

Research Article

The Association of Malnutrition and Chronic Stress Models Does Not Present Overlay Effects in Male Wistar Rats

Camila Gracyelle de Carvalho Lemes¹, Abraão Tiago Batista Guimarães¹, Wellington Alves Mizaél da Silva¹, Bruna de Oliveira Mendes¹, Dieferson da Costa Estrela¹, Adriana da Silva Santos², José Roberto Ferreira Alves Júnior², Iraci Lucena da Silva Torres³, André Talvani⁴, Guilherme Malafaia⁵

¹ Laboratório de Pesquisas Biológicas, Instituto Federal de Educação, Ciência e Tecnologia Goiano–Campus Urutaí, GO, Brazil

² Departamento de Ciências Veterinárias, Instituto Federal de Educação, Ciência e Tecnologia Goiano–Campus Urutaí, GO, Brazil

³ Departamento de Farmacologia, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), RS, Brazil

⁴ Departamento de Ciências Biológicas, Universidade Federal de Ouro Preto (UFOP), Brazil

⁵ Departmetno de Ciências Biológicas, Laboratório de Pesquisas Biológicas, Instituto Federal de Educação, Ciência e Tecnologia Goiano–Campus Urutaí, GO, Brazil

Corresponding Author: Guilherme Malafaia; email: guilhermeifgoiano@gmail.com

Received 19 April 2016; Accepted 31 July 2016

Academic Editor: Yusuf Tutar

Copyright © 2016 Camila Gracyelle de Carvalho Lemes et al.. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract. Chronic stress and protein-energy malnutrition (PEM) are both social problems resulting in physiological and behavioral alterations. In this present study an associative effects of PEM and chronic stress were evaluated through in Wistar rats. Four groups were established: standard diet– 19% of protein (Std); Std + stress; PEM–6% of protein and PEM + stress. In these groups were assessed physical, nutritional, hematological, histological parameters and anxiety-like behavior. There were a reduction of food intake, body mass and relative weight of the heart and thymus in the PEM group. The liver of the PEM animals presented a degenerative condition with steatosis and Kupffer cell hypertrophy and, additionally, a significant decrease in hematocrit percentages, in the number of red blood cells and in the concentration of hemoglobin and total protein. In those animals under stress and Std diet, there was observed an increase of the relative adrenal weights, an acute condition of leukocytosis with a predominance of neutrophils and an increase in the anxiety-like behavior. There was no overlapping/interaction among the anthropometric, biochemical, hematological and histological effects using PEM and stress in Wistar rats. The effects observed under experimental condition were those related to either PEM or stress, independently.

Keywords: protein deficiency, stress, anxiety-like behavior, steatosis, Wistar rats

1. Introduction

Considerable changes have been observed in human behavior as a response to the dynamic stimuli of the modern world

and, consequently, the busy life [1, 4, 5]. Chronic stress emerges as a frequent social problem that results from a certain condition and/or lifestyle, leading to an ample group

of behavioral alterations [2, 3, 6, 7]. Among these, changes in eating behavior stand out as a consequence of the interaction between the body physiological state and environmental conditions [3].

However, eating more does not mean eating well. The improper supply of nutrients, as evidenced in situations of protein-energy malnutrition (PEM) still has consisting a serious health problem. The study of Monte [8] pointed out that despite a recent reduction in the world prevalence, child PEM is a major public health problem in developing countries. In developing countries, an estimated 50.6 million children less than 5 years old are malnourished, and those who are severely malnourished, presenting a severe illness leading to hospitalization, face a case-fatality rate exceeding 20% [9]. Mortality rates of severely malnourished children treated as patients have been unchanged for the last five decades. In the early 1990s, prospective studies conducted in Asia and Africa, using data from eight communities, Pelletier et al. [10] estimated a relative risk for mortality associated with different degrees of child PEM as 2.5, 4.6 and 8.4 for mild, moderate, and severe malnutrition respectively. PEM is responsible, directly or indirectly, for 54% of the 10.8 million deaths per year in children less than 5 years old and contributes to second cause of death (53%) associated with infectious diseases among children less than 5 years old in developing countries [11].

More recent studies have demonstrated that PEM still haunts different parts of the world and different population groups [12–15], especially in developing countries. Moreover, chronic stress experienced by populations is not restricted to developing countries and, therefore, coexists with PEM in many populations, persisting as a worrying social factor that leads to both physiological and behavioral alterations in humans [16] and animal models [17].

In spite of this, studies on the combined effects of chronic stress and PEM situations on organic parameters in model animals are still scarce. Most of them analyze social behavioral aspects [18] instead of organic changes, in particular physiological, histological, biochemical and behavioral alterations [19]. Thus, there is a lack in knowledge regarding the factors that can possibly be altered in situations where there is a stress/PEM overlap.

Specifically regarding PEM, negative impacts have been evidenced on the immune response to infections [20–22], to the responsiveness to vaccines [23, 24] and to medication [25]. So, based on these previously data, new investigations are needed to understand the combination of a persistence stress and PEM. In this sense, the objective of the present study was to evaluate the effect of combination of PEM and chronic stress model on behavioral, anthropometric, biochemical, hematological, histological in confined Wistar rats.

2. Material and Methods

Thirty-day old male Wistar rats (45–65 g), were randomized by weight and housed, individually, in polypropylene material cages (49 × 34 × 16 cm) at Animal facility of the Laboratory for Biological Research of the *Instituto Federal Goiano–Campus Urutaí*, Goiás, Brazil. All animals were kept on a standard 12-hour light/dark cycle (lights on at 7.00 a.m. and lights off at 7.00 p.m.), in a temperature-controlled environment ($22 \pm 2^\circ\text{C}$) and food and water were offered *ad libitum*.

All procedures were approved by the Institutional Committee for Animal Care and Use of the *Instituto Federal Goiano*, Goiás, Brazil (protocol n. 003/2014) in accordance with the Guide for the Care and Use of Laboratory Animals, 11th edition and with Brazilian guidelines involving use of animals in research. Vigorous attempts were made to minimize animal suffering and decrease external sources of pain and discomfort, as well as to use only the number of animals that was essential to produce reliable scientific data.

From weaning at 21 days of life to the 30th day, the animals were fed with rodent standard feed (Nuvilab–CR1), containing 19% protein. After that, the animals were distributed in four experimental groups: standard diet (Std); Std + stress; PEM diet (PEM) and PEM + stress.

PEM and PEM + stress animals started this new diet at 30th day of life and Std and Std + stress groups were fed with rodent standard feed (Nuvilab–CR1) containing 19% protein until the end of the experiment. The rodent standard feed rigorously followed the recommendations of the Reeves et al. [26] (AIN-93G-MX and AIN-93-GX). PEM and PEM + stress groups received the same diet as the control animals, but with a lower percentage of proteins (hypoproteic diet - 6% protein, produced by *PragSoluções Comércio e Serviços Ltda.–ME–Jáú*, São Paulo, Brazil). The other food constituents also obeyed the recommendations of the Reeves et al. [26] (AIN-93G-MX and AIN-93-GX). Each experimental group was composed of six animals, and two experiments were conducted independently, totalizing twelve animals per group.

