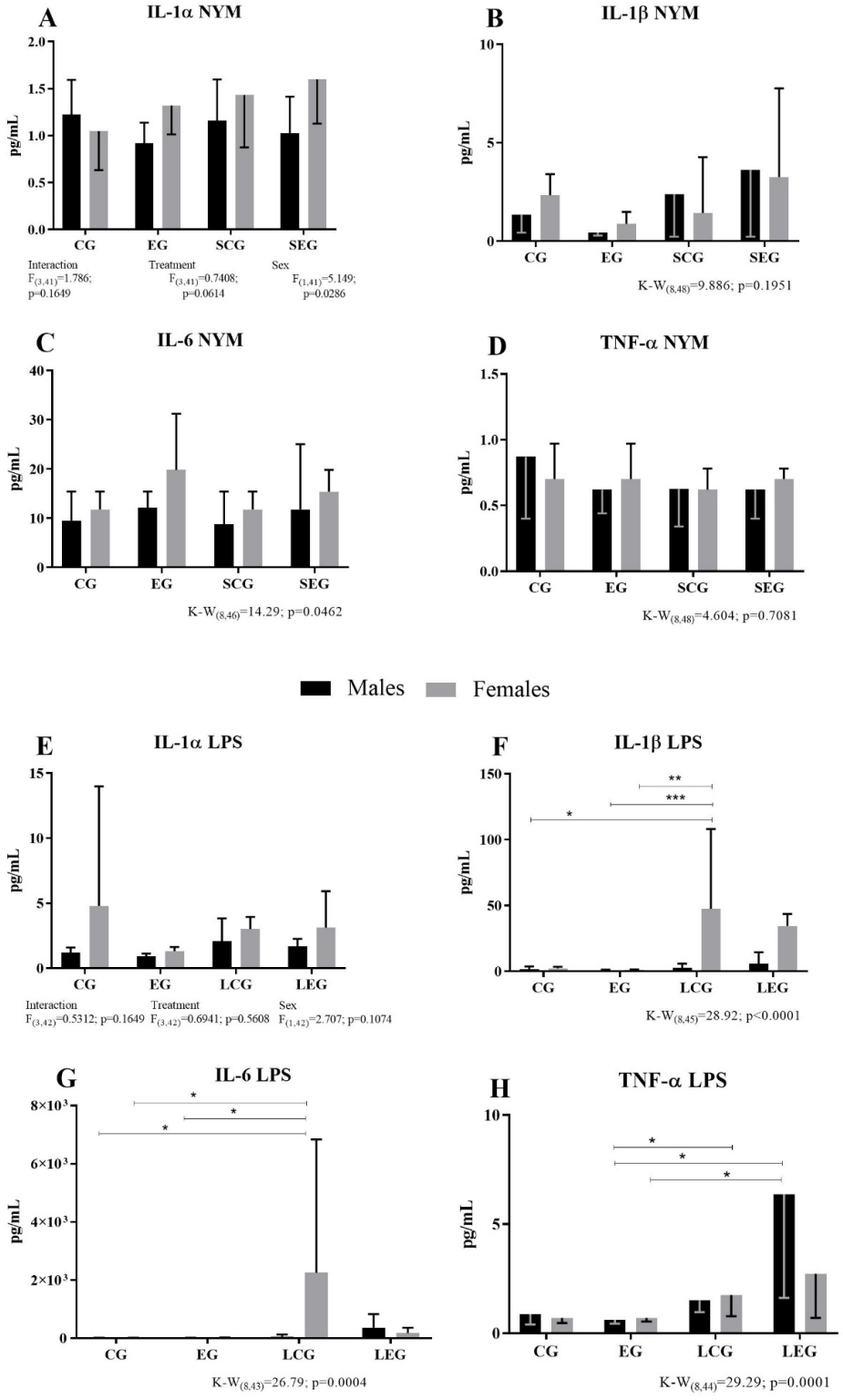

FIGURE 6 - Pro-inflammatory cytokine quantification in the serum of young male and female rats exposed to *Luffa operculata* dried aqueous fruit extract and the New York Metro (NYM) stress challenge or exposed to lipopolysaccharide (LPS) at postnatal day 60. **A.** IL-1α levels after NYM stress challenge; **B.** IL-1β levels after NYM stress challenge; **C.** IL-6 levels after NYM stress challenge; **D.** TNF-α levels after NYM stress challenge; **E.** IL-1α levels after LPS exposure; **F.** IL-1β levels after LPS exposure; **G.** IL-6 levels after LPS exposure; **H.** TNF-α levels after LPS exposure. Two-way ANOVA/Tukey's post-test or Kruskal-Wallis/ Dunn's post-test. Significance $\alpha<0.05$.

Liver and kidney studies

Microscopic alterations in the liver and kidneys

The livers and kidneys of the animals that underwent the NYM stress challenge showed no statistical difference in terms of tubular vacuolization ($K\text{-}W_{8,52}$=7.186; p=0.4097 and $K\text{-}W_{8,49}$=14.15; p=0.0486, possible error type II, respectively). Similarly, there was no statistical difference in tubular vacuolization in the liver and kidneys of animals exposed to LPS ($K\text{-}W_{8,53}$=4.754; p=0.6900 and $K\text{-}W_{8,47}$=6.957; p=0.4334). Figure 7 shows the histological features of the liver (males - Figures 7A to 7F, females - Figures 7G to 7L) and kidneys (males - Figures 7M to 7R, females - Figures 7S to 7X) removed from rats exposed to gestational EBN administration and stress challenge (Figures 7C, 7D, 7I, 7J, 7O, 7P, 7U, and 7V), or gestational EBN and exposure to LPS (Figures 7E, 7F, 7K, 7L, 7Q, 7R, 7W, and 7X, and Table I). The vehicle control groups can be seen in Figures 7A, 7G, 7M, and 7S, and the experimental groups in Figures 7B, 7H, 7N, and 7T.

