Feed restriction since lactation has reduced anxiety in adult Wistar rats

A restrição alimentar desde a lactação reduziu a ansiedade em ratos Wistar adultos

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A B S T R A C T

Objective

To evaluate the effects of macronutrients (protein, carbohydrate and/or lipid) in the diet of young adult (72 days) and adult (182 days) Wistar rats treated *ad libitum* and with 30% restriction from birth on anxiety in the elevated plus-maze.

Methods

We used 238 rats treated from birth, composing the groups: Control, Protein, Carbohydrate, Lipid, Carbohydrate and Lipid, Control Restriction, Protein Restriction, Carbohydrate Restriction, Lipid Restriction and Carbohydrate and Lipid Restriction. The animals were weighed at the beginning and at the end of the experiment and tested in the elevated plus-maze. Data were submitted to analysis of variance, followed by the Newman-Keuls Test ($p<0.05$).

Results

Among the animals treated *ad libitum*, the Control, Carbohydrate plus Lipid and Lipid gained more weight than the Carbohydrate and Protein; *ad libitum* animals gained more weight than those on restriction; among the restrictions, Carbohydrate Restriction rats were the ones that gained less weight. Diet-restricted animals exhibited reduced first-entry latency, greater percentage of entries and time spent, frequency of open arm extremity visits, head dipping (protected and unprotected), and length of stay in the central area of the elevated

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How to cite this article

plus-maze. The animals with 182 days presented greater latency for first entry, reduced frequency of false entries and visits to the ends of the open arms and protected head dipping.

Conclusion

Food restricted animals, regardless of the macronutrient present in the diet, were less anxious and/or increased their impulsivity and those at 182 days were more anxious and/or with reduced impulsivity.

Keywords: Anxiety. Caloric restriction. Carbohydrates. Diet, high-fat. Diet, high-protein. Nutrients.

R E S U M O

Objetivo

Este estudo buscou avaliar os efeitos dos macronutrientes (proteína, carboidrato e/ou lipídeos) na alimentação de ratos Wistar adultos jovens (72 dias) e adultos (182 dias) tratados ad libitum *e com restrição a 30% desde o nascimento quanto à ansiedade através do labirinto em cruz elevado.*

Métodos

Foram utilizados 238 ratos tratados desde o nascimento, compondo os grupos Controle, Proteína, Carboidrato, Lipídeos, Carboidrato e Lipídeos, Restrição Controle, Restrição Proteína, Restrição Carboidrato, Restrição Lipídeos e Restrição Carboidrato e Lipídeos. Os animais foram pesados no início e no final do experimento e testados no labirinto em cruz elevado. Os dados foram submetidos à análise de variância, seguida do teste de Newman-Keuls (p<0,05).

Resultados

Entre os animais tratados ad libitum, *os dos grupos Controle, Carboidrato e Lipídeos, e Lipídeos ganharam mais peso do que os Carboidrato e Proteína; os* ad libitum *ganharam mais peso do que os de restrição; os Restrição Carboidrato foram os que ganharam menos peso. Animais alimentados com restrição de dieta apresentaram menor latência de primeira entrada, maior porcentagem de entradas e de tempo gasto, frequência de visitas às extremidades dos braços abertos, de mergulho (protegido e desprotegido) e tempo de permanência na área central do labirinto em cruz elevado. Os animais com 182 dias demonstraram maior latência para primeira entrada, menor frequência de falsas entradas e visitas às extremidades dos braços abertos e mergulho protegido.*

Conclusão

Os animais em restrição alimentar, independentemente do macronutriente presente na dieta, foram menos ansiosos e/ou mais impulsivos, e os com 182 dias foram mais ansiosos e/ou menos impulsivos.

Palavras-chave: *Ansiedade. Restrição calórica. Carboidratos. Dieta hiperlipídica. Dieta rica em proteínas. Nutrientes.*

I N T R O D U C T I O N

Data published by the United Nations Children's Fund, the World Health Organization and the World Bank [1] showed that 150.8 million (22.2%) children under 5 years of age were malnourished and 38.3 million (5.6%) were overweight in 2017. Early life experiences, including nutrition, play an important role in the development of an organism and early exposure to inadequate nutritional conditions can cause the development of chronic non-communicable diseases, including metabolism disorders and long-term behavioral changes [2,3].

