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Predictive behaviors for anxiety and depression in female Wistar rats subjected to cafeteria diet and stress

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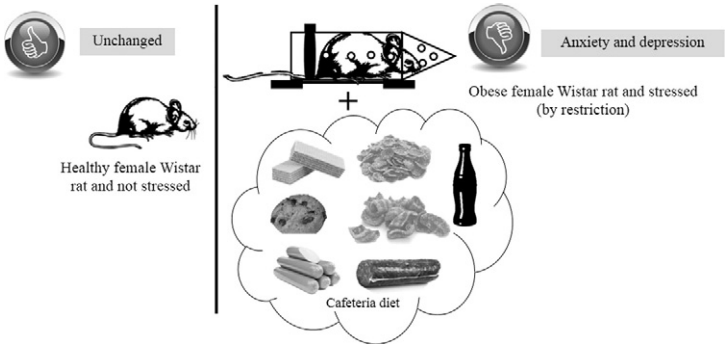
HIGHLIGHTS

- Cafeteria diet used in this study is effective in inducing obesity in female Wistar rats.
- Chronic stress induced in the animals does not increase obesity in females of the chosen rodent lineage.
- Obese and stressed female rats presented a higher anxiety index and predictive behavior for depression.

GRAPHICAL ABSTRACT



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ABSTRACT

Obesity and chronic stress have been considered important public health problems that affect millions of people worldwide. Our aim was to analyze the effect of obesity associated with chronic stress on neurobehavioral parameters in female rats, considering that the association of these syndromes can enhance the negative effects on homeostasis. The animals were distributed into standard diet (Std), standard diet + stress (Std + stress), cafeteria diet (Cafe), and cafeteria diet + stress (Cafe + stress) groups. The animals of groups Std and Std + stress were fed with rodent standard feed. Groups Cafe and Cafe + stress, additionally to the standard feed, were offered palatable and calorie-rich processed food and cola-type soft drink ad libitum. From the eighth experimental week, groups Std + stress and Cafe + stress were subjected to restraint chronic stress model (50 days). After the stress protocol, predictive anxiety (open-field and elevated plus-maze tests) and depression (forced swim) were applied. The cafeteria diet was effective in inducing obesity. The ratio locomotion in the central quadrants/total locomotion evaluated during the open field test was not indicative of anxiogenic or anxiolytic effect in the

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animal's behavior. However, the elevated plus maze test showed that obese and stressed animals were prone to higher anxiety levels. In addition, the obese and stressed animals display less climbing behavior than all the other groups, which can be considered an indicator of depression-like behavior. Nevertheless, it is suggested that the mechanisms involved in effects of obesity associated with chronic stress be better investigated in female rats, considering the organic complexity related to these modern illnesses.

1. Introduction

Obesity is considered epidemic and one of the main public health problems of the world [26,40,98]. It is defined as the accumulation of excess body fat to the extent that it results in other health complications, thus reducing life expectancy [3,30,31,48]. In humans, co-morbidities associated with obesity include psychological distress, osteoarthritis, type 2 diabetes mellitus, hypertension, hyperlipidemia, fatty liver (steatosis), cardiovascular disease and certain types of cancer [39,83]. The steady increase in life expectancy due to advanced medical treatment may be reversed by negative impacts of obesity on youth today in Westernized countries [66].

In clinical practice, body fat is most commonly and simply estimated by using a formula that combines weight and height [45]. The underlying assumption is that most variation in weight for persons of the same height is due to fat mass, and the formula most frequently used in epidemiological studies is the body-mass index (BMI). A graded classification of overweight and obesity using BMI values provides valuable information about increasing body fatness. It allows meaningful comparisons of weight status within and between populations and the identification of individuals and groups at risk of morbidity and mortality. It also permits identification of priorities for intervention at an individual or community level and for evaluating the effectiveness of such interventions. It is important to appreciate that, owing to differences in body proportions; BMI may not correspond to the same degree of fatness across different populations. Nor does it account for the wide variation in the nature of obesity between different individuals and populations. A World Health Organization (WHO) expert committee has proposed the classification of overweight and obesity that applies to both men and women and to all adult age groups (BMI ($kg\ m^{-2}$) = 25.0–29.9, overweight; 30.0–39.9, obesity and ≥40.0, morbid obesity [99]. However, it is important to emphasize that data presented reflect knowledge acquired largely from epidemiological studies in developed countries.

Another health problem that has affected millions of people is stress exposure, which is related to modern world dynamics [61]. As discussed by Lazarus [49] and Taylor and Stanton [85], stress can result from a certain condition and/or lifestyle and lead to an ample series of behavioral alterations. Among them, there are changes in eating habits, which reflect an interaction between the organism's physiological state and environmental conditions [67,79,90]. Chronic stress is associated with metabolic disorders and changes in energy homeostasis [5], which can induce to compensatory pleasant and compulsive behaviors, such as the intake of palatable and calorie-rich foods, and consequently lead to obesity [9,15,37].

Although the importance of the analysis of the joint effects of obesity and stress is recognized [21–23,25,56–58,86,96], studies involving the association of these conditions in neurobehavioral aspects are lacking. In addition, some studies have pointed to different results when males and females are evaluated in studies involving obesity [2] and stress [4,32,42,52]. Especially on stress, various stressor agents and protocols have been used. According to Franceschelli et al. [32], the chronic mild stress model is one of the most extensively investigated animal models of chronic stress. However, only a limited number of studies have been conducted in female rodents. In relation to obesity, although some work has been conducted using female rats [71,17,38,81,82], the neurobehavioral aspects that can indicate neuropsychiatric disorders, such as locomotors alterations, anxiety and depression have not been the focus of these studies.

Obesity is considered an "extreme" linked to nutrition, whose effects can be enhanced when in association with chronic stress. Obesity (induced in murine models by introducing cafeteria diets) has caused damage to several organic functions [92,27,84,93]. However, there is still the necessity of specific investigations on the factor stress linked to obesity, once stress alone is an impacting factor to the organism. The objective of our study is to analyze the effect of obesity associated with chronic stress on neurobehavioral parameters in rats, considering that the association of these diseases can increase the negative effects on homeostasis. Our hypothesis is that obesity associated with chronic stress by restraint induces anxiety-like and depression-like behaviors in female Wistar rats.

2. Material and methods

2.1. Animals and experimental groups

Female Wistar rats from the matrices obtained from the *Biotério Central* (Animal Facility) of the Federal University of *Goiás* (Goiás, Brazil) were kept in the animal facility of the Laboratory for Biological Research of the *Instituto Federal Goiano*, Urutaí, Goiás, Brazil. The animals were subjected to a natural photoperiod (approximately 12:12 h), and offered food and liquids ad libitum. Forty-five day old animals were used (age corresponding to the final stage of puberty, according to Andrade et al. [1], distributed in the following experimental groups: standard diet (Std); standard diet + stress (Std + stress); cafeteria diet (Cafe) and cafeteria diet + stress (Cafe + stress). We emphasize that the diet started at this age (forty-five days old) and then subsequent manipulations were performed when the animals were young adults. Each experimental group was composed of six animals, and two experiments were conducted independently, totalizing twelve animals per group. The animals were maintained individually in separate cages. 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, 8th edition (2011). Animal handling and all experiments were performed in accordance with the International Guidelines for Animal Welfare.