After 56 days, Std + stress and PEM + stress groups were subjected to a protocol of chronic stress by restriction, as proposed by Ely et al. [27]. In order to limit the animal's movements, a plastic tube (25 cm × 7 cm) was used, with the frontal part open to allow breathing. The animals were subjected to the stressor agent for an hour in the afternoon (from 1 p.m. to 3 p.m.), for five days a week, during 50 days. The selection of this model was based on several recent studies [28–32].

Body weight was the parameter taken as indicative of PEM and was measured weekly by means of a semi-analytical digital scale. The daily consumption was calculated by subtracting the leftovers from the total amount of food offered per day. Liquid intake was not measured.

At the end of the stress protocol, according to the schedule shown in Figure 2, the animals were subjected to tests for the assessment of the anxiety-like behavior by elevated plus-maze test. The maze was composed of two open arms (50 × 10 cm) (with “rims” on the edges) and two closed arms of the same size with 30-cm high walls. The whole maze was 50 cm high from the floor. Two lamps illuminated the apparatus indirectly and light intensity was approximately 110 lx in the open arms. The rats were placed individually in the center zone of the maze, facing an open arm, and allowed five minutes of free exploration. All rats were tested just once. Before each test, the arena was cleaned with 70% ethanol. The anxiety index was calculated according to Estrela et al. [32] as follows: Anxiety Index = $1 - \frac{[(\text{Open arm time}/\text{Test duration}) + (\text{Open arms entries}/\text{Total number of entries})]/2}$.

After the behavioral test, the animals were anesthetized with an intraperitoneal injection of 40 mg/kg pentobarbital, to collect blood. Blood samples were placed in tubes without anticoagulant, after fasting for 12 hours, being 3 mL collected after breaking the brachial artery. The serum total protein was determined by the biuret method, using the commercial kit from Labtest Diagnóstica S.A.®, Cat. 99 (Lagoa Santa, MG, Brazil). Blood glucose was determined using test strips (ACCU-CHECK Advantage II, Roche) coupled to a portable digital blood glucose meter.

The erythrogram and total and differential leukocyte counts were determined using the automatized ABX Micros 60 hematology analyzer, according to Estrela et al. [33]. For these analyses, 1 mL blood was collected in 5 ml test tube containing EDTA-type anticoagulant.

Subsequently, the animals were euthanized and the brain, spleen, heart, pancreas, liver, thymus, lungs, adrenal glands and kidney relative mass were weighed. The mass of the organs was normalized to the body weight using the following formula: weight of the organs (g)/body weight (g). As a stress parameter, we analyzed the relative adrenal gland weight, according to Macedo et al. [31].

For the histopathological evaluations, liver (important metabolic organ) and stomach (based on the hypothesis that chronic stress and PEM can induce the appearance of erosive lesions and ulcerated gastric mucosa associated with these stressful conditions) were collected and fixed in 10% buffered formalin, embedded in paraffin blocks and thin-sliced to 5 μm thickness [34]. The hematoxylin and eosin staining technique (HE) was used, following Behmer et al. [35]. The thin-session descriptions were carried out using a bright-field Carl Zeiss® optical microscope, model Jenaval, in order to compare the tissue structures of the organs removed from animals of the different experimental groups.

For the analysis of body weight variations along the experimental period, the repeated measures analysis of variance (ANOVA) was used, with Tukey post-test at 5% probability, when indicated. The data related the other parameters were subjected to ANOVA, according to the factorial model 2 × 2 (two-way ANOVA), the factors being

“nutrition” (Std and PEM) and “condition” (non-stress and stress) and Tukey post-test was performed at 5% probability, when indicated. The residual normality was checked with the Shapiro-Wilk test. Bartlett test was used to check the residual homoscedasticity. The software ASSISTAT was used in the statistical analyses.

3. Results

Regarding body weight from the second week, those PEM and PEM + stress groups presented less weight gain in comparison to Std and Std + stress groups (Figure 3A). This difference remained to the end of the experiment without significant difference between Std and Std + stress and PEM and PEM + stress groups, showing that the stress condition imposed to the animals did not interfere in gain or loss of body weight. Malnourished groups (PEM and PEM + stress) presented a decrease in intake of the diet imposed to them (Figure 3B).

Regarding the relative organs weight, there was statistically significant interaction between the factor 1 “nutrition” and factor 2 “condition”. However, there was a decrease in relative thymus weight and increase in relative heart weight in malnourished animals (groups PEM and PEM + stress) (Table 1).

The malnourished animals presented a decrease in red blood cell counts and hemoglobin concentrations, as well as in the hematocrit percentage. The effects of the factor 2 “condition” or of the interaction of the factors were not observed (Table 2). Thus, the increase of heart mass could be related to a compensatory mechanism to volumetric overload, common result of chronic anemia.

During the collection of the biological samples the a macroscopic observation of the liver from PEM and PEM + stress indicated a paler and yellowish organ. In addition, a fat accumulation in intracellular vacuoles with the characteristic displacement of the hepatocyte nucleus to the cell periphery was observed under the microscope, point to hepatic steatosis (Figures 3C and 3D). Other frequent observations during the histological analysis of the liver in those PEM animals were Kupffer cell hypertrophy (Figure 3C–detail) and higher amounts of mononuclear infiltrate composed by lymphocytes, plasma cells and macrophages (Figure 3D–detail), when compared to the control group (Figures 3A and 3B). Qualitatively, there was no histological alteration of the liver in groups PEM and PEM + stress (Figures 3C and 3D), nor in groups Std and Std + stress (Figures 3A and 3B). Regarding the stomach, no differences were observed in the experimental groups, being maintained the integrity of the tissue structures of the animals subjected to the experimental conditions of this study (Figure 3E-3H).

Regarding the elevated plus-maze test, the statistical analysis showed only effect of the factor 2 “condition” for anxiety index ($F_{(1,40)} = 14.531, p = 0.002$) and percentage of entries into the open arms ($F_{(1,40)} = 23.802, p <$

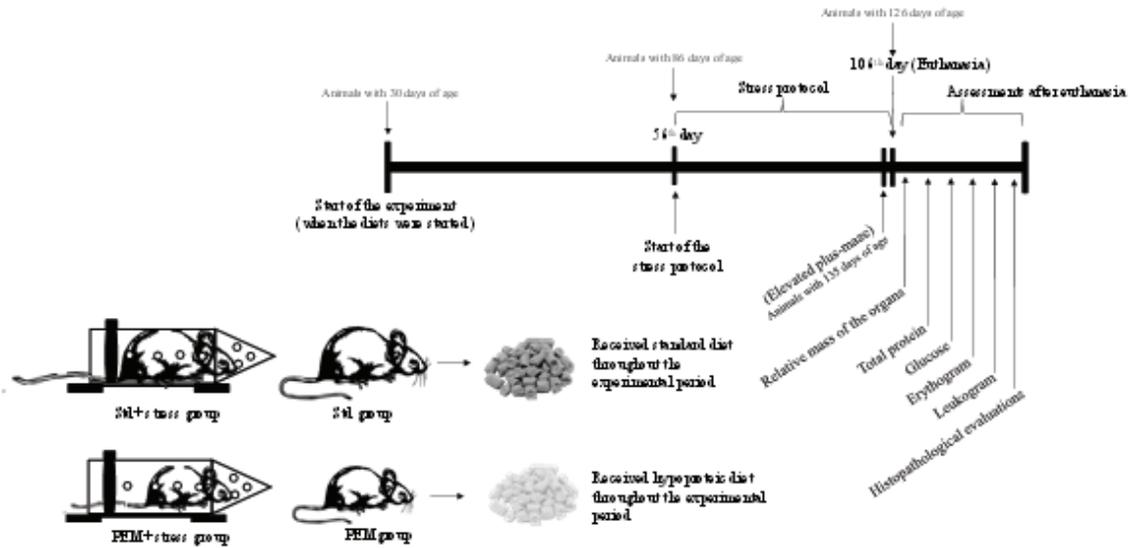


Figure 1: Schedule prepared for the experiment carried out with male Wistar rats subjected to standard and hypoproteic diets (PEM), subjected or not to chronic stress by restriction. The colors are merely illustrative.