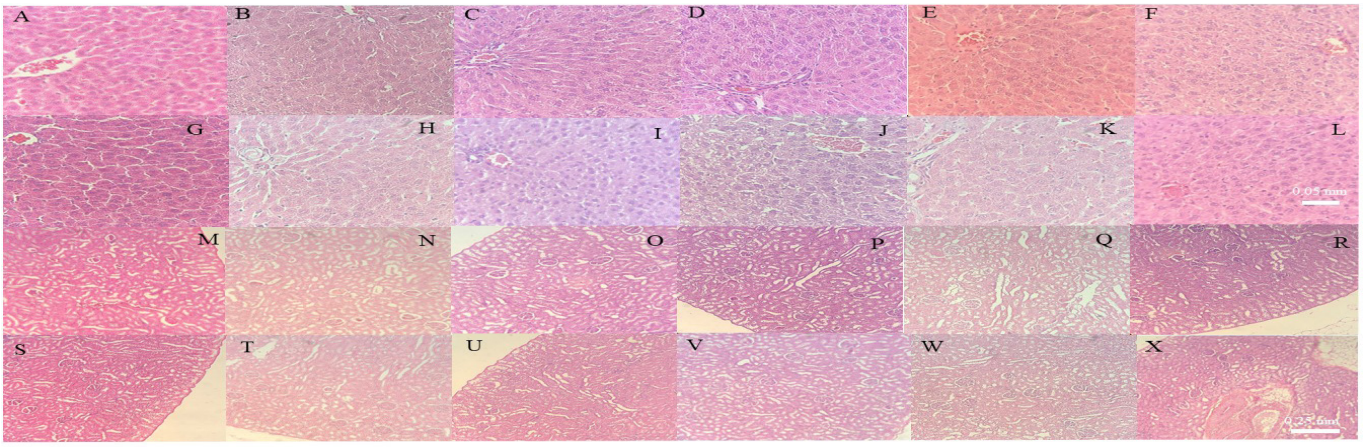

FIGURE 7 - Histological aspects of the liver (males - Figures 7A to 7F, females - Figures 7G to 7L) and kidneys (males - Figures 7M to 7R, females - Figures 7S to 7X) removed from rats exposed to gestational EBN administration and stress challenge (Figures 7C, 7D, 7I, 7J, 7O, 7P, 7U, and 7V), or gestational EBN and exposure to LPS (Figures 7E, 7F, 7K, 7L, 7Q, 7R, 7W, and 7X). The histology of the vehicle control groups is shown in Figures 7A, 7G, 7M, and 7S, and the experimental groups in Figures 7B, 7H, 7N, and 7T.

TABLE I - Scores obtained from the histometric analysis performed with kidneys as liver from male and female young Wistar rats treated with the aqueous extract of Luffa operculata and a late stress challenge or LPS administration

Liver parameters NYM stress challenge							
Cell nucleus							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
3	3	3	3	3	3	3	3
3	3	3	3	3	3	3	3
3	3	3	3	3	3	3	3
3	3	3	3	3	3	3	3
3	3	3	3	3	3	3	3
3	3	3		3	3	3	
3		3		3		3	
3							

TABLE I - Scores obtained from the histometric analysis performed with kidneys as liver from male and female young Wistar rats treated with the aqueous extract of Luffa operculata and a late stress challenge or LPS administration

Liver parameters NYM stress challenge							
3							
Nucleus in regeneration							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1
3	2	1	1	1	2	1	1
1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1
1	1	1		1	2	1	
1		1		1		1	
1							
1							
Chromatin condensation							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0		0	0	0	
0		0		0		0	
0							
0							
Picnosis							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0		0	0	0	
0		0		0		0	
0							

TABLE I - Scores obtained from the histometric analysis performed with kidneys as liver from male and female young Wistar rats treated with the aqueous extract of Luffa operculata and a late stress challenge or LPS administration

Liver parameters NYM stress challenge							
0							
Caryorhex							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0		0	0	0	
0		0		0		0	
0							
0							
Cariolysis							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
0	0	0	0	0	0	1	1
0	1	0	0	0	1	1	0
2	0	0	1	0	1	0	0
2	2	0	2	2	2	0	0
0	0	1	0	2	0	1	1
1	1	1		2	2	1	
1		0		0		1	
0							
0							
Vacuole							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
1	0	0	0	0	0	1	0
1	1	0	1	0	0	0	0
0	1	0	1	0	1	0	0
2	1	1	1	2	0	0	1
1	0	1	0	2	0	1	0
2	1	1		1	1	1	
1		0		0		0	
0							

TABLE I - Scores obtained from the histometric analysis performed with kidneys as liver from male and female young Wistar rats treated with the aqueous extract of Luffa operculata and a late stress challenge or LPS administration

Liver parameters NYM stress challenge							
1							
eosinophilly							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
0	0	0	0	3	2	0	1
1	1	0	0	1	1	2	1
1	0	0	2	0	1	0	0
1	1	0	1	1	1	0	0
0	0	0	0	1	0	1	1
1	1	2		1	1	1	
1		0		3		1	
1							
0							
dilatation							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
1	0	0	1	0	2	1	1
1	1	0	0	1	1	2	2
2	1	0	2	0	1	0	1
2	2	0	2	1	1	1	0
1	0	0	0	2	1	2	1
2	1			1	1	1	
1		0		0		2	
Liver parameters LPS administration							
Cell nucleus							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
3	3	3	3	3	3	3	3
3	3	3	3	3	3	3	3
3	3	3	3	3	3	3	3
3	3	3	3	3	3	3	3
3	3	3	3	3	3	3	3
3	3	3	3	3	3	3	3
3				3		3	
3							
3							

TABLE I - Scores obtained from the histometric analysis performed with kidneys as liver from male and female young Wistar rats treated with the aqueous extract of Luffa operculata and a late stress challenge or LPS administration

Liver parameters NYM stress challenge							
Nucleus in regeneration							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1
3	2	1	1	1	2	1	1
1	1	2	1	1	1	1	1
1	1	1	1	1	1	1	1
1	1	2	1	1	2	1	1
1				1		1	
1							
1							
Chromatin condensation							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0				0			
0							
0							
Picnosis							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0				0			
0							
0							

TABLE I - Scores obtained from the histometric analysis performed with kidneys as liver from male and female young Wistar rats treated with the aqueous extract of Luffa operculata and a late stress challenge or LPS administration