2.2. Experimental design

Standard diet groups (stress or no stress) were fed with rodent standard feed (Nuvilab — CR1®) (Table 1). Cafeteria diet groups (stress or no stress) were fed with cafeteria diet, which consisted of palatable foods with expressive levels of lipids. This food composition is nowadays prevalent in urban societies and associated with the pandemic of obesity. Cafe and Cafe + stress groups (in separate feeders) were offered daily three varieties of palatable foods ad libitum (randomly selected from the foods listed in Table 2 — all values were expressed by the amount (in grams) informed on the food package), besides rodent standard feed. Cafe and Cafe + stress groups received the same variety of palatable food, in addition to standard diet (Fig. 1). Std and Std + stress groups received standard diet only.

The cafeteria and standard diets were weighed before and after consumption. The daily consumption was calculated by subtracting the

Table 1
Nutritional information from the rodent standard food (Nuvilab — CR1).

Ingredients of the standard food[a]	
Ground whole corn, soybean bran, wheat bran, calcium carbonate, dicalcium phosphate, sodium chloride, mixture of vitamins, minerals and amino acids.	
Specifications[a]	Warranty levels per kg of the product (g/kg)[a]
Moisture	125
Casein[b]	220
Ether extract	40
Mixture of salts	90
Fibrous matter	70
Calcium	10
Phosphorus	8
kcal	2930
Enrichment per kg of the product[c]	
Vitamins: Vitamin A 13,000 IU; Vitamin D3 2000 IU; Vitamin E 34 IU; Vitamin K3 3 mg; Vitamin B1 5 mg; Vitamin B2 6 mg; Vitamin B6 7 mg; Vitamin B12 22 μg; Niacin 60 mg; Calcium pantothenate 20 mg; Folic acid 1 mg; Biotin 0.05 mg; Choline 1900 mg. Minerals: Zinc 60 mg; Copper 10 mg; Iodine 2 mg; Selenium 0.05 mg; Cobalt 1.5 mg; Fluorine 80 mg. Amino acids: Lysine 12 g; Methionine 4000 mg. Additives: BHT 100 mg.	

[a] Information taken from food packaging [64].
[b] The protein content of the casein used was approximately 80%.
[c] Adapted from Reeves et al. [74] (AIN-93-GX) and Reeves et al. [74] (AIN-93G-MX).

leftovers from the total amount of food offered per day. The palatable food chosen had different shape and color from the standard diet to facilitate differentiation of leftovers used to calculate daily consumption. The following parameters were calculated daily: Total feed intake (in grams), percentage and amount (in grams) of proteins, carbohydrates and total lipids consumed. In addition, the animals of the Cafe groups received natural water; water plus sucrose (300 g/L concentration adopted from [60]), and cola-type soft drink ad libitum. Liquid intake was not measured. The calculation of the daily caloric (kcal) intake was based on food intake separately multiplied by their caloric content.

After eight weeks, Std + stress and Cafe + stress groups were subjected to a protocol of chronic stress by restriction, as proposed by Ely et al. [28]. 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 2 p.m. to 3 p.m.), for five days a week, during 50 days. The selection of this model was based on several studies [28,33–35,80,69,25,56–58]. We associate it with the cafeteria diet as an attempt to simulate the daily life of the general population, at times when people undergo low intensity stresses.

Body mass was the parameter taken as indicative of obesity and was measured weekly by means of a semi-analytical digital scale. In the eighth experimental week, the naso-anal length and waist circumference were also measured. As proposed by Levin et al. [50], the animals considered obese were those that gained 15% or more weight than the animals that were not fed with the cafeteria diet, and increased in naso-anal length and waist circumference.

From the 15th week on and according to the schedule shown in Fig. 1, the animals were subjected to predictive behavioral tests for the assessment of the following neuropsychiatric disorders: anxiety and depression. After the behavioral tests, the animals were anesthetized with an intraperitoneal injection of 40 mg/kg pentobarbital, followed by cervical dislocation. Visceral, retroperitoneal fat mass and relative mass of adrenal were weighed. Mass of the organs were normalized to body weight using the following formula: mass of the organs (g)/body weight (g). As a stress parameter, we analyzed the relative adrenal gland mass, according to Macedo et al. [58].

2.3. Open field test

Open field test consisted of a lit (110 lx) circular arena, 120 cm in diameter, surrounded by a 45 cm-high circular wall, divided in 12 quadrants. Animals were individually placed in the center of the arena and filmed during 5 min (with a camera mounted vertically over the apparatus). The ratio (in percentage) locomotion in the central quadrants/total locomotion was calculated. According to Prut and Belzung [72], a lower percentage of locomotion in the central quadrants and consequently a high percentage of locomotion in the lateral quadrants can be used as an index of anxiety. The measured parameters were treated using the analysis of variance according to the factorial model (two-way ANOVA), considering the factors "nutrition" (standard and cafeteria diet) and "condition" (no stress and stress), with 12 animals in each group (using the software ASSISTAT, version 7.7 beta — freeware).

2.4. Elevated plus-maze

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 5 min 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 Cohen et al. [16] and Contreras et al. [18] as follows: Anxiety Index = 1 − [([Open arm time / Test duration] + [Open arms entries / Total number of entries]) / 2]. The measured parameter was also treated using the analysis of variance according to the factorial model (two-way ANOVA), considering the factors "nutrition" (standard and cafeteria diet) and "condition" (no stress and stress), with 12 animals in each group (using the software ASSISTAT, version 7.7 beta — freeware).

2.5. Forced swim test

The apparatus was mounted according to the specifications in Porsolt et al. [70]. A plexiglass cylinder (65 cm in height and 35 cm in diameter) was filled with water (25 ± 2 °C) up to 55 cm, so that the animal could not stand at the bottom of the cylinder. The animals were subjected to a forced swim test on two consecutive days, respecting the same time schedule. The first day consisted of a training session, in which each animal stayed in the cylinder for 15 min (establishing the inescapable aversive situation); on the second day (test session), the animal remained in the cylinder for only 5 min. To record the animal's behavior during the test session, it was filmed with a digital camera. At the end of the test, the animal was taken from the cylinder and dried with a towel. Between the sessions, the water in the cylinder was drained and the cylinder sanitized with alcohol (70%).

The following time intervals were measured: floating (complete immobility or smooth movements, only enough to keep the nose/head above the water surface); climbing (vigorous movements with the forepaws above water surface or against the cylinder wall), and swimming (the animal moving inside the cylinder with the body lying horizontally, without breaking the water surface with the forepaws), as specified in Fernandes et al. [29]. Swimming was also considered when the animal kept the body under the water surface. The measured parameters were treated using the analysis of variance according to the factorial model (two-way ANOVA), considering the factors "nutrition" (standard and cafeteria diet) and "condition" (no stress and stress), with 12 animals in each group (using the software ASSISTAT, version 7.7 beta — freeware).

We emphasize that two trained observers carried out record of behavioral parameters during all tests. The same video was analyzed twice, resulting in an inter-observer concordance of more than 85%.

Table 2
List of palatable food offered to the animals of the Cafe groups along the experimental period, with nutritional information.