Table 1: Relative weight of organs (per gram of body mass) of Wistar rats subjected or not to malnutrition (valeria a pena especificar PEM por extenso) and induced or not to chronic stress by restriction.

Parameters	Std	Std + stress	PEM	PEM + stress	“Nutrition” factor		“Condition” factor		Interaction between factors	
					F-value	P-value	F-value	P-value	F-value	P-value
Brain	0.0043a	0.0044a	0.0053a	0.0055a	7.37	0.11	0.01	0.89	0.01	0.89
Heart	0.0032b	0.0031b	0.0037a	0.0038a	18.34	<0.001	0.03	0.84	0.22	0.64
Spleen	0.0013a	0.0012a	0.001a	0.0014a	2.45	0.13	0.32	0.57	0.86	0.36
Pancreas	0.0022a	0.0024a	0.0018a	0.0021a	2.22	0.14	1.25	0.27	0.28	0.59
Liver	0.0284a	0.0263a	0.0265a	0.0261a	0.60	0.44	0.81	0.37	0.38	0.53
Thymus	0.0009a	0.0007a	0.0011b	0.0013b	9.56	0.005	0.01	0.90	1.42	0.24
Lungs	0.0040a	0.0049a	0.0051a	0.0048a	2.32	0.14	0.71	0.40	2.86	0.10
Adrenal glands	0.0001b	0.0002a	0.0001a	0.0002b	0.65	0.42	3.26	0.03	0.0039	0.95
Kidneys	0.0065a	0.0065a	0.0079a	0.0065a	1.56	0.22	1.74	0.19	1.83	0.18

Legend: standard diet (Std); standard diet + stress (Std + stress); PEM diet (PEM) and PEM + stress (PEM + stress). Averages followed by the same letter do not differ from one another by the analysis of variance (two way ANOVA), followed by the Tukey post-test at 5% probability. “Nutrition” factor: Std and PEM; “Condition” factor: non-stress and stress.

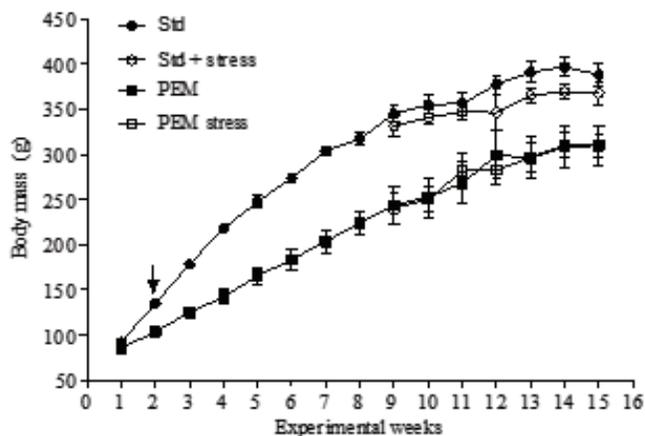
0.001) (Figure 3). Thus, the stressed animals (Std or PEM) presented increase of the anxiety-like behavior characterizing an anxiogenic effect of stress.

4. Discussion

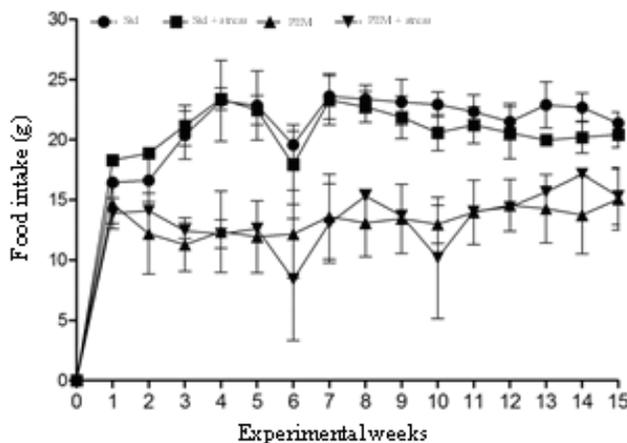
These present data concerning chronic stress and deficient nutritional status reinforce previously studies where special attention was done to the existence of a direct association between malnutrition status and body mass, because of adaptive processes that take place so that the organism can adjust to adverse nutritional conditions [33]. It is possible that the decrease in body weight observed in malnourished

groups (PEM and PEM + stress) is related to the decrease in intake of the diet imposed to them. As discussed by Alves et al. [36], the skeletal muscle tissue can be an important protein reservoir and becomes a depletion target in protein deficit scenario, causing significant muscle mass loss and consequently body weight loss [37]. Besides, less diet intake leads to a deficiency in calories and micronutrients, even when a hypoproteic isocaloric diet is used instead of the control diet (rodent standard feed).

On the other hand, in this study, no differences were observed between stressed and non-stressed groups under Std or PEM diets, showing that exposure to the chronic stress had no influence in body weight and in food intake. These



(A)



(B)

Figure 2: (A) Changes in body weight observed in male Wistar rats submitted or not to PEM and induced chronic stress by restriction and (B) food intake during the experimental period. Data expressed in mean \pm standard deviation of two experiments conducted independently with 6 rats per group (total, n=12). The statistical analysis was performed using two-way ANOVA with repeated measures with Tukey’s test at 5% probability. The arrow in “A” indicates significant difference from the second week on.

data contrast with some studies that showed that stress caused decrease in weight gain and in food intake [38, 39]. However, stress effects depend on the type of stress, frequency and duration of the stressor, and also on the age and specie of the animal [40]. Outbred rats are resistant to diet-induced obesity [diet- resistant rats], and when exposure to association of standard feed/hypercaloric diet and restraint stress do not present weight gain. However, when fed with standard feed and subjected to stress, the body weight reduction observed in stressed animals was 66%, when compared to the non-stressed animals [40]. According to Rybkin et al. [41], another factor that influences the physiological effects on stressed animals is the period of the day in which the stress model is applied. These authors demonstrated that stress by

restriction could cause a major effect on the metabolism and energy equilibrium when it is applied in the morning.