Liver parameters NYM stress challenge							
Caryorhex							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0				0			
0							
0							
Cariolysis							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
0	0	1	1	0	0	2	1
0	1	0	1	0	1	0	1
2	0	1	0	0	1	1	0
2	2	2	1	2	2	0	1
0	0	0	1	2	0	0	1
1	1	1	0	2	2	0	0
1				0		1	
0							
0							
Vacuole							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
1	0	0	1	0	0	2	1
1	1	1	1	0	0	1	1
0	1	1	0	0	1	1	0
2	1	1	1	2	0	1	1
1	0	0	1	2	0	0	1
2	1	1	0	1	1	1	1
1				0		0	
0							
1							

TABLE I - Scores obtained from the histometric analysis performed with kidneys as liver from male and female young Wistar rats treated with the aqueous extract of Luffa operculata and a late stress challenge or LPS administration

Liver parameters NYM stress challenge							
eosinophilly							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
0	0	0	0	3	2	1	1
1	1	0	1	1	1	0	2
1	0	1	0	0	1	1	0
1	1	0	0	1	1	0	1
0	0	0	0	1	0	1	1
1	1	0	0	1	1	0	0
1				3		1	
1							
0							
dilatation							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
1	0	1	1	0	2	1	1
1	1	0	1	1	1	0	1
2	1	1	0	0	1	2	0
2	2	0	0	1	1	0	1
1	0	0	1	2	1	2	1
2	1	0	0	1	1	0	0
1				0		1	
Kidney parameters NYM challenge							
glomerular hypercellularity							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0				0	0
0	0	0					
0							
glomerular degeneration							

TABLE I - Scores obtained from the histometric analysis performed with kidneys as liver from male and female young Wistar rats treated with the aqueous extract of Luffa operculata and a late stress challenge or LPS administration

Liver parameters NYM stress challenge							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0				0	0
0	0	0					
0							
tubular vacuolization							
CG.M	EG.M	SCG.M	SEG.M	CG.F	EG.F	SCG.F	SEG.F
0	0	0	0	0	2	2	1
0	0	0	2	0	2	0	0
0	0	0	0	0	0	0	1
0	0	0	0	0	0	1	1
0	0	0	0	0	0	0	0
0	2	0				2	1
0	0	0					
0							
Kidney parameters LPS administration							
glomerular hypercellularity							
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0				0	
0	0						
0							
glomerular degeneration							
CG.M	EG.M	LCG.M	LEG.M	CG.F	EG.F	LCG.F	LEG.F
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0

TABLE I - Scores obtained from the histometric analysis performed with kidneys as liver from male and female young Wistar rats treated with the aqueous extract of Luffa operculata and a late stress challenge or LPS administration

			Liver parameters NYM stress challenge				
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0				0	
0	0						
0							
tubular vacuolization							
CG.M	EG.M	LCG.M	LEG.M	CG.F	EG.F	LCG.F	LEG.F
0	0	0	0	0	2	0	0
0	0	0	1	0	2	0	2
0	0	0	0	0	0	0	1
0	0	0	0	0	0	0	0
0	0	1	0	0	0	1	0
0	2	1				0	
0	0						
0							

Biochemistry of liver and kidneys

Figure 8 shows the biochemical data from the liver and kidney analyses after gestational exposure of F1 male and female Wistar rats to EBN and a posterior challenge with the NYM stress challenge or exposure to LPS at PND60. In the groups challenged with NYM stress, there was no difference in ALT serum levels [Figure 8A; interaction between factors ($F_{3,38}$=1.285; p=0.2935), treatment ($F_{3,38}$=1.81; p=0.1618), and sex ($F_{1,38}$=2.783; p=0.1035)] or AST levels (Figure 8B; interaction between factors ($F_{3,37}$=0.4146; p=0.7435), treatment ($F_{3,37}$=0.9205; p=0.4405), and sex ($F_{1,37}$=5.518; p=0.0243, probable error type II)). However, creatinine levels (K-$W_{8,44}$=19.84; p=0.0059; Figure 8C) were higher in the male and female EG groups relative to the male SCG group (p=0.0498 for both intergroup comparisons). In the LPS group, none of the biochemical parameters showed significant differences (ALT – K-$W_{8,43}$=11.28; p=0.1269; AST – K-$W_{8,44}$=2.892; p=0.8948, and creatinine – K-$W_{8,42}$=17.21; p=0.0161, possible type II error; Figures 8D, 8E, and 8F).