Processed foods and portion (in grams) as regards their nutritional composition	kcal per portion	Carbohydrates		Protein		Total lipids	
		(g)	% DV	(g)	% DV	(g)	% DV
1. Bacon cracker (São João Sabor e Nutrição®) (30 g portion)	120	19.8	6.6	1.8	2.4	3.6	6.6
2. White chocolate bonbon (Nestle® Brasil) (20 g portion)	93	14	5	0.7	1	3.6	7
3. Honey bread (Quero Quero) (30 g portion)	123	28	9	1.6	2	0.5	1
4. Salame (Friato®) (50 g portion)	112	2.5	1	6	8	8	15
5. Lemon wafer cookies (Parmalat®) (30 g portion)	165	19	6	1.1	2	9.5	17
6. Corn flakes (Skiny®) (25 g portion)	127	20	7	1.8	2	4.2	8
7. Coated peanuts (Dori®) (25 g portion)	109	20	7	1.8	2	2.4	4
8. Vanilla mini-cake (Bauducco®) (40 g portion)	164	20	7	2.2	3	8.4	15
9. Homemade dulce de leche (Parati®) (20 g portion)	70	17	4.4	1	1.6	12	1.3
10. Mini bread rolls (Pullman®) (50 g portion)	154	28	9	3.9	5	2.8	5
11. Brigadeiro sandwich biscuits (Mabel®) (30 g portion)	147	21	7	1.7	2	6.9	12
12. Toasted popcorn (São João®) (40 g portion)	64.4	12.4	4	2.1	3	7.5	14
13. Cookies (Bauducco®) (30 g portion)	151	19	6	2.1	3	7.5	14
14. Maisena biscuit (Parmalat®) (30 g portion)	132	22	7	2.4	3	3.7	7
15. Tapioca flour biscuit (Peta Caseira) (25 g portion)	117	18	6	0.9	1	0.93	4
16. Pizza flavored biscuit (Miliopã®) (30 g portion)	128	22	7	2.0	2	3.5	6
17. Caramel-filled bonbon (Nestlé®) (20 g portion)	92	15	5	0.6	1	3.3	6
18. Strawberry sandwich biscuit (Amanda®) (30 g portion)	125	16	5	1	2	4	3
19. Cheese flavored chips (Miliopã®) (30 g portion)	130	22	7	2.2	2	3.8	7
20. Provolone cheese (Vale Orizona®) (30 g portion)	103	0	0	8.7	12	76	14
21. Hot dog sausage (Sadia®) (50 g portion)	121	2.0	1	6.8	9	9.5	17
22. Paio sausage (Sadia®) (50 g portion)	187	0	0	8.5	11	17	31
23. Bacon flavored chip (Miliopã®) (25 g portion)	105	18	6	1.8	2	2.5	5
24. Mini chocolate cake (Bauducco®) (40 g portion)	164	20	7	2.2	3	8.4	15
25. Prestígio bonbon (Nestlé®) (18.4 g portion)	85	11	4	0	0	3.8	7
26. Mortadella (Friato®) (50 g portion)	112	2.5	1	6	8	8	15
27. Smoked Calabrian sausage (Sadia®) (50 g portion)	182	0.8	0	8.7	12	16	29
28. Lemon wafer cookies (Amanda®) (30 g portion)	125	16	5	1	2	4	3
29. Sugar peanut (Dori®) (15 g portion)	73	8.9	3	1.8	2	3.4	6
30. Cream biscuits (Maranata®) (30 g portion)	190	20	7	2	3	5	9
31. Chocolate donut (Mabel®) (30 g portion)	125	22	7	1.8	2	3.5	6
32. Carrot cake (Ana Maria®) (40 g portion)	159	23	7	2.0	3	5.6	10
33. Ready crackling (Sabor D'Abadia®) (10 g portion)	68	0	0	2.6	3	6.2	11
34. Brigadeiro filled roll (Bauducco®) (30 g portion)	133	18	6	1.7	2	6.0	11
35. Baconzitos – bacon chips (Elma Chips®) (25 g portion)	126	15	5	1.9	3	6.7	12
36. Cashew (Dunorte®) (15 g portion)	88	3.4	1	3.0	4	6.9	12
37. Chicken sausage (Perdigão®) (50 g portion)	106	2.0	1	6.0	8	8.2	15
38. Mortadella (Sadilar®) (40 g portion)	121	3.4	1	4.0	6	10	18
39. Gomets (Dori®) (20 g portion)	72	18	6	0	0	–	–
40. Mini strawberry cake (Bauducco®) (40 g portion)	146	20	7	2.2	3	6.4	12
41. Baked cheese chips (Cheetos®) (20 g portion)	100	11	4	0.9	2	4.4	8
42. Wavy potato chip (Crony®) (25 g portion)	153	1.4	5	1.7	2	10	18
43. Apresuntado ham (Sadia®) (30 g portion)	36	0.8	0	3.9	5	1.9	3
44. Sweet corn flakes (Ki gostoso®) (25 g portion)	69	16	5	1.3	2	0	0
45. Peanuts (Paulista®) (15 g portion)	90	16	5	1.3	2	0	0
46. Bis — chocolate wafer (Lacta®) (30 g portion)	149	19	6	1.8	2	7.1	13
47. Passatempo — strawberry filled sandwich biscuit (Nestlé®) (30 g portion)	135	21	7	1.9	3	4.8	9
48. Fandangos (Elma Chips®) (22 g portion)	100	16	5	1.5	2	3.5	6
49. Teens Bauny – rolls (Marilam®) (30 g portion)	134	20	7	2.4	3	4.8	9

Legend: (–): information not provided by food packaging.

3. Results and discussion

The evaluation of the body parameters revealed that, from the fifth experimental week on, the animals fed with cafeteria diet presented a significant increase in body mass from animals fed with the standard diet (Fig. 2A), and this difference remained until the end of the experiment (Fig. 2B). No difference was observed between the body masses of stressed and non-stressed animals fed with cafeteria diet, indicating that stress had no effect in this parameter. In the eighth experimental week, we observed the effect of only the factor "nutrition" on retroperitoneal ($F_{(1,40)} = 71.202$, $p < 0.001$) and visceral fat mass ($F_{(1,40)} = 32.887$, $p < 0.001$) (Fig. 3A and B). These results show that the cafeteria diet was effective in inducing obesity in the animals.

Daily calorific intake (kcal) of the Cafe group, up to the 8th experimental week, was higher when compared to the Std diet group ($p = 0.002$), according to Student's t-test at 5% probability (Fig. 4A). However, when starting the stress protocol, we observed the effect of Factor 1 "nutrition" ($F_{(1,40)} = 14.578$, $p < 0.001$) and Factor 2 "condition" ($F_{(1,40)} = 5.446$, $p = 0.021$) on the daily calorific intake (Fig. 4B), but did not observe interaction of factors ($F_{(1,40)} = 0.007$, $p = 0.930$).

On the other hand, no difference was observed between the total daily food intakes (in grams) (up to eight experimental weeks) ($p = 0.533$, according to Student's t-test at 5% probability), or effect of the factors (1, 2 or interaction) after the start of the stress protocol (Factor 1 ($F_{(1,40)} = 2.206$, $p = 0.139$); Factor 2 ($F_{(1,40)} = 0.266$, $p = 0.606$) and interaction ($F_{(1,40)} = 0.034$, $p = 0.853$) (figures not shown).