According to Macedo [42], under chronic stress conditions there is continuous stimulation of the adrenal by adrenocorticotrophic hormone leading to hypertrophy of these glands. In this present study, we used the weight of adrenal as indirect stress parameter to determine the stress condition in the animals. The effect of factor 2 “condition” (stress exposure) was observed in the relative adrenal gland weight, with increased relative adrenal weights in stressed animals compared with no stressed animals. This result corroborates previous studies that used similar protocols to ours, and showed that exposure to daily restraint stress can cause adrenal gland hypertrophy [31, 43–45]. Besides, focusing on

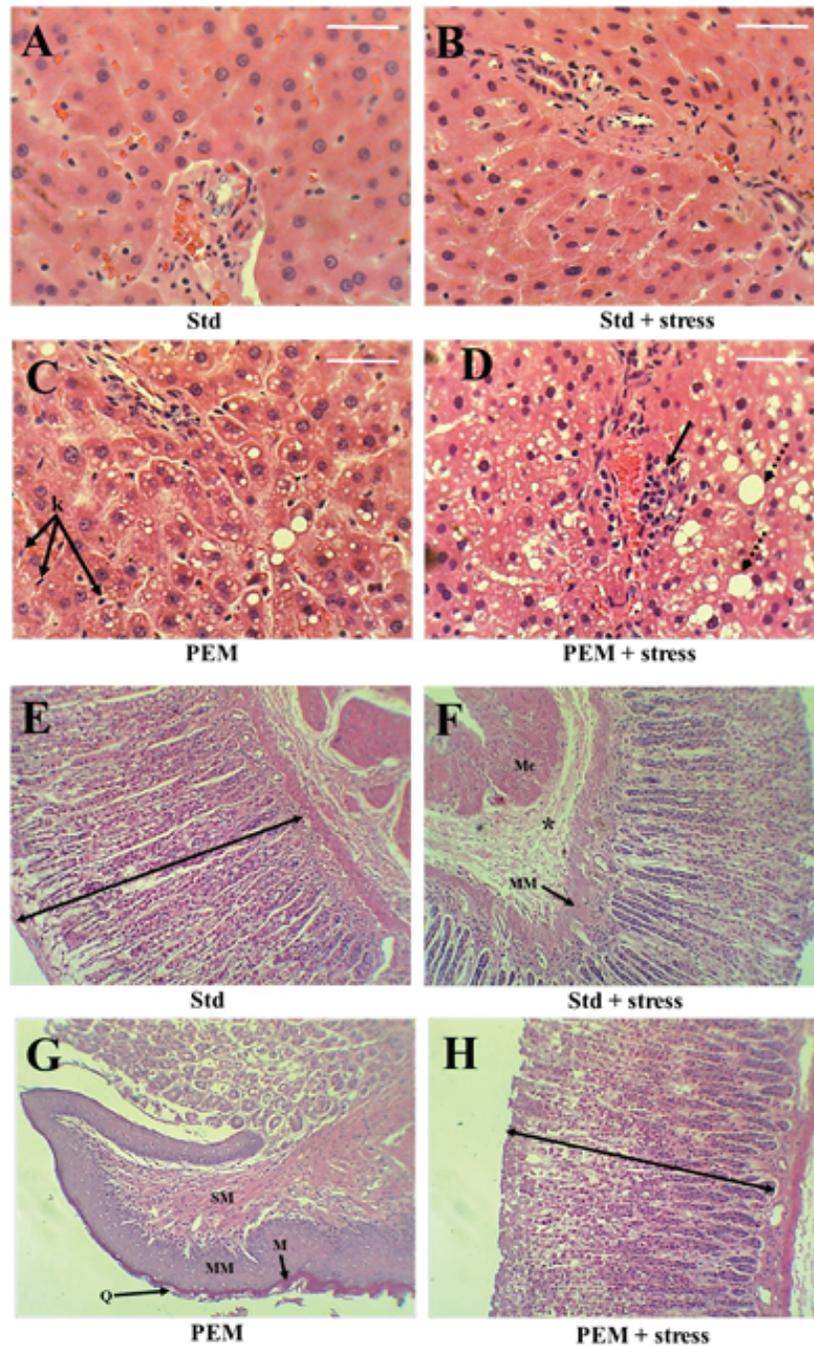


Figure 3: Photomicrographs representative of distinct liver (A-D) and stomach (E-H) regions of Wistar rats submitted or not to malnutrition and induced chronic stress by restriction. (A) non-stressed control group (Std); (B) stressed control group (Std + stress); (C) non-stressed malnourished group (PEM)—full arrows indicate Kupffer cells; (D) stressed malnourished group (PEM + stress)—full arrow indicates inflammatory infiltrate and dashed arrows indicate fat accumulation inside some periportal cell vacuoles; (E) non-stressed control group (Std)—glandular region of the gastric mucosa, with a double arrow showing the mucosa thickness; (F) stressed control group (Std + stress)—glandular region of the mucosa, showing the muscularis mucosae (MM), tunica submucosa (*) and the lamina muscularis (Mc); (G) non-stressed malnourished group (PEM)—detail of the aglandular region of the gastric mucosa, showing the keratin (Q), mucosa (M), muscularis mucosae (MM) and submucosa (SM); (H) stressed malnourished group (PEM + stress)—glandular region of the gastric mucosa, showing the mucosa thickness (double arrow). Bar = 100 μ m. A-D = 400 X magnification; E-F = 10 X magnification. HE staining.

Table 2: Erythrogram, leukogram, serum total protein concentrations observed in male Wistar rats subjected or not to malnutrition (idem acima) and induced or not to chronic stress chronic stress by restriction.

Parameters	Std	Std + stress	PEM	PEM + stress	“Nutrition” factor		“Condition” factor		Interaction between factors		
					F-value*	P-value	F-value*	P-value	F-value*	P-value	
Erythrogram											
Red blood cells (tera/L)	34.90a	32.78a	22.86b	25.95b	29.73	<0.001	0.01	0.91	2.00	0.17	
Hematocrit (%)	34.5a	31.9a	24.18b	25.65b	35.94	<0.001	0.09	0.75	2.73	0.11	
Hemoglobin (g/dL)	14.17a	14.27a	12.15b	12.78b	42.21	<0.001	1.99	0.17	1.05	0.31	
Leukogram											
Leukocytes (/mm ³)	11057.14b	14685.71a	11233.33b	16671.43a	0.23	0.63	5.11	0.04	0.16	0.68	
Segmented (/mm ³)	2372.71b	4136.42a	2463.83b	4709.85a	0.14	0.70	5.72	0.02	0.07	0.78	
Total neutrophils (/mm ³)	2594.71b	4198.57a	2519.00b	4802.57a	0.08	0.77	4.80	0.03	0.14	0.70	
Lymphocytes (/mm ³)	7256.42a	8781.42a	7212.33a	10134.43a	0.28	0.59	4.33	0.06	0.32	0.57	
Monocytes (/mm ³)	1336.71a	1694.57a	1442.5a	1618.14a	0.001	0.96	0.51	0.48	0.05	0.80	
Total Protein											
Total protein (g/dL)	6.96a	6.20a	5.63b	5.6b	19.52	0.003	3.34	0.11	2.80	0.13	

Legend: standard diet (Std); standard diet + stress (Std + stress); PEM diet (PEM) and PEM + stress (PEM + stress). In the rows, averages followed by the same letter do not differ from one another by the analysis of variance (two-way ANOVA), followed by the Tukey post-test at 5% probability. “Nutrition” factor: std and PEM; “Condition” factor: non-stress and stress.

lymphoid organ, the decrease in the relative weight of the thymus observed in PEM and PEM + stress groups indicates thymus atrophy, one of the most striking characteristics of thymic commitment in PEM, with consequent depletion of cortical thymocytes of phenotypes CD₄⁺CD₈⁺ [46].