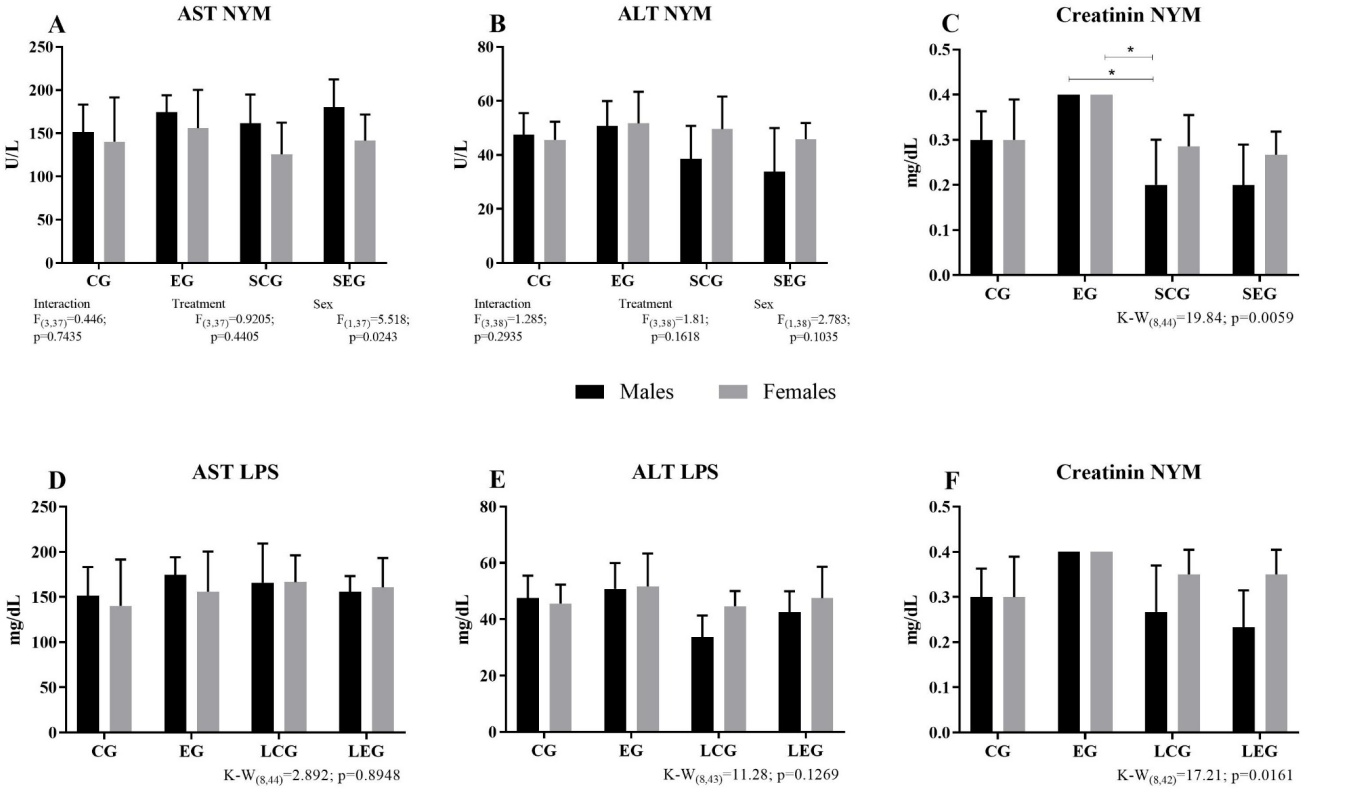

FIGURE 8 - Biochemical parameter quantification (alanine transaminase, ALT; aspartate transaminase, AST; and creatinine) in the serum of young male and female rats exposed to *Luffa operculata* dried aqueous fruit extract and then the New York Metro (NYM) stress challenge or exposed to lipopolysaccharide (LPS) at postnatal day 60. **A.** ALT levels after the NYM stress challenge; **B.** AST levels after the NYM stress challenge; **C.** Creatinine levels after the NYM stress challenge; **D.** ALT levels after LPS exposure; **E.** AST levels after LPS exposure; **F.** Creatinine levels after LPS exposure. Two-way ANOVA/Tukey's post-test or Kruskal-Wallis/Dunn's post-test. Significance $\alpha<0.05$.

Figure 9 shows the PCA obtained from the 18 variables and 64 cases related to the locomotion, hormonal, and cytokine parameters. The PCA yielded a cumulative percentage of 66.354 on the second axis and 83.893 on the third axis, which allowed a gradient formation to be observed by the green (males) and blue (females) colors in the fourth and first quadrants. The formation of the gradient indicates the sexual differences in response to the 18 parameters represented by the vectors. The green and blue sub-groups in the first quadrant are more influenced by ACTH and TNF-α. In contrast, the expressive green and blue sub-groups in the fourth quadrant corroborate the sexual dimorphism observed in the expression of the locomotion parameters, hormones, and cytokines in the pups that received EBN.

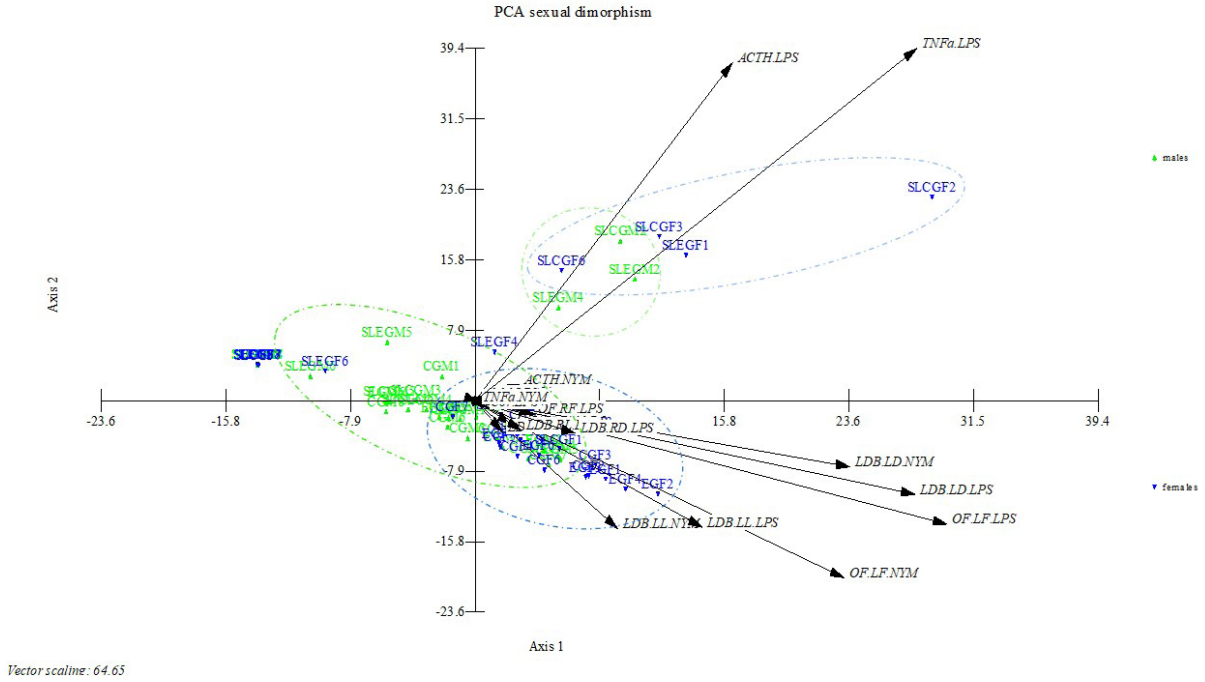

FIGURE 9 - Principal component analysis in the evaluation of the 18 locomotion, hormonal, and cytokine parameters of rat behavior after gestational exposure to EBN and stress challenge or lipopolysaccharide exposure at postnatal day 60.

The presence of curcumin in EBN

The partitioning technique was used to separate the curcumins from EBN, as shown in Figure 10, which depicts the bitter principles present in EBN from TLC analyses.