The detailed daily consumption of proteins, carbohydrates, and total lipids was assessed during the first eight experimental weeks and after the start of the stress protocol. During the first eight experimental weeks the animals fed with cafeteria diet presented lower daily protein intake (% and amount, in grams) ($p < 0.001$, for both); lower consumption of carbohydrates (%) ($p < 0.05$) and higher consumption of lipids (% and amount, in grams) ($p < 0.001$, for both) (Fig. 5A–F). After the start of the stress protocol, we observed the effect of Factor 1 "nutrition" on the daily protein intake (% and amount, in grams) ($F_{(1,40)} = 3581.881$, $p < 0.001$ and $F_{(1,40)} = 49.001$, $p < 0.001$,

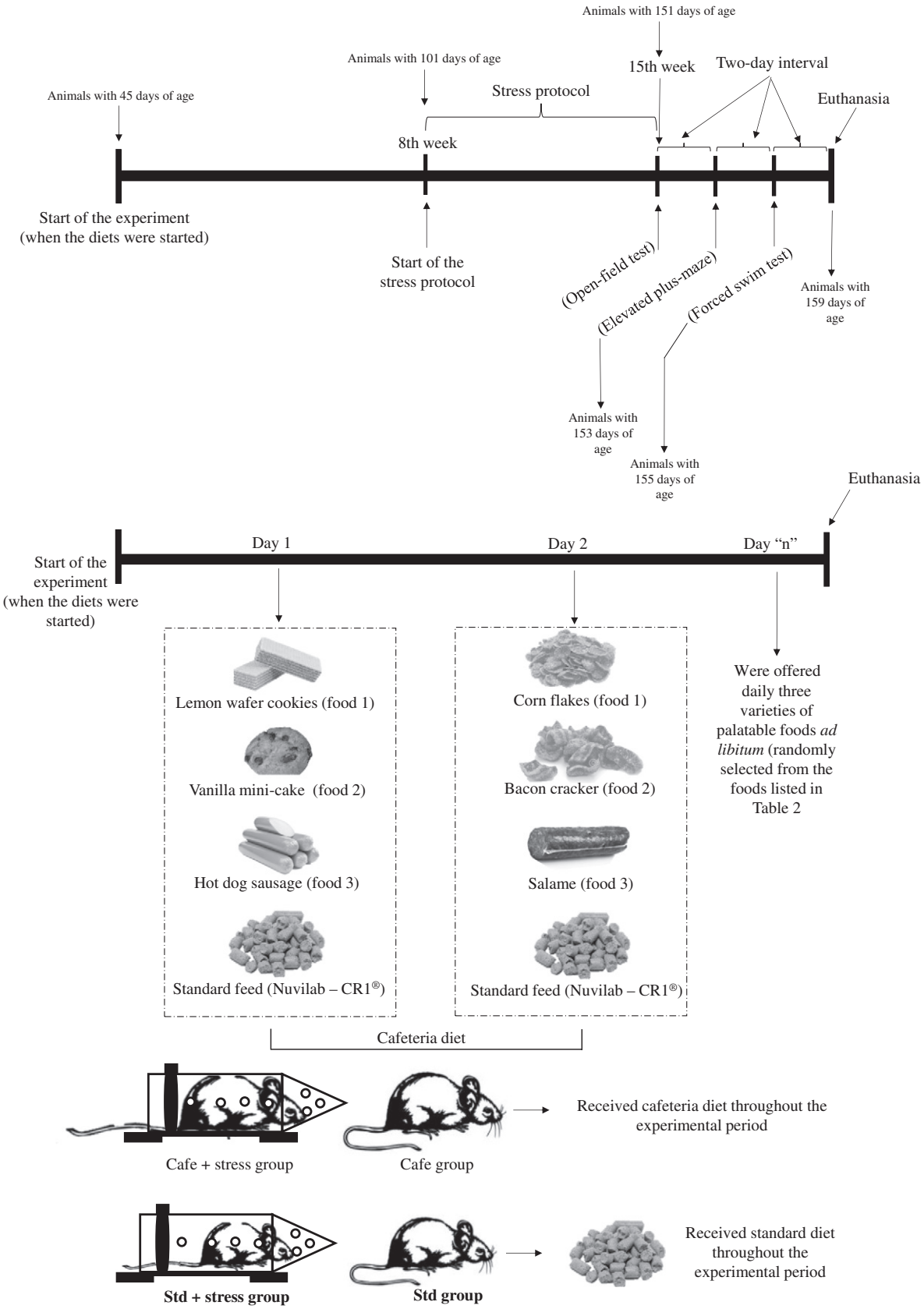

Fig. 1. Schedule prepared for the experiment carried out with Wistar rats subjected to standard and cafeteria diets, subjected or not to chronic stress by restriction. The colors are merely illustrative.