Interestingly, the increase in the relative weight of the heart in malnourished animals has not been a consensus among different authors. Kothari et al. [47], studying variations in left ventricular mass and functions in healthy and undernourished children (one to five years old), observed that the left ventricular mass was lower associated to low nutritional status. However, the authors suggest that the significant increase of the left ventricular mass/body mass ratio in the malnourished children may be a compensatory response for the preservation of the systolic functions in atrophic hearts. Instead, Fioretto et al. [48] observed heart, ventricular and body weight loss in young rats, undernourished from birth. Nonetheless, the systolic function in these animals was preserved indicating that malnutrition adverse effects do not affect the heart. Cury et al. [49] also observed that, despite a direct relationship exists between a hypoproteic diet and body and heart weight in Wistar rats, the relative weight of the heart does not change. In this present study, we believe that the increase in the relative weight of the heart in malnourished animals also can be related to the anemia observed in these animals, indexed by the decrease of parameters of the erythrogram.

Few studies proposed the assessment of these hematological parameters in experimental conditions similar to those

adopted in our study. However, Amaral [50] showed that PEM causes decreases this parameters in Wistar rats. It is important to note that the hematological profile in nutritional deficiency conditions depends on the duration of PEM, on the protein content and on the micronutrient concentrations of the diets. Recently, we showed that the food restriction imposed to Wistar rats for a short period was not enough to change the same hematological parameters evaluated in this present study [33].

Regarding white blood cell counts, the stress condition was responsible to reduce total leukocytes, and segmented neutrophils, in particular in those Std + stress and PEM + stress groups. Different studies have proposed that chronic stress can influence the immunological system in different ways and varied activation and regulation routes [51]. Our data point to leukocytosis by neutrophilia, which also was observed in studies involving stress conditions. Prasse et al. [52], Tvedten [53] and Kociba [54] reported that in stress situations in which fear e excitation are detected, such as venipuncture procedure, an immunological alterations are associated, for example, by the presence of leukocytosis and neutrophilia. Similar effects have also been observed in other chronic stress animal models [55, 57–59, 65]. However, it seems that there is no overlap of the PEM and stress effects of leukocyte parameters analyzed.

Protein-energy malnutrition isolated or under stress condition presented smaller serum total protein concentrations. This data also corroborates with other studies, which point to the importance of a good nutrition in the maintenance

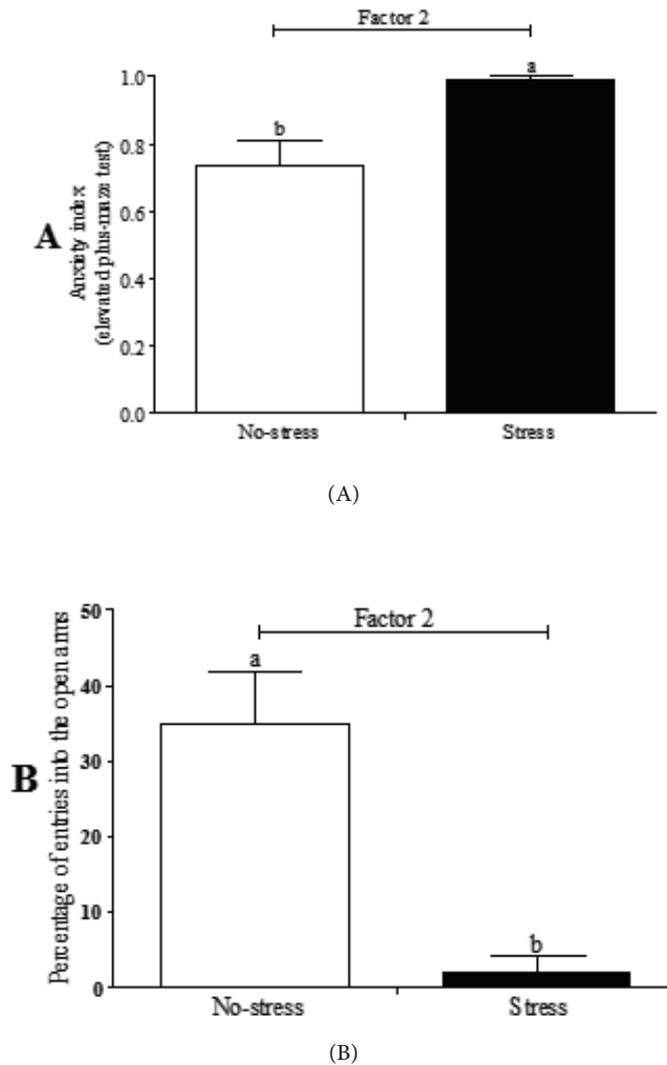


Figure 4: (A) Anxiety index and (B) percentage of entries into the open arms for male Wistar rats of the no stress and stress groups ($n = 24$ per group). Comparison between no stress and stress (Factor 2) by two-way ANOVA, at 5% probability ($n=24$).

of normal plasma protein levels [60–64]. The analysis of total protein can provide important information that reflects the general state of the organism, as well as in relation to nutritional status and the developing of illness. As discussed by Fontoura et al. [65], the use of serum protein as a tool to assess PEM is considered an important and reliable meter.

The analysis of the liver reveals the presence of histopathological indicators associated to the PEM, which allow us to infer that the malnutrition protocol was sufficient to promote hepatic alterations in the Wistar rats. However, Coelho [66] have shown some alterations in his study, such as hydropic degeneration, hyperemia of the portal space, sinusoidal congestion, as well as granulomatous reaction, which were not observed in our study. We believe that the time of the PEM diet, as well as the experimental model used, were important factors that explain the particular patterns of our study.

The elevated plus-maze test is based on the exploratory behavior of rodents and their natural aversion for open spaces, which normally cause fear and anxiety [67, 68]. This well-established paradigm has a long and successful history in assessing anxiety-like behavior in rodents [67, 69–71]. The test takes advantage of the natural tendency of rat to explore novel environments. The rodent is given the choice of spending time in open, unprotected maze arms or enclosed, protected arms, all elevated approximately 1 m from the floor. Rodents tend to avoid the open areas, especially when they are brightly lit, favoring darker, more enclosed spaces. This approach-avoidance conflict results in the behaviors that have been correlated with increases in physiological stress indicators [72]. In contrast, administration of benzodiazepines and other anxiolytic treatments results in increased exploration of the open arms, without affecting general motivation or locomotion [70].

Our data converge to studies demonstrating that stress in fact induces increase of the anxiety-like behavior in experimental models [73, 74] and in human [75]. In particular, the gender can be an important factor linked to the effects of stress in the organism. Niknazar et al. [74] showed that females presented more robust stress induction on behavioral and hormonal measures than males, and also reduced the expression of BDNF but not TrkB following stress.

These findings suggest that under stress, biological characteristics associated to the gender can promote the differentiation of BDNF. Methylation may be root cause of decreased BDNF levels in females and may underlie susceptibility to pathology development. Therefore, it is suggested that further studies should be developed associating malnutrition and stress, involving males and females and different stressors agents.