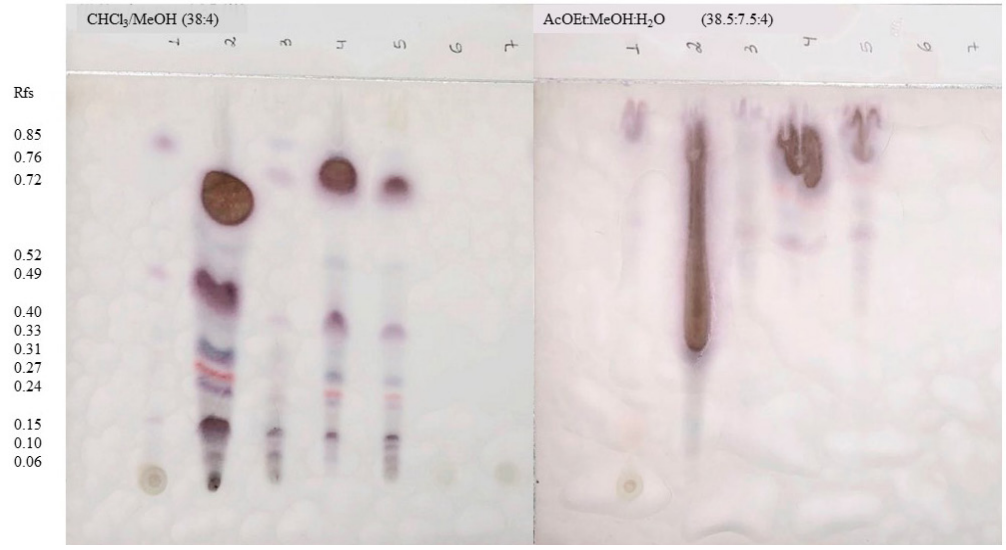

FIGURE 10 - Thin layer chromatogram in silica gel GF_{254} observed under natural light, obtained from elution with **A.** chloroform:methanol (19:2); and **B.** ethyl acetate:methanol:water (38.5:7.5:4). Spots were observed after reaction with vanillin phosphate reagent VP41 followed by application of heat at 100°C (Wagner, Bladt, 1996). 1. Aqueous extract from *Luffa operculata* fruits; 2. Partition fraction dichloromethane (DCM); 3. Partition fraction ethyl acetate (AcOEt); 4. Solid-liquid extraction fraction DCM; 5. Solid-liquid extraction fraction AcOEt; 6. Partition fraction H_2O; 7. Solid-liquid extraction fraction H_2O.

DISCUSSION

Buchinha-do-norte is a fruit widely used in Brazil to treat sinusitis and as an abortifacient. Previous studies have shown that motor and behavioral impairments occur after administration of the aqueous extract obtained from the fruits of *L. operculata* (Alves *et al*., 2018, 2021; Frias *et al*., 2021) on Wistar rats. Nonetheless, such important findings have not been reported in the context of sexual dimorphism. In this study, we analyzed how some behavioral and stress-expression parameters were influenced after male and female young Wistar rats had been orally administered EBN, followed by a stress challenge or LPS administration.

In the present study, the locomotion and rearing frequencies were significantly diminished in both male and female young Wistar rats in the OF. However, significant differences in locomotion frequency were observed between female rats of the CG group and male rats of the SCG groups, which may indicate a possible influence of the stress challenge on male behavior. Based on these findings, we propose that stress is a more critical factor in reducing locomotion in male rats than the EBN treatment. Several studies have reported that female rats typically explore the OF more than male rats (Scholl *et al*., 2019; Simpson *et al*., 2012; Fernandes *et al*., 1999). However, Scholl *et al*. (2019) did not report these differences in the OF. In addition, these authors observed no interference of the estrous cycle on the OF test. Regarding gestational exposure to EBN, no differences between male and female rats were observed in the OF parameters relative to the CG groups.

To evaluate the influence of EBN and posterior challenges on spatial exploration ability, the rearing frequency was analyzed. Stress exposure was associated with less spatial exploration in the male SCG group and the female SEG group, compared to the male CG group. Previously, we showed that stress reduced rearing behavior in male adult rats but that EBN treatment did not, and this is corroborated by the present findings. Thus, prenatal exposure to EBN did not affect the rearing behavior of male rats, although stress did. In the female rats, we observed that both EBN and the stress challenge diminished rearing frequency compared to the males of the control group, which indicates sexually-related differences in this parameter.

Self-grooming was also assessed. Grooming is a relevant rodent behavior related to stress-coping responses directly influenced by an internal or external stimulus. Self-grooming is performed by rodents after stress exposure and is thought to play a role in awakening (Kalueff *et al*., 2016). Our findings are consistent with this concept. Grooming behavior was increased in female rats, after the EBN administration and stress challenge, suggesting a central role of stress influencing this behavior. In addition, females in the SEG group, but not males, exhibited more grooming than females of the EG group. Thus, stress has a relevant role in increasing grooming behavior. Finally, although stress was the most pertinent factor contributing to the increase in grooming, EBN treatment corresponded with diminished grooming in young female rats after the stress challenge.

LPS is a type of endotoxin naturally found in Gram-negative bacteria that is used to emulate infection when inoculated into an organism or biological system. It stimulates the immune system to produce and release cytokines such as IL-1β, TNF-α, and IL-6 (Aderem,Ulevitch, 2000). These specific cytokines are involved in neurodevelopmental losses (Yu *et al*., 2004; Pang, Cai, Rhodes, 2003; Hornig *et al*., 1999) and are related to the response to some diseases in adulthood (Boisse, 2004). LPS reduces exploratory behavior in the open field, which is a relevant sign of sickness behavior (Chen *et al*., 2013).

According to the theory describing sickness behavior as an adaptive strategy, the reduced behavioral responsiveness to immune activation observed in females may be linked to the risks associated with sickness during gestation (Avitsur,Yirmiya, 1999). It is known that infection during the gestational period enhances the risk of spontaneous abortions, preterm labor, stillbirth, and neurodevelopmental losses (Bernardi *et al*., 2014; Lynch,Ghidini, 1993; Johnson, 1994). Females undergoing sexual activity while sick diminish their chances of self-recovery and consequently developing viable offspring (Avitsur,Yirmiya, 1999). LPS modifies female behavior by leading to a protective mechanism that reduces the possibility of conception during a period of sickness.

Regarding the influence of LPS exposure on the behavioral parameters of male and female young rats, we observed that females in the LCG group showed high levels of two inflammatory cytokines, IL-1β and IL-6, which confirmed the effective induction of sickness behavior. Still, the higher amount of TNF-α in young male rats in the LEG group indicated that the males were less sensitized by the anti-inflammatory activity of the EBN cucurbitacins and somehow demonstrated sexually-specific particularities.