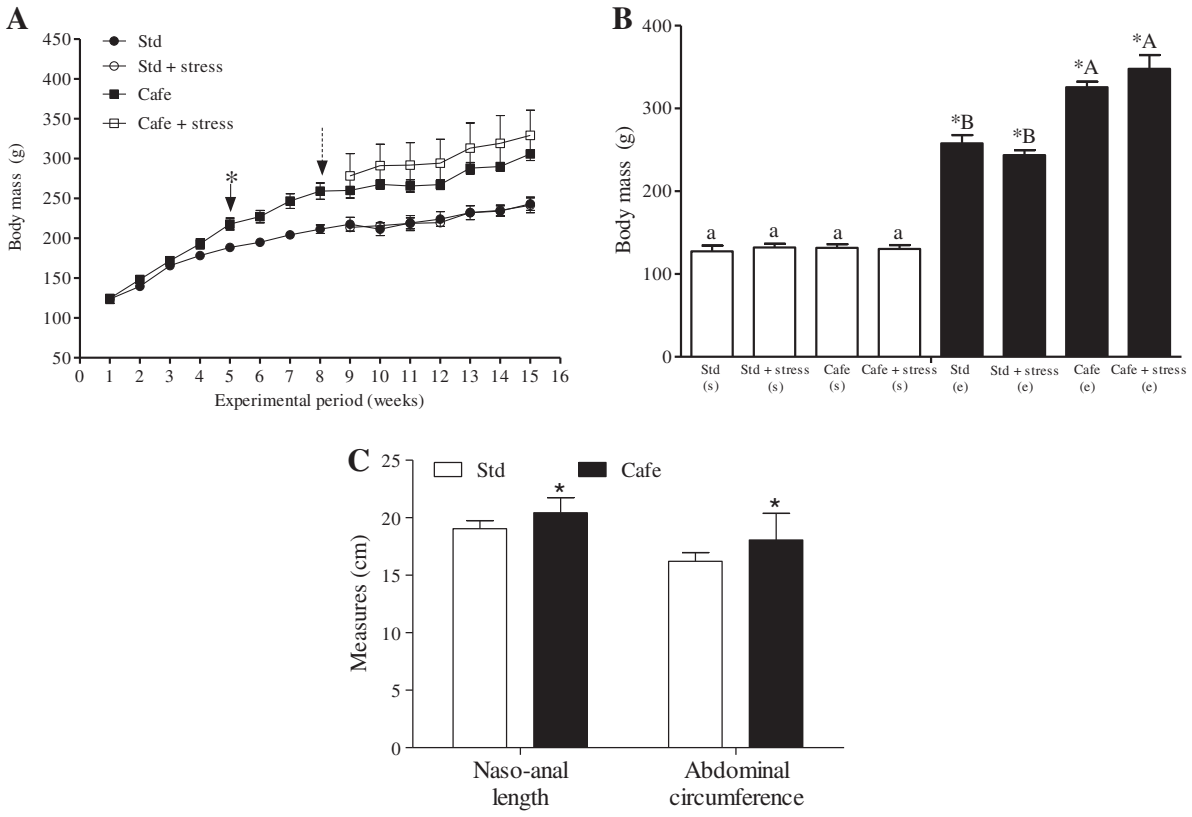

Fig. 2. (A) Weekly and (B) initial vs. final body mass, and (C) naso-anal length and abdominal circumference of Wistar rats subjected to standard and cafeteria diets, exposed or not to chronic stress by restriction, in the eighth experimental week. Std: standard diet group; Cafe: cafeteria diet group; Std + stress: standard diet group with stress; Cafe + stress: cafeteria diet group with stress. Data expressed in means ± standard deviation in A and in means ± standard deviation in B and C. The data result from two experiments (n = 12) performed independently. For the analysis of the weekly and initial vs. final body masses, the analysis of variance for repeated measures (ANOVA RM) and Tukey's post-test were used. For the analysis of the data between experimental groups, the factorial model (two-way ANOVA) was used with Tukey's post-hoc test at 5% probability. Distinct letters indicate statistical difference ($p < 0.05$) between experimental groups. Lowercase letters compare groups at the beginning of the experiment. Uppercase letters compare groups at the end of the experimental period (15 weeks). In (A), the arrow with an asterisk indicates the week from which the body mass of the animals fed with cafeteria diet was higher than the body mass of animals fed with standard diet. The dashed arrow indicates the week in which the chronic stress protocol started. In (B), the asterisks (*) indicate significant differences ($p < 0.05$) between the experimental groups, considering the beginning and the end of the experiment. In (C), the asterisks (*) indicate significant differences ($p < 0.05$) between the Std and Cafe groups, according to Student's t-test at 5% probability.

respectively) (Fig. 6A–B), carbohydrates (% and amount, in grams) ($F_{(1,40)} = 13.064$, $p < 0.001$ and $F_{(1,40)} = 8.170$, $p = 0.004$, respectively) (Fig. 6C–D), and lipids (amount, in grams) ($F_{(1,40)} = 34.254$, $p < 0.001$) (Fig. 6E). Thus, stress condition did not influence the parameters related to consumption, in relation to the nutritional detailing of diets. We believe that these results are directly related to the nutritional composition of the food offered to the animals.

Few studies have assessed the effect of obesity associated with stress on the detailed consumption of protein, carbohydrate and lipid of the diets in female rats. The most similar study to ours was that of Wang [95], who investigated the effects of brief (20 min), acute (2 h) and chronic restraint stress (2 h/day for five days) at the time of dark onset on macronutrient selection in female Wistar rats (aged 6–12 months). In the brief and acute paradigms, the intake of

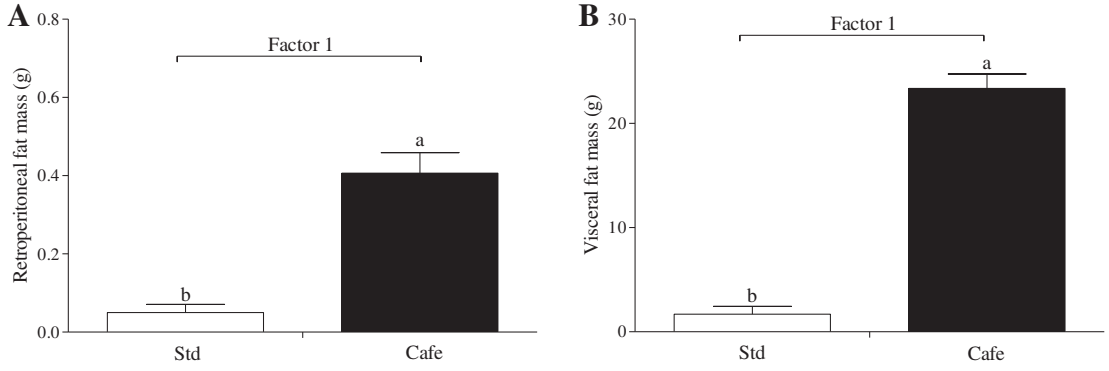

Fig. 3. (A) Retroperitoneal and (B) visceral fat mass of Wistar rats subjected to standard and cafeteria diets, exposed or not to chronic stress by restriction. Bars indicate means ± standard deviation of the data from two experiments (n = 12), performed independently. Comparison between Std and Cafe groups by two-way ANOVA of the factor 1, at 5% probability. Distinct lowercase letters indicate statistically significant differences between experimental groups. Std: standard diet group; Cafe: cafeteria diet group.

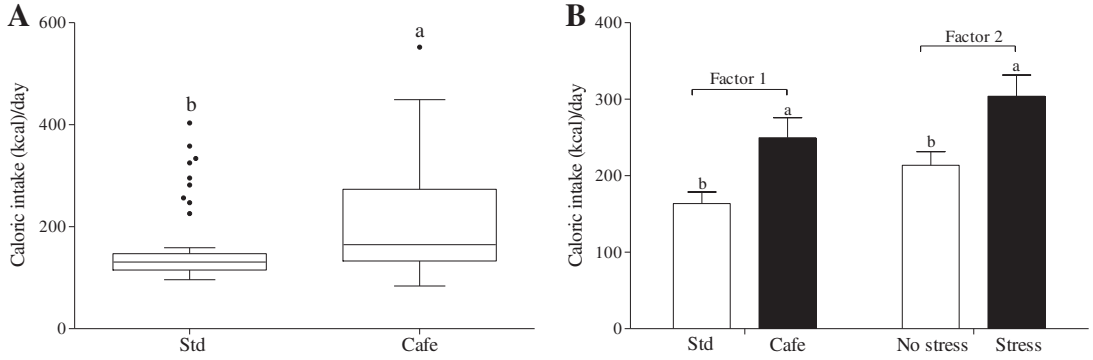

Fig. 4. (A) Caloric intake (kcal) per day by female Wistar rats fed with standard and cafeteria diets up to 8 experimental weeks (n = 24 per group) by Student's t test at 5% probability. (B) Comparison between Std and Cafe groups (Factor 1) and Std + stress vs. Cafe + stress (Factor 2) by two-way ANOVA, at 5% probability (n = 12). In "A", horizontal bars indicate medians, boxes indicate interquartile ranges, whiskers indicate minimum and maximum values, and circles indicate outliers (values 1.5 times higher or lower than the first and third quartiles, respectively). Different letters are used to indicate differences in statistical significance ($p < 0.05$), according to Student's t test in "A" and two-way ANOVA in "B". Std: standard diet group; Cafe: cafeteria diet group; Std + stress: standard diet group with stress; Cafe + stress: cafeteria diet group with stress.