Several tests have been developed to assess anxiety in animals, including the Elevated Plus-Maze (EPM) test that generates an ethological conflict by contrasting the animals' natural tendency to explore new environments with the aversive characteristics of an open, lighted and elevated space. This conflict of approach-avoidance results in behaviors correlated with anxiety, that is, the enhanced exploration of the open arms, which is interpreted as an anxiolytic effect (reduction of anxiety) while exploration reduction is interpreted as an anxiogenic effect (anxiety increase) [4].

The assessment of anxiety in animals treated with obesogenic diets (rich in carbohydrates [5,6], lipids [7-17] and carbohydrates and lipids [18,19]) is controversial. For some authors, the diet did not change the behavior in the EPM [5-7,9,10,16,20], for others it increased [8,11,12,14,15,17-19] or reduced anxiety [13].

The beneficial effects of dietary restriction are widely reported in the literature in different organic systems, including the central nervous system, with increased survival and decreased morbidity. Chronic moderate food restriction is the only known and reproducible intervention associated with increased longevity [21] demonstrated in short-lived animals [22].

Pifferi *et al.* [23] evaluated the effect of moderate chronic food restriction (30%) and demonstrated that the animals showed an increased longevity, a reduction in diseases associated with aging such as cancer, preserved the white matter, but accelerated the gray matter atrophy in much of the brain, indicating a negative impact of food restriction on the integrity and functioning of the central nervous system. The results of food restriction in the EPM are as contradictory as those of excess macronutrients. While some authors found no change [24], others found a reduction [25] or an increase in anxiety [26-28].

There is a wide variation in the type and amount of nutrient (carbohydrate, lipid and protein), time of treatment and stage of life of the animals that can contribute to the outcome disparities; in addition, the understanding of how early unhealthy nutritional practices hinder the development of the central nervous system and behavior, evaluating the simultaneous role of each macronutrient (in excess and restricted) in the performance of young and adult rats is important to prevent or delay the onset of mental illnesses, such as anxiety disorders. Thus, the objective of this study was to evaluate the effects of macronutrients (protein, carbohydrate and/or lipid) in the diet of young adult (72 days) and adult (182 days) Wistar rats treated *ad libitum* and with 30% restriction since birth in the elevated plus-maze.

METHODS

A total of 32 litters of *Rattus novergicus* (Wistar lineage) from the central bioterium of the University of *São Paulo – Ribeirão Preto Campus*, were used. The litters (dam with 8 male pups) were kept in polypropylene cages (41x40x17cm), under 12-hours light-dark cycles (beginning of the light cycle at 6 am), temperature 21±2°C and natural humidity throughout the study. On the 21st day of age the puppies were weaned and placed in individual cages, receiving the same nutritional treatment as their mothers and water *ad libitum* until the end of the experiment.

The dams were discarded and only the puppies made up the study sample that included ten nutritional groups that were subdivided into two subgroups, one tested at 72 days of age and another at 182 days of age. Each group included 10 to 16 animals each, totaling 238 rats: (1) Control (C): AIN-93 diet *ad libitum*; (2) Protein (P): AIN-93 diet added with 30% calories from protein (casein) *ad libitum*; (3) Carbohydrate (G): AIN-93 diet added with 30% calories from simple carbohydrates (sugar) *ad libitum*; (4) Lipid (L): AIN-93 diet plus 30% calories from unsaturated lipids (lard) *ad libitum*; (5) Carbohydrate and Lipid (GL): AIN-93 diet plus 30% calories from simple carbohydrates (sugar) and unsaturated lipids (lard) *ad libitum*; (6) Restriction Control (rC): 70% of the AIN-93 diet ingested by C; (7) Protein Restriction (rP): 70% of the AIN-93 diet plus 30% calories from protein (casein)

ingested by P; (8) Carbohydrate Restriction (rG): 70% of the AIN-93 diet plus 30% calories from simple carbohydrates (sugar) ingested by G; (9) Lipid Restriction (rL): 70% of the AIN-93 diet plus 30% calories from unsaturated lipids (lard) ingested by L; and (10) Carbohydrate and Lipid Restriction (rGL): 70% of the AIN-93 diet plus 30% calories from simple carbohydrates (sugar) and unsaturated lipids (lard) ingested by Carbohydrate and Lipid.

The diets were prepared according to AIN-93, modified [29] and their compositions are shown in Table 1. All diets were stored under refrigeration until the animals were fed (between 7 and 9 hours). Weight gain was calculated by the difference between the final weight (72 or 182 days) and the initial weight (21 days).