The behavior evaluation performed with the LDB apparatus showed that latency to the light side and time spent on both sides of the apparatus did not result in behavior differences between male and female rats. Exposure to LPS in male rats increased the latency to the light side and reduced the locomotion frequency on the dark side. These effects can be attributed to LPS-induced sickness behavior (Dantzer *et al*., 1998). Nonetheless, some degree of behavioral difference related to both sexes was observed in the increased number of attempts to enter the light side, fewer side changes, locomotion and rearing frequencies on the light side, and reduced grooming in the females that received the stress challenge. Female rats in the LDB were more sensitive to stress induced by the aversive side, demonstrating a behavioral specificity related to sex.

LPS exposure induced the sickness behavior in the rats. The young male rats showed an increased latency to enter the light side compared to the female rats, as well as impaired locomotion on the dark side of the apparatus, indicating that the behaviors are related to the sex. As shown in Figures 4B, 4C, 4E, and 4F, no significant differences were observed in the LEG groups, including those related to the differences between sexes. Nonetheless, the results indicate a differentiated sensitization of the control male rats in comparison to control female rats, as seen in the time spent on the dark side (female CG and male LCG $p=0.0324$), rearing frequency on the light side (male CG and male LCG $p=0.0103$; female EG and male LCG $p=0.0117$), and the rearing frequency on the dark side (female EG and male LCG $p=0.0058$), indicating that this model is adequate to prospect for sexual-dimorphic behaviors.

The pro-inflammatory cytokine levels did not differ between the groups of rats submitted to EBN and NYM stress challenge. Nonetheless, increased levels of IL-1β and IL-6 were observed in the female LCG group, confirming that the rats were induced into a sickness behavior state, as described elsewhere (Dantzer *et al*., 1998). The induction of sickness behavior seemed more effective in the female rats, as they had higher levels of IL-1β and IL-6 compared to the male rats.

In the present work, the corticosterone and cytokine levels were not modified in the stress challenge groups analyzed in the OF and LDB apparatus. Corticosterone is the primary neuroendocrine mediator that responds to a stress stimulus. The corticosterone levels in young male rats exposed to LPS in the LEG group was higher than those in the female LCG group, indicating an inferior recovery of the male rats compared to the female rats that received EBN. The ACTH levels were increased in females in the SEG group relative to females of the EG group, indicating that the stress challenge influenced the improvement of ACTH in the young female rats, while LPS exposure did not.

Melatonin levels were higher in the male EG group relative to the females in the CG and EG groups for rats receiving the stress challenge and those exposed to LPS. These results are concordant with the increased levels of TNF-α observed in the male rats exposed to LPS.

The temporal analysis of corticosterone levels showed that the onset of hormone release occurs between 0.5 and 2 hours of restraint stress. In our experiment, blood collection was performed within a maximum of 10 minutes of the behavior evaluations (Odio, Maickel, 1985). The lack of difference in corticosterone after the OF procedure could be derived from the temporal analysis of the hormone. The same reason could be proposed to explain the absence of interference with cytokine levels. The release sequence of a hypothalamic hormone – such as glucocorticoids - triggered by some stress conditions may play a reducing role in defense mechanisms, particularly in those committed to the inflammatory cell-immune response (Dhabhar, 2014). LPS exposure activates the hypothalamus-pituitary-adrenal axis, releasing corticosterone. However, female rats are protected against stress, as already described.

Although time has likely influenced adequate corticosterone quantification after the stress challenge, the ACTH levels were elevated in the females in the LEG group compared to the females in the EG group.

It is known that ACTH may modulate corticosterone synthesis via melanocortin-2-receptors, also known as MC2 receptors (Gehrand *et al*., 2020), which may be indicative of the anxiety-like behavior demonstrated by the female LEG group.

Higher melatonin levels were observed in the male and female experimental groups, indicating that the gestational administration of the extract may have sensitized the animals for melatonin production. Melatonin is related to the circadian cycle; its production is stimulated by the absence of light and reduced during the daytime (Carlomagno *et al*., 2018).

It is also known that melatonin is involved in systemic inflammation and other physiological functions, by interfering with the neuro-immuno-endocrine system (Fernandes *et al*., 2017). Interleukin levels were not elevated after the stress challenge in male and female animals and were associated with the lower levels of melatonin in the stress-challenged animals. Males and females exposed to LPS have also shown sex dimorphism: there was a subtle but statistically significantly higher melatonin in the male EG group compared to the female group. Also, the females of the LCG group had elevated IL-1β and IL-6 levels, but not melatonin, due to temporal regulation of the sickness behavior, induced by the LPS exposure. Kirsten *et al*., (2013), added that male rats remained more on the dark side of the LDB and showed elevated TNF-α levels, a cytokine intimately involved in the melatonin cycle.

The present work showed a wide range of behavioral, hormone and inflammatory parameters that evidence that the young Wistar rat receiving EBN and stress or LPS treatments have sex-specific differences in behavior at a young age. To highlight such findings, we performed a PCA analysis which has enabled us to observe all of the parameters that resulted in the sex-specific responses, by observing the discrimination of the green and the blue groups that corresponded to the male and female pup rats, respectively.

CONCLUSIONS

Female young Wistar rats that receive a gestational administration of EBN and a second stimulus of stress challenge or LPS exposure at a young age are susceptible to pro-inflammatory cytokine and grooming increases, and behavior diminishments, while young males are susceptible to behavior, corticosterone, melatonin, and TNF-α alterations. Animals of both sexes showed reduced locomotor and rearing frequencies. No histological alterations in the liver or kidneys, nor in the AST, ALT or creatinine levels were observed.

ACKNOWLEDGMENTS

Fundação de Amparo à Pesquisa do Estado de São Paulo Grants # 2017/03470-9 and 2019/12202-3 granted to IBS; CNPq Productivity Scholarship #304699/2018-7 and #309628/2021-0 awarded to IBS; UNIP Scholarships awarded to CSA, and HVF. In honor of the best Supervisor, Professor, and Friend, Dr. Elfriede Marianne Bacchi.

DECLARATION OF INTEREST

None

REFERENCES

Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature. 2000;406(6797):782–787.