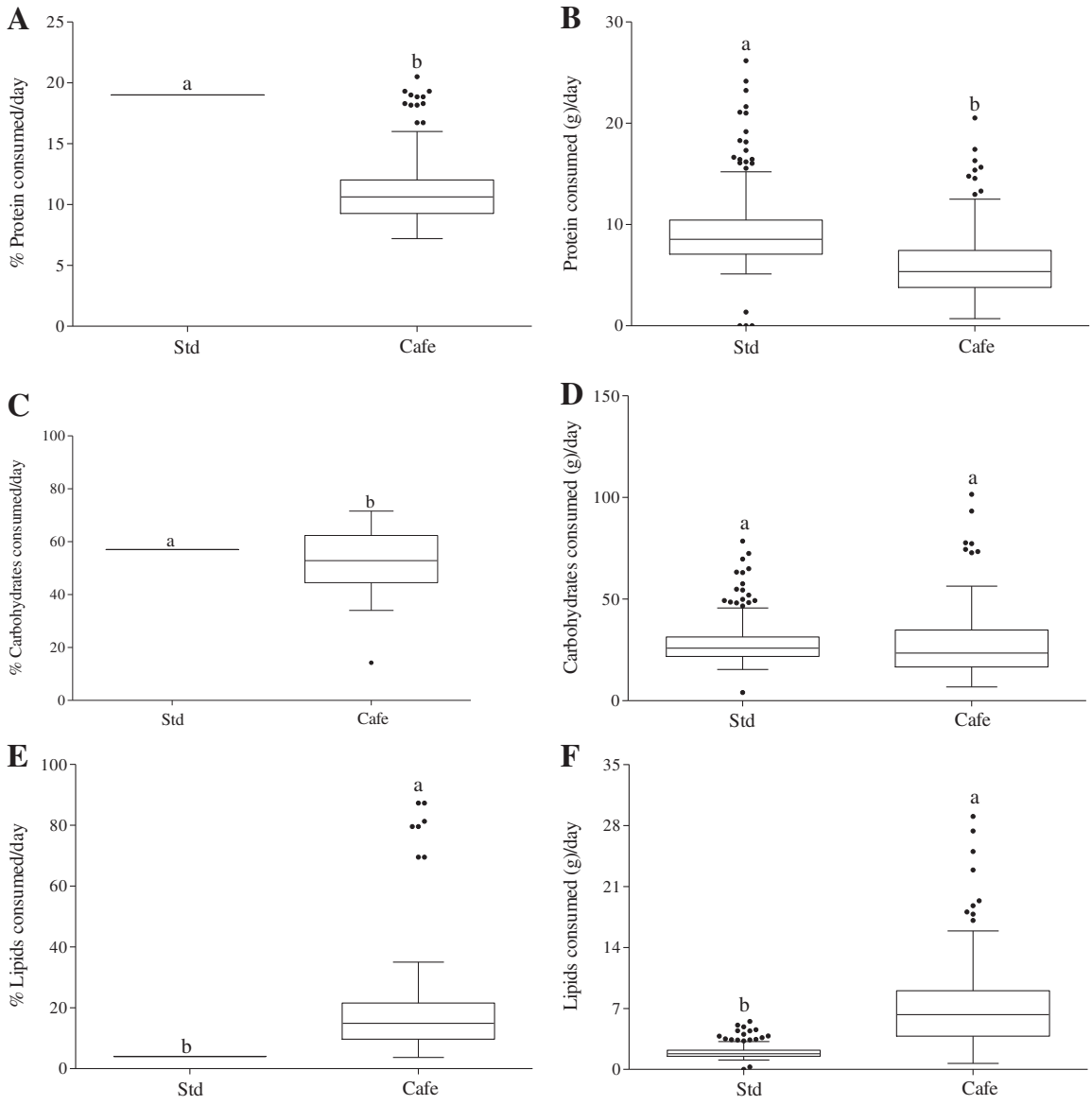

Fig. 5. Percentage (A, C and E) and grams (B, D and F) of protein, carbohydrates and lipids consumed per day, respectively, by female Wistar rats fed with standard and cafeteria diets up to 8 experimental weeks (n = 24 per group). Different letters are used to indicate differences in statistical significance ($p < 0.05$), according to repeated measures ANOVA at 5% probability. Horizontal bars indicate medians, boxes indicate interquartile ranges, whiskers indicate minimum and maximum values, and circles indicate outliers (values 1.5 times higher or lower than the first and third quartiles, respectively). Std: standard diet group; Cafe: cafeteria diet group.

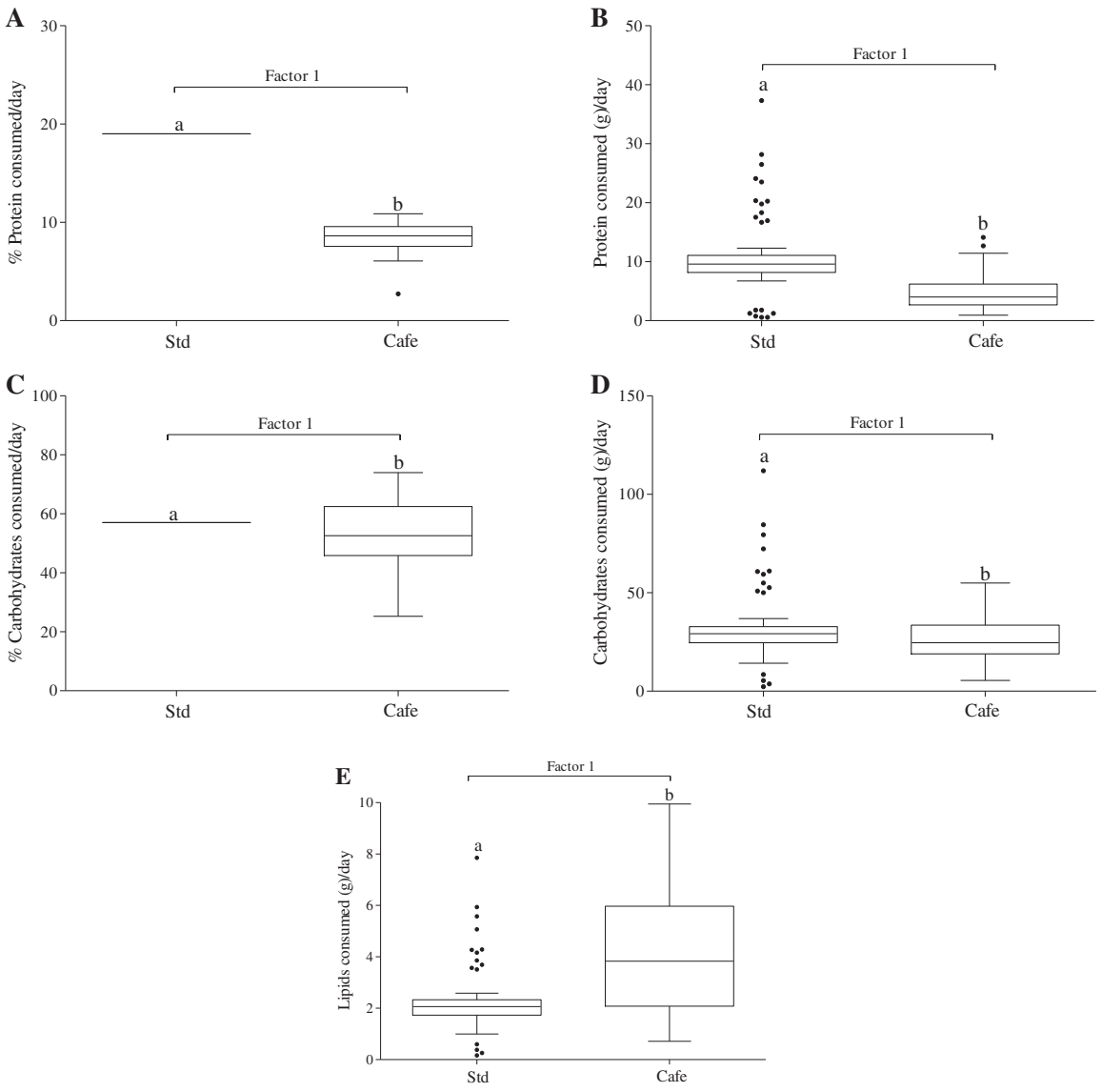

Fig. 6. Percentage (A and C) and amount (in grams) (B and D) of the protein and carbohydrates; (E) amount (in grams) of the lipids consumed per day by female Wistar rats fed with standard and cafeteria diets up to 8 experimental weeks. Comparison between Std and Cafe groups (Factor 1) by repeated measures ANOVA at 5% probability (n = 12). Horizontal bars indicate medians, boxes indicate interquartile ranges, whiskers indicate minimum and maximum values, and circles indicate outliers (values 1.5 times higher or lower than the first and third quartiles, respectively). Different letters are used to indicate differences in statistical significance. Std: standard diet group; Cafe: cafeteria diet group.

pure carbohydrate, protein and fat was measured 40 min after a single exposure to stress; in the chronic model, nutrient intake was assessed 40 min after the final restraint stress session on day 5. This author showed that neither protein nor total intake was significantly altered by restraint in the brief, acute or chronic restraint experiments, whereas carbohydrate consumption was suppressed by acute and chronic restraint, and fat intake was suppressed by brief, acute and chronic restraint. Therefore, further studies are needed to better understand the effect of stress on the detailed food intake, in terms of nutritional composition.