The animals with 72 and 182 days of life were tested in the elevated plus maze, as described by Soares *et al.* [29]. The Elevated Plus-Maze consists of an apparatus with two closed arms (50x10x40cm) perpendicular to two open arms (50x10cm) and a central area (10x10cm), raised 50 cm from the floor of the room. A camera (Sony®, Japan, Handcam model) was placed above the maze, connected to a computer (LG®, United States) with a video capture program GeoVision GV–800 System (Copyright 2006 – Multicam System/Multicam Surveillance System 8.01), installed in an adjacent room to enable the monitoring and recording of images for further assessments.

Each rat was placed in the central area with the head facing one of the closed arms and left for five minutes for free exploration. The number of entries (with the four paws) and the time spent in the open and closed arms, latency of first entry in the open arms, frequency of rearing, protected and unprotected head dipping, false entries and visits to the extremities of the open arms were recorded.

With these data at hand, the following was calculated: time in the open arms: time in the open arms/(time in the open + closed arms)x100; % frequency of entry in the open arms; number of entries in the open arms/(number of entries in the open + closed arms) x100; time in the center; test duration (300s) - (time in the open + closed arms); total dive: protected + unprotected dive. Data on body weight gain and behavior was tested using ANOVA, with two factors: diet (with

Table 1. Centesimal composition of diets prepared according to AIN-93, modified [23]. *Ribeirão Preto*, Brazil, 2018.

| Diets	Control	Protein	Carbohydrate	Lipid	Carbohydrate and Lipid
Ingredients					
Casein (g)	17.56	38.54	13.45	15.46	14.39
Methionine (g)	0.04	0.08	0.03	0.03	0.03
Starch (g)	68.47	51.00	52.45	60.28	56.09
Sugar (g)	-	-	23.40	-	12.51
Oil (g)	7.85	5.85	6.01	6.91	6.43
Lard (g)	-	-	-	11.95	5.56
Mixed salts (g)	4.91	3.66	3.76	4.32	4.02
Mixed vitamins (g)	0.98	0.73	0.75	0.86	0.80
Choline (g)	0.20	0.15	0.15	0.17	0.16
Composition					
Calories (kcal)	414.93	411.13	411.41	472.82	439.99
Protein (%)	16.97	37.57	13.11	13.10	13.11
Carbohydrate (%)	66.01	49.62	73.75	51.00	62.37
Lipid (%)	17.03	12.81	13.15	35.90	24.53

10 levels: C, P, G, L, GL, rC, rP, rG, rL and rGL) and age (with two levels: 72 and 182 days), followed by the Newman-Keuls Test ($p<0.05$), after finding the normal distribution. The methodology complied with the ethical principles of animal experimentation and the project was approved by the Ethics Committee on the Use of Animals of *Ribeirão Preto* (*Universidade de São Paulo*) Medical School, registered under number 090/2017.

RESULTS

There was a diet effect ($F_{(9.214)}=62.07$, $p<0.0001$) in weight gain. Animals in groups C, GL, L and P gained more weight than G, rC, rGL, rL and rP, which in turn gained more than rG; the C, GL and L gained more weight than P; L gained more weight than C. There was an interaction effect between diet and age ($F_{(9.214)}=7.20$, $p<0.0001$) on weight gain. Animals in all groups gained more weight at 182 days than at 72 days of life. At 72 days, C, GL and L gained more weight than G, P and all the animals on restriction; P gained more than rG, rGL, rL and rP; G, rC, rGL, rL and rP gained more than rG. At 182 days, GL and L gained more than C, G and P; C more than G; rL more than rC, rP and rG; rC, rGL and rP more than rG; all animals that received an *ad libitum* diet gained more weight than all animals in restriction. The animals of the rG, rGL and rP groups entered the EPM closed arms more often than the C ($F_{(9.211)}=3.74$, $p<0.0005$) and those with 72 days more than those with 182 days of life ($F_{(1.211)}=71.50$, $p<0.0001$) (Figure 1).