Alves CDS, Frias, HV, Bonamin LV, Bondan EF, Coelho CP, Martins MFM, et al. *Luffa operculata* (L.) Cogn. Gestational Exposition Induces Anxiety-like Behavior and Interferes with Melatonin and Inflammation in Young Female Rats. Pharmacogn Res. 2023;15(3):524-536. DOI: 10.5530/pres.15.3.055

Alves CS, Frias HV, Bonamin LV, Correia MSF, Corrêa MG, Bondan EF, et al. *Luffa operculata* at a late period of gestation dysregulates melatonin and cytokines interfering with weight of dams and their male offspring. J Ethnopharmacol. 2021;275:113867, DOI:10.1016/j.jep.2021.113867

Alves CDS, Frias HV, Kirsten TB, Cordeiro F, Bernardi MM, Suffredini IB. *Luffa operculata* fruit aqueous extract induces motor impairments, anxiety-like behavior, and testis damage in rats. J Ethnopharmacol. 2018;222:52–60. DOI:10.1016/j.jep.2018.04.044

Avitsur R, Yirmiya R. The immunobiology of sexual behavior: gender differences in the suppression of sexual activity during illness. Pharmacol Biochem Behav. 1999;64(4):787–796.

Barilli SLS, Santos ST, Montanari T. An experimental investigation of *Luffa operculata* (L.) Cogn.(*buchinha-do-norte*) as abortifacient plant. Reprod Climat. 2007;22(4):165–168.

Belovicova K, Bogi E, Csatlosova K, Dubovicky M. Animal tests for anxiety-like and depression-like behavior in rats. Interdiscip Toxicol. 2017;10(1):40-43. DOI:10.1515/intox-2017-0006.

Bernardi MM, Teixeira LP, Ligeiro-De-Oliveira AP, Tavares-De-Lima W, Palermo-Neto J, Kirsten TB. Neonatal lipopolysaccharide exposure induces sexually dimorphic sickness behavior in adult rats. Psychol Neurosci. 2014;7(2):113–123.

Boelter R. Plantas medicinais usadas na medicina veterinária. 2nd Edition. São Paulo: Andrei. 2003.

Boisse L. Long-Term alterations in neuroimmune responses after neonatal exposure to lipopolysaccharide. J Neurosci. 2004;24(21):4928–4934.

Borzouie SM, Rathgeber BM, Stupart C, MacIsaac J, MacLaren LA. Effects of dietary inclusion of seaweed, heat stress and genetic strain on performance, plasma biochemical and hematological parameters in laying hens. Animals (Basel). 2020;10(9):1570. DOI:10.3390/ani10091570.

Carlomagno G, Minini M, Tilotta M, Unfer V. From implantation to birth: insight into molecular melatonin functions. Int J Mol Sci. 2018;19(9):2802. DOI:10.3390/ijms19092802.

Chen Q,Tarr AJ, Liu X, Wang Y, Reed NS, Demarsh CP, et al. Controlled progressive innate immune stimulation regimen prevents the induction of sickness behavior in the open field test. J Inflamm Res. 2013;6:91–98.

Corrêa MG, Pires PR, Ribeiro FV, Pimentel SP, Cirano FR, Napimoga MH, et al. Systemic treatment with resveratrol reduces the progression of experimental periodontitis and arthritis in rats. PLoSOne. 2018;13(10):e0204414. DOI:10.1371/journal.pone.0204414.

Crawley J, Goodwin FK. Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. Pharmacol Biochem Behav. 1980;13(2):167–170. DOI:10.1016/0091-3057(80)90067-2.

Dantzer R, Bluthé RM, Layé S, Bret-Dibat JL, Parnet P, Kelley KW. Cytokines and sickness behavior. Ann N Y Acad Sci. 1998;840:586–590. DOI:10.1111/j.1749-6632.1998.tb09597.x.

DeSantis DT, Schmaltz LW. The mother-litter relationship in developmental rat studies: cannibalism vs caring. Dev Psychobiol. 1984;17(3):255–262. https://doi.org/10.1002/dev.420170306.

Dhabhar FS. Effects of stress on immune function: the good, the bad, and the beautiful. Immunol Res. 2014;58(2-3):193-210. DOI:10.1007/s12026-014-8517-0.

Dhabhar FS, McEwen BS.Acute stress enhances while chronic stress suppresses cell-mediated immunity *in vivo*: A potential role for leukocyte trafficking. Brain Behav Immun. 1997;11(4):286-306. DOI:10.1006/brbi.1997.0508.

Eng J. Sample size estimation: How many individuals should be studied? Radiology, 2003;227:309-313. DOI:10.1148/radiol.2272012051

Estork DM, Gusmão DF, Paciencia MLB, Frana as, Díaz IEC, Varella AD, et al. *Casinga-cheirosa* organic extract impairment over balb-c male mice behavioral phenotype. Braz J Pharmacogn. 2016;26(2):216–224. DOI:/10.1016/j.bjp.2015.10.004

Estork DM, Gusmão DF, Paciencia ML, Díaz IE, Varella AD, Younes RN, et al. First chemical evaluation and toxicity of *casinga-cheirosa* to balb-c male mice. Molecules. 2014;19(4):3973–3987. DOI:10.3390/molecules19043973.

Faria JA, Kinote A, Ignacio-Souza LM, de Araújo TM, Razolli DS, Doneda DL, et al. Melatonin acts through MT1/MT2 receptors to activate hypothalamic Akt and suppress hepatic gluconeogenesis in rats. Am J Physiol Endocrinol Metab. 2013;305(2):E230-42. DOI:10.1152/ajpendo.00094.2013.

Fernandes C, González MI, Wilson CA, File SE. Factor analysis shows that female rat behaviour is characterized primarily by activity, male rats are driven by sex and anxiety. Pharmacol Biochem Behav. 1999;64(4):731–738. DOI: 10.1016/s0091-3057(99)00139-2

Fernandes PA, Tamura EK, D'Argenio-Garcia L, Muxel SM, da Silveira Cruz-Machado S, Marçola M, et al. Dual effect of catecholamines and corticosterone crosstalk on pineal gland melatonin synthesis. Neuroendocrinology. 2017;104(2):126-134, DOI:/10.1159/000445189.

Flora do Brasil. Jardim Botânico do Rio de Janeiro. Available at: Flora do Brasil - *Luffa operculata* (L.) Cogn.(jbrj.gov.br) (accessed 23 February 2023).