According to Macedo [55], under chronic stress conditions there is continuous stimulation of the adrenal by adrenocorticotropic hormone leading to hypertrophy of these glands. Thus, as we did not evaluate the glucocorticoid levels, we used the weight of adrenal as indirect stress parameter showing that the animals were stressed. The effect of factor 2 "condition" (stress exposure) was observed on relative adrenal gland mass ($F_{(1,40)} = 6.681$ $p = 0.032$), with increased adrenal weights in stressed animals compared with control (no stress) (Fig. 7). This result corroborates previous studies that used similar protocols to ours, and showed that exposure to daily restraint stress can cause adrenal gland hypertrophy [10,58,59,97].

Anyway, it is important to emphasize that the measured glucocorticoids levels may not indicate that the animals were stressed. It is well known that repeated exposure to the same stressor agent can lead to a habituation of the hypothalamic–pituitary–adrenal (HPA) axis [56,87]. Therefore, chronically stressed animals do not show the same behavior and do not experience the same consequences as animals exposed to acute stress [43,51]. Torres et al. [87], using adult male Wistar rats, showed that corticosterone levels were increased after exposure to acute restraint. Contrarily, Macedo et al. [56] showed no changes in the levels of corticosterone after repeated chronic stress (6 weeks), suggesting adaptation of HPA axis. It is important to highlight that these studies used male Wistar rats, whereas we used females. Corroborating our result, Macedo et al. [58] showed increase in adrenal gland weight in male Wistar rats submitted to 12 weeks of restraint chronic stress.

Recently, Balog et al. [4] evaluated cardiometabolic risk-related biochemical markers and sexual hormone and leptin receptors in the

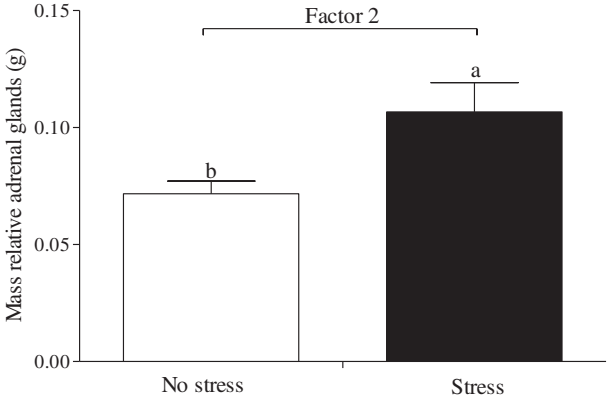

Fig. 7. Relative adrenal gland mass (g) of female Wistar rats of the no stress and stress groups (n = 24 per group). Comparison between no stress and stress (Factor 2) by two-way ANOVA, at 5% probability (n = 24).

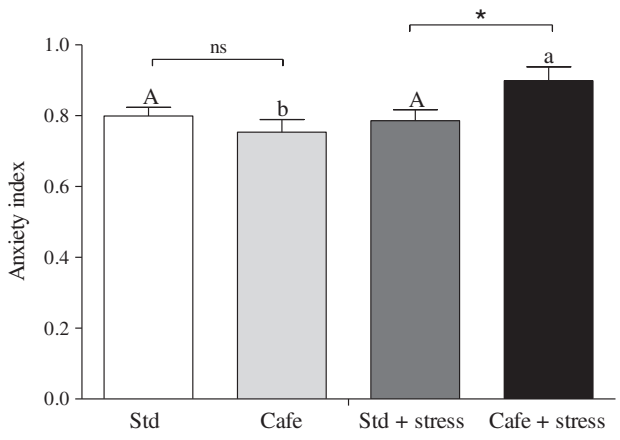

Fig. 8. Anxiety index for female Wistar rats subjected to standard and cafeteria diets, exposed or not to restraint chronic stress evaluated in the elevate plus-maze test. Lowercase letters compare Std vs. Std + stress groups. Uppercase letters compare Cafe vs. Cafe + stress groups. "ns" indicates that no difference was observed between Std and Cafe groups. Asterisk (*) indicates that there were statistical differences between Std + stress and Cafe + stress groups, at 5% probability (two-way ANOVA), by Tukey's test. Distinct letters indicate significant differences.

adrenal gland of rat males, non-ovariectomized females (NON-OVX) and ovariectomized females (OVX) (Sprague–Dawley rats), under chronic stress. In response to stress, early changes in sex specific pattern were detected in the adrenal gland. Changes upon chronic stress in the adrenal gland were related to a decrease in testosterone receptor in females and increase in estrogen receptor in males. Anyway, more studies are needed to better understand the hormonal dynamics in stressed female Wistar rats fed with cafeteria diet and its effects on the levels of glucocorticoids and structure and function of the adrenal glands.

Regarding neurobehavioral parameters, our results did not indicate the effects of the Factor 1 "nutrition" ($F_{(1,40)} = 3.505$ $p = 0.075$), Factor 2 ($F_{(1,40)} = 0.098$ $p = 0.756$) or the interaction between factors ($F_{(1,40)} = 0.073$ $p = 0.789$) on the parameter locomotion in the central quadrants/total locomotion. The ratio locomotion in the central quadrants/total locomotion evaluated during the open field test was not indicative of anxiogenic or anxiolytic effect in the animal's behavior. According to Prut and Belzung [72], a lower percentage of locomotion in the central quadrants (on the total locomotion), and consequently a high percentage of locomotion in the lateral quadrants can be used as an index of anxiety. The study of Bailey et al. [6] showed that males were significantly more active than females on horizontal activity and total distance traveled in the arena of the open field test. More recently, Sivanathan et al. [81] showed that chronic high fat feeding increases anxiety-like behavior of the adult female rats in the light dark and open field tasks compared to rats in the low fat diet group. Thus, we suggest the development of further studies in order to better understand the effects of obesity associated with stress on these parameters, including studies involving males and females.

Regarding the elevated plus maze test, the statistical analysis showed interaction between factors "nutrition" and "condition" for anxiety index ($F_{(1,40)} = 5.723$ $p = 0.021$), showing that obese and stressed animals presented a higher anxiety index (Fig. 8).

Studies that use female rats to analyze anxiety parameters in murine models subjected to chronic stress and/or to cafeteria diet are rare in the literature [81], which makes it difficult to make comparisons with our results. We found in recent literature some studies involving female rats that evaluate obesity effects [71,38,17,81] and stress [4,78] in a dissociated form, or in younger models [24,47,91], which differs from our work.