On the other hand, there are few studies regarding the relationship between PEM and anxiety. Regarding to humans, Mattar et al. [76] performed a systematic search of literature about the relationship between malnutrition and depression or anxiety, in humans. According to authors, evidence based data is very rare. From the seven reviewed studies, none of them draws the same conclusion. This is mainly due to the large differences in the samples' populations and the studies' protocols. Mattar et al. [76] suggests that future studies are needed to focus on the relationship between depression/anxiety symptoms and PEM. A more critical nutritional assessment should be undertaken with multiple psychological assessment scales. In experimental models, some studies indicate a correlation between PEM and increased anxiety-like behavior. Soares et al. [77] compared the effects of the tactile/handling stimulation (H) and environmental enrichment (EE) in well-nourished (C - 16% of protein) and malnourished (M - 6% of protein) rats tested in the elevated plus-maze at 36 and 37 days old. The results showed higher exploration of the open arms in the elevated plus-maze in M as compared with C animals, as well as lower index of risk assessment behaviors, and EE, but not H, reversed the alterations produced by malnutrition in the EPM. Biochemical analysis showed higher levels of corticosterone in M when compared with C rats. The non-stimulated animals presented higher levels of polyamines in the hippocampus when compared with the stimulated ones in both diet conditions. These authors also suggest that both the lower anxiety levels and the lower risk-assessment behaviors in the elevated plus-maze, as well as the higher levels of corticosterone, can be due to alterations in the activity of the hypothalamic-pituitary-adrenal axis as the result of early PEM.

More recently, Soares et al. [78] compared glucocorticoid receptor (GR) gene expression in the hippocampus of rats subjected to a low protein, "malnourished" diet (M; 6% protein) or a control, "well-nourished" diet (W; 16% protein), exposed to different environmental stimulation

(environmental enrichment, E; no enrichment, N) between postnatal day 8 (P8) and P35. The authors showed that MN rats (M - 6% protein and N- no enrichment) spent more time and made more entries into the open arms of the elevated plus-maze compared to W rats. On the other hand, ME rats spent a similar percentage of time, and made a similar number of entries, in the open arms as WN rats. Following the elevated plus-maze test, GR mRNA expression in the hippocampus was different in MN rats compared to W rats; expression was also different between M and ME rats; mRNA and expression of GR receptors in WN rats was similar to that observed in WE rats. These data also show that the effects of PEM on risk assessment in the EPM were reversed by E. Early PEM may alter GR expression in the hippocampus and environmental enrichment may exert a neuroprotective effect on malnutrition-induced brain injury.

On the other hand, we believe that further studies should be performed in order to better elucidate the relationship between malnutrition and anxiety-like behavior. Rocinholi & Landeira-Fernandez [78] showed that PEM alters anxiety-like behavior in weanling and young adult, male and female malnourished rats. Weanling malnourished rats exhibited reduced anxiety-like behavior and young adult male rats presented less anxiety-like behavior than young adult female rats in this experimental model.

5. Conclusions

In conclusion, there was no overlapping/interaction of the anthropometric, biochemical, hematological and histological effects from PEM and chronic stress association, under the experimental conditions used in this study. The effects observed in the Wistar rats were those related to either PEM or stress. The adaptive mechanisms exist that prevent the interaction between PEM and chronic stress in the model studied here, which could enhance negative effects of these illnesses in the organism.

Acknowledgments

The authors thank the *Fundação de Amparo à Pesquisa* of Goiás State for financing this study (Process number 201210267001124), the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) and the *Instituto Federal Goiano*, for granting the scholarship for GUIMARÃES AT and SILVA WM, respectively; the *Secretaria de Educação Superior do Ministério da Educação*, for the scholarship to LEMES CGC, and to the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) for the grants to MALAFAIA G and ESTRELA DC. The authors also thank Prof. Dr. Anderson Rodrigo da Silva for supervising the statistical analyses.

References

- [1] S. M. Monroe, Modern approaches to conceptualizing and measuring human life stress, *Annu Rev Clin Psychol*, **4**, 33–52, (2008).
- [2] M. AB. Almeida and L. F. Vitagliano, (2009).
- [3] J. H. Patrick, S. T. Stahl, and M. Sundaram, Disordered eating and psychological distress among adults, *Int J Aging Hum Dev*, **73**, 209–226, (2011).
- [4] R. S. Lazarus, Puzzles in the study of daily hassles, *J Behav Med*, **7**, 375–389, (1984).
- [5] S. E. Taylor and A. L. Stanton, Coping resources, coping processes, and mental health, *Annu Rev Clin Psychol*, **3**, 377–401, (2007).
- [6] J. EM. Vilela, J. A. Lamounier, M. A. Dellaretti Filho, J. R. Barros Neto, and G. M. Horta, [Eating disorders in school children], *J Pediatr (Rio J)*, **80**, 49–54, (2004).
- [7] J. R. Silva, Restraint eating and sensitivity to stress: preliminary experimental evidence, *Riv Psichiatr*, **46**, 300–304, (2011).
- [8] C. MG. Monte, Undernourishment: a century old challenge to infant nutrition, *J Pediatr*, **3**, 285–297, (2000).
- [9] D. L. Pelletier, E. A. Frongillo Jr, D. G. Schroeder, and J. P. Habicht, A methodology for estimating the contribution of malnutrition to child mortality in developing countries, *J Nutr*, **124**, Suppl, 2106S–2122S, (1994).
- [10] M. Blössner and M. Onis, in *Malnutrition – Quantifying the health impact at national and local levels*, WHO, Geneva, 2005.
- [11] J. Fletcher, Giving nutrition support to critically ill adults, *Nurs Times*, **111**, 12–16, (2015).
- [12] P. N. Mittwede, P. F. Bergin, J. S. Clemmer, and L. Xiang, Obesity, Malnutrition, and the Response to Critical Illness, *Crit Care Med*, **43**, p. e321, (2015).
- [13] A. MS. Fakir and M. WR. Khan, Determinants of malnutrition among urban slum children in Bangladesh, *Health Econ Rev*, **5**, p. 59, (2015).
- [14] C. Y. Wong, M. S. Zaililah, E. Y. Chua, S. Norhasmah, Y. S. Chin, and A. Siti Nur'Asyura, Double-burden of malnutrition among the indigenous peoples (Orang Asli) of Peninsular Malaysia, *BMC Public Health*, **15**, p. 680, (2015).
- [15] S. Linnemayr, H. Alderman, and A. Ka, Determinants of malnutrition in Senegal: individual, household, community variables, and their interaction, *Econ Hum Biol*, **6**, 252–263, (2008).
- [16] A. CD. Bello, T. R. Riul, and L. M. Oliveira, Desnutrição e estresse na gestação: medidas comportamentais das mães e dos filhotes durante a lactação, *Temas Psicol*, **13**, 34–44, (2005).
- [17] V. Michopoulos, D. Toufexis, and M. E. Wilson, Social stress interacts with diet history to promote emotional feeding in females, *Psychoneuroendocrinology*, **37**, 1479–1490, (2012).
- [18] M. EF. Almeida, R. S. Medeiros, F. J. Figueiredo, E. JB. Coelho, and M. PT. Sena, Efeitos do estresse auditivo e da dieta hipercalórica sobre o peso corporal, lipídios e glicemia de ratos Wistar, *Alimento Nutr*, **22**, 359–365, (2011).
- [19] G. Malafaia, Protein-energy malnutrition as a risk factor for visceral leishmaniasis: a review, *Parasite Immunol*, **31**, 587–596, (2009).
- [20] E. Carrillo, M. A. Jimenez, C. Sanchez, J. Cunha, C. M. Martins, A. da Paixão Sevá, and J. Moreno, Protein malnutrition impairs the immune response and influences the severity of infection in a hamster model of chronic visceral leishmaniasis, *PLoS One*, **9**, p. e89412, (2014).
- [21] H. Pickersgill, An immune response to malnutrition, *Sci Signal*, **7**, ec26–ec26, (2014).
- [22] G. Malafaia, T. D. Serafim, M. E. Silva, M. L. Pedrosa, and S. A. Rezende, Protein-energy malnutrition decreases immune response to *Leishmania chagasi* vaccine in BALB/c mice, *Parasite Immunol*, **31**, 41–49, (2009).
- [23] R. Haque, C. Snider, Y. Liu, J. Z. Ma, L. Liu, U. Nayak, J. C. Mychaleckyj, P. Korpe, D. Mondal, M. Kabir, M. Alam, M. Pallansch, M. S. Oberste, W. Weldon, B. D. Kirkpatrick, and W. A. Petri Jr, Oral polio vaccine response in breast fed infants with malnutrition and diarrhea, *Vaccine*, **32**, 478–482, (2014).
- [24] E. D. McCollum, C. King, R. Hollowell, J. Zhou, T. Colbourn, B. Nambiar, D. Mukanga, and D. C. Burgess, Predictors of treatment failure for non-severe childhood pneumonia in developing countries—systematic literature review and expert survey—the first step towards a community focused mHealth risk-assessment tool? *BMC Pediatr*, **15**, p. 74, (2015).
- [25] P. G. Reeves, F. H. Nielsen, and G. C. Fahey Jr, AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet, *J Nutr*, **123**, 1939–1951, (1993).
- [26] D. R. Ely, V. Dapper, J. Marasca, J. B. Corrêa, G. D. Gamaro, M. H. Xavier, M. B. Michalowski, D. Catelli, R. Rosat, M. B. Ferreira, and C. Dalmaç, Effect of restraint stress on feeding behavior of rats, *Physiol Behav*, **61**, 395–398, (1997).
- [27] I. C. Macedo, L. F. Medeiros, C. Oliveira, C. M. Oliveira, J. R. Rozisky, V. L. Scarabelot, A. Souza, F. R. Silva, V. S. Santos, S. G. Cioato, W. Caumo, and I. L. Torres, Cafeteria diet-induced obesity plus chronic stress alter serum leptin levels, *Peptides*, **38**, 189–196, (2012).
- [28] C. de Oliveira, V. L. Scarabelot, A. de Souza, C. M. de Oliveira, L. F. Medeiros, I. C. de Macedo, P. R. Marques Filho, S. G. Cioato, W. Caumo, and I. L. Torres, Obesity and chronic stress are able to desynchronize the temporal pattern of serum levels of leptin and triglycerides, *Peptides*, **51**, 46–53, (2014).