Frias HV, Alves CDS, Flório JC, Bondan EF, Bonamin LV, Coelho CP, et al. Vertical exposition to *Luffa operculata* extract deregulates behavior and hypothalamus neurotransmitters in juvenile rats. J Ethnopharmacol. 2021;264:113265. DOI:10.1016/j.jep.2020.113265

Gehrand AL, Phillips J, Malott K, Raff H. Corticosterone, adrenal, and the pituitary-gonadal axis in neonatal rats: Effect of Maternal Separation and Hypoxia. Endocrinology. 2020;161(7):bqaa085. DOI:10.1210/endocr/bqaa085.

Gusmão DF, Estork DM, Paciencia MLB, Díaz IEC, Montero EFS, Varella AD, et al. Influence of the intraperitoneal

This is an open-access article distributed under the terms of the Creative Commons Attribution License.

administration of antitumor *Abarema auriculata* extract on mice behavior. Braz J Pharmacogn. 2013a;23(6):903–912. DOI:10.1590/S0102-695X2013000600007.

Gusmão DF, Estork DM, Paciencia MLB, Díaz IEC, Frana SA, Rodrigues PA, et al. Preliminary evaluation of the acute toxicity related to *Abarema auriculata* to mice and investigation of cytotoxicity of isolated flavonones. PharmacologyOnline. 2013b;1:113–127.

Hornig M, Weissenböck H, Horscroft N, Lipkin WI. An infection-based model of neurodevelopmental damage. Proc Natl Acad Sci USA. 1999;96(21):12102–12107.

JHU.2020. http://web.jhu.edu/animalcare/procedures/rat.html, accessed at 10/10/2020.

Johnson R. Infections during pregnancy. Adv Neurol. 1994;64:153–162.

Kalueff AV, Stewart AM, Song C, Berridge KC, Graybiel AM, Fentress JC. Neurobiology of rodent self-grooming and its value for translational neuroscience. Nat Rev Neurosci. 2016;17(1):45–59. DOI: 10.1038/nrn.2015.8.

Kirsten TB, Lippi LL, Bevilacqua E, Bernardi MM. LPS exposure increases maternal corticosterone levels, causes placental injury and increases IL-1B levels in adult rat offspring: relevance to autism. PLoSOne. 2013;8(12):e82244. DOI:10.1371/journal.pone.0082244.

Lynch L, Ghidini A. Perinatal infections. Curr.Opinion Obstetr.Gynecol., 1993;5(1), 24–32.

Menon-Miyake MA Saldiva PHN Lorenzi-Filho G, et al. *Luffa operculata* affects on the epithelium of frog palate: histological features. Braz. J. Otorhinolaryngol. 2005a;71(2):132-8.

Menon-Miyake MA, Carvalho de Oliveira R, Lorenzi-Filho G, Saldiva PH, et al. *Luffa operculata* affects mucociliary function of the isolated frog palate. Am J Rhinol. 2005b;19(14):353-357.

Nunes AMM, Silva VA. O uso de plantas abortivas no Nordeste brasileiro: uma revisão. Ethnoscientia. 2021;6(2):100-112. DOI:10.18542/ethnoscientia.v6i2.10368.

Odio MR, Maickel RP. Comparative biochemical responses of rats to different stressful stimuli. Physiol Behav. 1985;34(4):595–599.

Pang Y, Cai Z, Rhodes PG. Disturbance of oligodendrocyte development, hypomyelination and white matter injury in the neonatal rat brain after intracerebral injection of lipopolysaccharide. Brain Res Devel Brain Res. 2003;140(2):205–214.

Revilla J. Plantas úteis da bacia Amazônica 1. Manaus: INPA/SEBRAE.pp.391. 2002.

Scholl JL, Afzal A, Fox LC, Watt MJ, Forster GL. Sex differences in anxiety-like behaviors in rats. Physiol Behav. 2019;211:112670. DOI: 10.1016/j.physbeh.2019.112670.

Shimada T, Matsumoto K, Osanai M, Matsuda H, Terasawa K, Watanabe H. The modified light/dark transition test in mice: Evaluation of classic and putative anxiolytic and anxiogenic drugs. Gen Pharmacol. 1995;26(1):205–210. https://doi.org/10.1016/0306-3623(94)00148-G

Simpson J, Ryan C, Curley A, Mulcaire J, Kelly JP. Sex differences in baseline and drug-induced behavioural responses in classical behavioural tests. Prog Neuropsychopharmacol Biol Psychiatry. 2012;37(2):227–236. DOI: 10.1016/j.pnpbp.2012.02.004

Suffredini IB, Paciencia MLB, Díaz IEC, Frana as, Bernardi MM. Mice behavioral phenotype changes after administration of anani (*Symphonia globulifera*, Clusiaceae), an alternative Latin American and African medicine. Pharmacogn Mag. 2017;13(52):617-626. DOI:10.4103/pm.pm_168_17.

Takao K, Miyakawa T. Light/dark transition test for mice. J Visualized Exp – JoVE. 2006;(1):104. DOI: 10.3791/104.

The Plant List. http://www.theplantlist.org/tpl1.1/search?q=luffa+operculata. Accessed 18 February 2023.

Udo MS, Sandini TM, Reis TM, Bernardi MM, Spinosa HS. Prenatal exposure to a low fipronil dose disturbs maternal behavior and reflex development in rats. Neurotoxicol Teratol. 2014;45:27–33. DOI:10.1016/j.ntt.2014.05.010.

Wagner H, Bladt S. Plant Drug Analysis. 2nd Ed. Berlin: Springer Berlin Heidelberg. 384p. 1996.

Witschi E. Rat Development. In: Growth Including Reproduction and Morphological Development. Altman PL, Dittmer DS., Eds. Washington DC: Fed Am Soc Exp Biol. 1962; pp.304-314.

Yu H-M, Yuan T-M, Gu W-Z, Li J-P. Expression of glial fibrillary acidic protein in developing rat brain after intrauterine infection. Neuropathology. 2004;24(2):136–143. DOI: 10.1111/j.1440-1789.2003.00539.x.

Zar JH. Biostatistical Analysis. 4th ed. New Jersey: Prentice-Hall Inc. 929 p. 1999.

Received for publication on 03rd June 2023
Accepted for publication on 22nd January 2024