In our study, the elevated plus maze test shows the overlap of the effects caused by diet and stress on the predictive parameters for anxiety in rodents. However, during the open field test, this interaction was not observed, which can be explained by different nature of the tests. As discussed by Carola et al. [14], the open field test is more appropriate to evaluate indices of motor activities (mechanical components) and less sensitive to evaluate psychomotor behaviors in rodents, when compared to the elevated plus maze test, although both tests can be used to evaluate anxiety-like behavior. Komada et al. [44] point to the fact that, rather than leading to behaviors related to anxiety, the open field test measures the anxiety of animals in open spaces (open-space anxiety-like behavior), which is not the case of the elevated plus maze test.

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 [68,94]. This well-established paradigm has a long and successful history in assessing anxiety-like behavior in rodents [7,68,75,76]. The test takes advantage of the natural tendency of mice 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 behaviors that have been correlated with increases in physiological stress indicators [41]. In contrast, administration of benzodiazepines and other anxiolytic treatments results in increased exploration of the open arms, without affecting general motivation or locomotion [76].

In humans, emotional problems are generally perceived as consequence of obesity, despite conflicts and self-concept psychological problems can precede the development of this condition [89]. In this case, anxiety is one of the most common symptoms in obese individuals [36]. Besides, it has been reported that emotionally unstable obese patients fall into an anxiogenic state when undergoing nutritional education processes, accompanied by high-calorie food restriction [89].

Anyway, it has been a consensus in the literature that anxiety disorders have a complex origin, being associated with changes in several neurotransmission systems [11]. However, in chronic stress-induced animal models, it has already been demonstrated a decrease in molecular links to benzodiazepine receptors in the frontal cortex, hippocampus, and hypothalamus [62], aspect that can possibly explain the results obtained in our study.

Regarding forced swimming, which is a validated predictive test for depression in laboratory animals Porsolt et al. [70], interaction between the factors "nutrition" and "condition" for the parameter climbing ($F_{(1,40)} = 6.093$ $p = 0.009$), and the effect of the Factor 1 "nutrition" alone for the parameter floating ($F_{(1,40)} = 7.039$ $p = 0.015$) were observed (Fig. 9). No effect of the factors was observed on the swimming parameter [Factor 1 ($F_{(1,40)} = 1.638$ $p = 0.215$), Factor 2 ($F_{(1,40)} = 0.015$ $p = 0.901$) and interaction ($F_{(1,40)} = 3.645$ $p = 0.070$)].

Our data suggest that the floating parameter, commonly used as an indicator of depression in testing models for depression [19,20] could

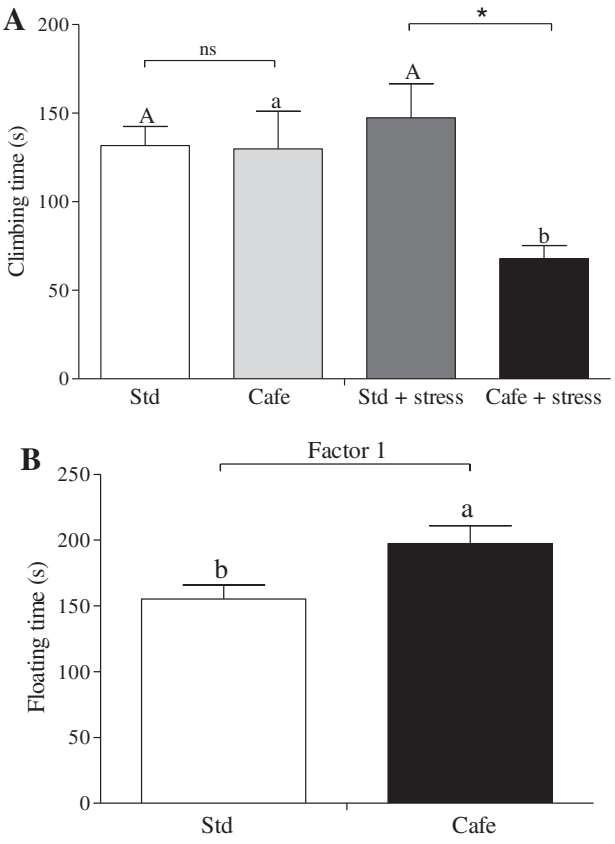

Fig. 9. (A) Climbing time recorded for female Wistar rats subjected to standard and cafeteria diets, exposed or not to restraint chronic stress (n = 12), during the forced swimming test. (B) Comparison between floating time of Std and Cafe groups (Factor 1), by two-way ANOVA, at 5% probability (n = 24). In "A", lowercase letters compare Std vs. Std + stress groups. Uppercase letters compare Cafe vs. Cafe + stress groups. "ns" indicates that no difference was observed between Std and Cafe groups. Asterisk (*) indicates that there were statistical differences between Std + stress and Cafe + stress groups, at 5% probability (two-way ANOVA), by Tukey's test. Distinct letters indicate significant differences.

be a result of a mobility impairment caused by the increased weight, and not due to a depressive-like state in these animals. The study of Calil et al. [12] supports this hypothesis, showing that the meaning of floating (helplessness or adaptation) depends on the experimental model. Nishimura et al. [63] also corroborate this hypothesis.

In this study, the findings were interpreted as further evidence for the notion that floating in forced-swimming rats does not necessarily imply "behavioral despair", but rather an emotional reaction to an inescapable stressor. In our study the finding that obese and stressed animals display less climbing behavior than all the other groups is very interesting, considering that rodents, when exposed to an aversive situation with no possibility of escaping, tend to give up escaping and remain motionless [13], plus the fact that climbing is a good index of fighting to escape.

Although we have not assessed corticosterone levels, it is well established that hormones of the adrenal medulla (epinephrine) and adrenal cortex (corticosterone in rats, cortisol in humans) are released during and immediately after stressful stimulation of the kind used in emotionally arousing learning tasks. The degree to which these hormonal systems are activated depends on the severity as well as type of stressor employed [46]. As removal of endogenous hormones by adrenalectomy impairs memory consolidation for emotionally arousing experiences [65,8,73,77]. Such evidence indicates that stress hormones released by the training experience may act as endogenous modulators of memory consolidation. In addition, Uysal et al. [88] showed that acute-footshock-stress during the adolescent period increased hippocampal VEGF and BDNF levels, cell numbers of CA1 and GD (gyrus dentatus) without altering apoptosis, and enhanced spatial learning and memory in males independent of stress level. However, the level of stress seems to be important in adolescent females.

Finally, it should be noted that our study is not exhaustive and other investigations must be conducted in order to better understand the cognitive and physiologic aspects of obesity and stress. Regarding stress, Luine et al. [53] reported sex-dependent behavioral and neural changes in rats depending on the duration of chronic stress and age. In addition, changing levels of estradiol in the sexes over the lifespan appear to contribute to the differences in response to stress [54].

Thus, theories of stress dependent modulations in central nervous system function – developed solely in male models, focused on peripheral physiological processes and tested in adults – may require revision when applied to a more diverse population (age- and sex-wise) at least in relation to the neural functions of cognition and anxiety. Moreover, these results suggest that other stressors and neural functions should be investigated to determine whether age, sex and gonadal hormones also have some effects.

4. Conclusions

Based on our results and according to the experimental conditions, we conclude that the cafeteria diet used in this study is effective in inducing obesity in female Wistar rats. However, chronic stress induced in the animals does not increase obesity in females of the chosen rodent lineage. In addition we observed that obese and stressed female rats presented a higher anxiety index and predictive behavior for depression.

Nevertheless, it is suggested that the mechanisms involved in effects of obesity associated with chronic stress be better investigated in female rats, considering the organic complexity related to these modern illnesses.

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