- [29] I. C. Macedo, J. R. Rozisky, A. M. Battastini, M. F. M. Ribeiro, and I. L. S. Torres, Obesity and chronic stress modulate adenine nucleotide hydrolysis in rat blood serum, *Int J Pharm Sci Rev Res*, **3**, 28–40, (2014).
- [30] I. C. Macedo, J. R. Rozisky, C. Oliveira, C. M. Oliveira, G. Laste, Y. Nonose, V. S. Santos, P. R. Marques, M. F. Ribeiro, W. Caumo, and I. L. S. Torres, Chronic stress associated with hypercaloric diet changes the hippocampal BDNF levels in male Wistar rats, *Neuropeptides*, **51**, 75–81, (2015).
- [31] D. C. Estrela, et al., Predictive behaviors for anxiety and depression in female Wistar rats subjected to cafeteria diet and stress, *Physiol Behav*, (2015).
- [32] A. D. C. Estrela, C. G. C. Lemes, A. T. B. Guimarães, and G. Malafaia, Effects of short-term malnutrition in rats, *Sci Plena*, **10**, 1–13, (2014).
- [33] L. G. Luna, in *Manual of histologic staining methods of the Armed Forces Institute of Pathology*, McGraw-Hill, New York, 13th ed edition, 1968.
- [34] A. O. Behmer, E. M. C. Tolosa, and A. G. F. Neto, in *Manual de técnicas para histologia normal e patológica*, Ed. Art/Edusp, São Paulo, 1976.
- [35] A. P. Alves, A. R. Damaso, and V. Dal-Pai, Efeito da desnutrição proteica pré e pós-natal sobre a morfologia, a diferenciação e o metabolismo do tecido muscular estriado esquelético em ratos, *J Pediatr*, **84**, 264–271, (2008).
- [36] F. L. C. Oliveira, A. S. B. Oliveira, B. Schmidt, and O. M. S. Amancio, Desnutrição energética intra-uterina em ratos: alterações músculo-esqueléticas na 1ª e 2ª gerações, *J Pediatr*, **75**, 350–356, (1999).
- [37] R. A. Harris, Introduction to special section: stress triggers, stress shadows, and implications for seismic hazard, *JGR Solid Earth*, **103**, 24347–24358, (1998).
- [38] M. T. Marin, F. C. Cruz, and C. S. Planeta, Chronic restraint or variable stresses differently affect the behavior, corticosterone secretion and body weight in rats, *Physiol Behav*, **90**, 29–35, (2007).
- [39] C. Michel, B. E. Levin, and A. A. Dunn-Meynell, Stress facilitates body weight gain in genetically predisposed rats on medium-fat diet, *Am J Physiol Regul Integr Comp Physiol*, **285**, R791–R799, (2003).
- [40] I. I. Rybkin, Y. Zhou, J. Volaufova, G. N. Smagin, D. H. Ryan, and R. B. Harris, Effect of restraint stress on food intake and body weight is determined by time of day, *Am J Physiol*, **273**, R1612–R1622, (1997).
- [41] I. C. Macedo, 2010.
- [42] A. M. Magariños and B. S. McEwen, Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons: involvement of glucocorticoid secretion and excitatory amino acid receptors, *Neuroscience*, **69**, 89–98, (1995).
- [43] C. L. Wellman, Dendritic reorganization in pyramidal neurons in medial prefrontal cortex after chronic corticosterone administration, *J Neurobiol*, **49**, 245–253, (2001).
- [44] E. B. Bloss, W. G. Janssen, B. S. McEwen, and J. H. Morrison, Interactive effects of stress and aging on structural plasticity in the prefrontal cortex, *J Neurosci*, **30**, 6726–6731, (2010).
- [45] F. M. Susana, P. Paula, and N. Slobodianik, Dietary modulation of thymic enzymes, *Endocr Metab Immune Disord Drug Targets*, **14**, 309–312, (2014).
- [46] S. S. Kothari, T. M. Patel, A. N. Shetalwad, and T. K. Patel, Left ventricular mass and function in children with severe protein energy malnutrition, *Int J Cardiol*, **35**, 19–25, (1992).
- [47] J. R. Fioretto, S. S. Querioz, C. R. Padovani, L. S. Matsubara, K. Okoshi, and B. B. Matsubara, Ventricular remodeling and diastolic myocardial dysfunction in rats submitted to protein-calorie malnutrition, *Am J Physiol Heart Circ Physiol*, **282**, H1327–H1333, (2002).
- [48] J. C. S. Cury, A. E. Liberti, and E. F. Gama, Estudo histomorfométrico de cardiomiócitos ventriculares de ratos Wistar submetidos a desnutrição proteica, *FIEP Bull On-line*, **76**, 161–163, (2006).
- [49] D. A. Amaral, 2005.
- [50] A. Vitlic, J. M. Lord, and A. C. Phillips, Stress, ageing and their influence on functional, cellular and molecular aspects of the immune system, *Age (Dordr)*, **36**, p. 9631, (2014).
- [51] K. W. Prasse, M. L. Kaeberle, and F. K. Ramsey, Blood neutrophilic granulocyte kinetics in cats, *Am J Vet Res*, **34**, 1021–1025, (1973).
- [52] H. Tvedten, in *Small animal clinical diagnosis by laboratory methods. Pennsylvania*, H. Tvedten and G. Turnwald, Eds., 63–91, Saunders Company, Willard, MD, 1999.
- [53] G. J. Kociba, in *Tratado de medicina interna veterinária: doenças do cão e do gato*, S. J. Ettinger and E. C. Feldman, Eds., 1941–1956, Guanabara Koogan, Rio de Janeiro, 2004.
- [54] Y. Yagi, H. Shiono, Y. Chikayama, A. Ohnuma, I. Nakamura, and K. Yayou, Transport stress increases somatic cell counts in milk, and enhances the migration capacity of peripheral blood neutrophils of dairy cows, *J Vet Med Sci*, **66**, 381–387, (2004).
- [55] M. G. Kerr, in *Exames laboratoriais em medicina veterinária: bioquímica clínica e hematologia*, Rocca, São Paulo, 2nd ed edition, 2003.
- [56] K. L. Overall, in *Clinical behavioral medicine for small animals*, Mosby, St. Louis, 1997.
- [57] W. R. Klemm, in *Fisiologia dos animais domésticos*, W. H. D. Swenson and W. O. D. Reece, Eds., 825–841, Guanabara Koogan, Rio de Janeiro, 1996.
- [58] A. L. P. Fam, R. M. V. Rocha, C. T. Pimpão, and M. A. Cruz, Alterações no leucograma de felinos domésticos (*Felis catus*) decorrentes de estresse agudo e crônico. *Rev. Acad., Ciên, Anim*, **8**, 299–306, (2010).

- [59] J. C. Waterlow and G. AO. Alleyne, in *Má nutrição proteica em crianças. Evolução do conhecimento nos últimos dez anos*, L.P.M. Editora, São Paulo, 1974.
- [60] C. J. Laing and D. R. Fraser, Changes with malnutrition in the concentration of plasma vitamin D binding protein in growing rats, *Br J Nutr*, **88**, 133–139, (2002).
- [61] J. LA. Couto, H. S. Ferreira, D. B. da Rocha, M. EL. Duarte, M. L. Assunção, and E. M. Coutinho, Structural changes in the jejunal mucosa of mice infected with *Schistosoma mansoni*, fed low or high protein diets, *Rev Soc Bras Med Trop*, **35**, 601–607, (2002).
- [62] M. A. de Abreu, L. LM. Weckx, and C. HW. Hirata, Histological and ultrastructural aspects of the tongue in undernourished rats, *Rev Bras Otorrinolaringol (Engl Ed)*, **72**, 523–527, (2006).
- [63] V. Santhiago, A. SR. Silva, C. A. Gobatto, and M. AR. Mello, Treinamento físico durante a recuperação nutricional não afeta o metabolismo muscular da glicose de ratos, *RBME*, **12**, 76–80, (2006).
- [64] C. SM. Fontoura, D. O. Cruz, L. G. Londero, and R. M. Vieira, Avaliação nutricional de paciente crítico, *RBTI*, **18**, 298–306, (2006).
- [65] L. F. Coelho, 2011.
- [66] S. Pellow, P. Chopin, S. E. File, and M. Briley, Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat, *J Neurosci Methods*, **14**, 149–167, (1985).
- [67] A. A. Walf and C. A. Frye, The use of the elevated plus maze as an assay of anxiety-related behavior in rodents, *Nat Protoc*, **2**, 322–328, (2007).
- [68] R. J. Rodgers and A. Dalvi, Anxiety, defence and the elevated plus-maze, *Neurosci Biobehav Rev*, **21**, 801–810, (1997).
- [69] R. J. Rodgers, N. J. Johnson, J. Carr, and T. P. Hodgson, Resistance of experientially-induced changes in murine plus-maze behaviour to altered retest conditions, *Behav Brain Res*, **86**, 71–77, (1997).
- [70] L. J. Bertoglio and A. P. Carobrez, Prior maze experience required to alter midazolam effects in rats submitted to the elevated plus-maze, *Pharmacol Biochem Behav*, **72**, 449–455, (2002).
- [71] A. Holmes, J. W. Kinney, C. C. Wrenn, Q. Li, R. J. Yang, L. Ma, J. Vishwanath, M. C. Saavedra, C. E. Innerfield, A. S. Jacoby, J. Shine, T. P. Iismaa, and J. N. Crawley, Galanin GAL-R1 receptor null mutant mice display increased anxiety-like behavior specific to the elevated plus-maze, *Neuropsychopharmacology*, **28**, 1031–1044, (2003).
- [72] A. Longo, A. Oberto, P. Mele, L. Mattiello, M. G. Pisu, P. Palanza, M. Serra, and C. Eva, NPY-Y1 coexpressed with NPY-Y5 receptors modulate anxiety but not mild social stress response in mice, *Genes Brain Behav*, **14**, 534–542, (2015).
- [73] S. Niknazar, A. Nahavandi, A. A. Peyvandi, H. Peyvandi, A. S. Akhtari, and M. Karimi, Comparison of the adulthood chronic stress effect on hippocampal bdnf signaling in male and female rats, *Mol Neurobiol*, (2015)., Epub ahead of print.
- [74] C. A. Soria, C. Remedi, D. A. Núñez, L. D’Alessio, and E. J. Roldán, Impact of alprazolam in allostatic load and neurocognition of patients with anxiety disorders and chronic stress (GEMA): observational study protocol, *BMJ Open*, **5**, p. e007231, (2015).
- [75] L. Mattar, C. Huas, J. Duclos, A. Apfel, and N. Godart, Relationship between malnutrition and depression or anxiety in Anorexia Nervosa: a critical review of the literature, *J Affect Disord*, **132**, 311–318, (2011).
- [76] R. O. Soares, L. M. Oliveira, J. S. Marchini, J. Antunes-Rodrigues, L. L. Elias, and S. S. Almeida, Effects of early protein malnutrition and environmental stimulation on behavioral and biochemical parameters in rats submitted to the elevated plus-maze test, *Nutr Neurosci*, **16**, 104–112, (2013).
- [77] R. O. Soares, R. C. Rorato, D. Padovan, J. J. Lachat, J. Antunes-Rodrigues, L. L. Elias, and S. S. Almeida, Environmental enrichment reverses reduction in glucocorticoid receptor expression in the hippocampus of and improves behavioral responses of anxiety in early malnourished rats, *Brain Res*, **1600**, 32–41, (2015).
- [78] L. F. Rocinholi and J. Landeira-Fernandez, Anxiety-like behavior in weanling and young adult male and female malnourished rats, *Physiol Behav*, **102**, 13–16, (2011).