Animals in all food restricted groups exhibited shorter latency for first entry into one of the open arms compared with all the animals treated *ad libitum* ($F_{(9.208)}=7.55$, $p<0,0001$) (Figure 2A) and higher rate of entries ($F_{(9.211)}=10.86$, $p<0.0001$) (Figure 2B) and time spent in the open arms ($F_{(9.211)}=13.17$, $p<0.0001$) (Figure 2C) and higher frequency of visits to the extremities of the open arms ($F_{(9.208)}=14.81$, $p<0.0001$) (Figure 2D). 182-day-old animals showed greater latency in first entry into one of the open arms ($F_{(1.208)}=9.65$, $p<0.005$) (Figure 2A) and less frequency of visits to the extremities of the open arms ($F_{(1.208)}=5.38$, p<0.05) (Figure 2D).
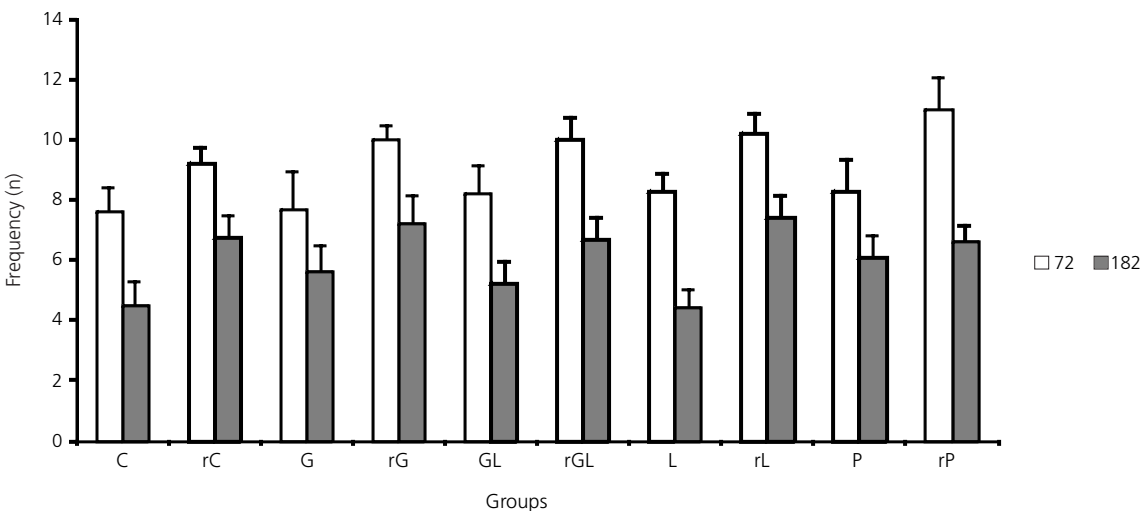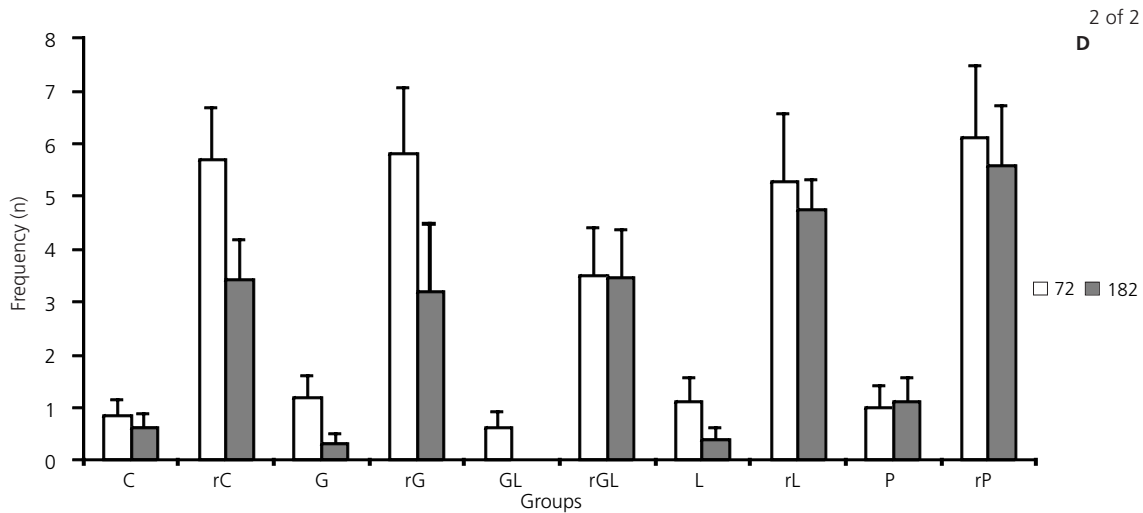

Figure 1. Frequency of entry into the closed arms of the Elevated Plus-Maze by the animals at 72 and 182 days.

Note: C: Control; G: Carbohydrate; GL: Carbohydrate and Lipid; L: Lipid; rC: Control Restriction; rG: Carbohydrate Restriction; rGL: Carbohydrate and Lipid Restriction; rL: Lipid Restriction; rP: Protein Restriction.

rG, rGL, rP>C; 72>182, by Newman-Keuls Test ($p<0.05$).
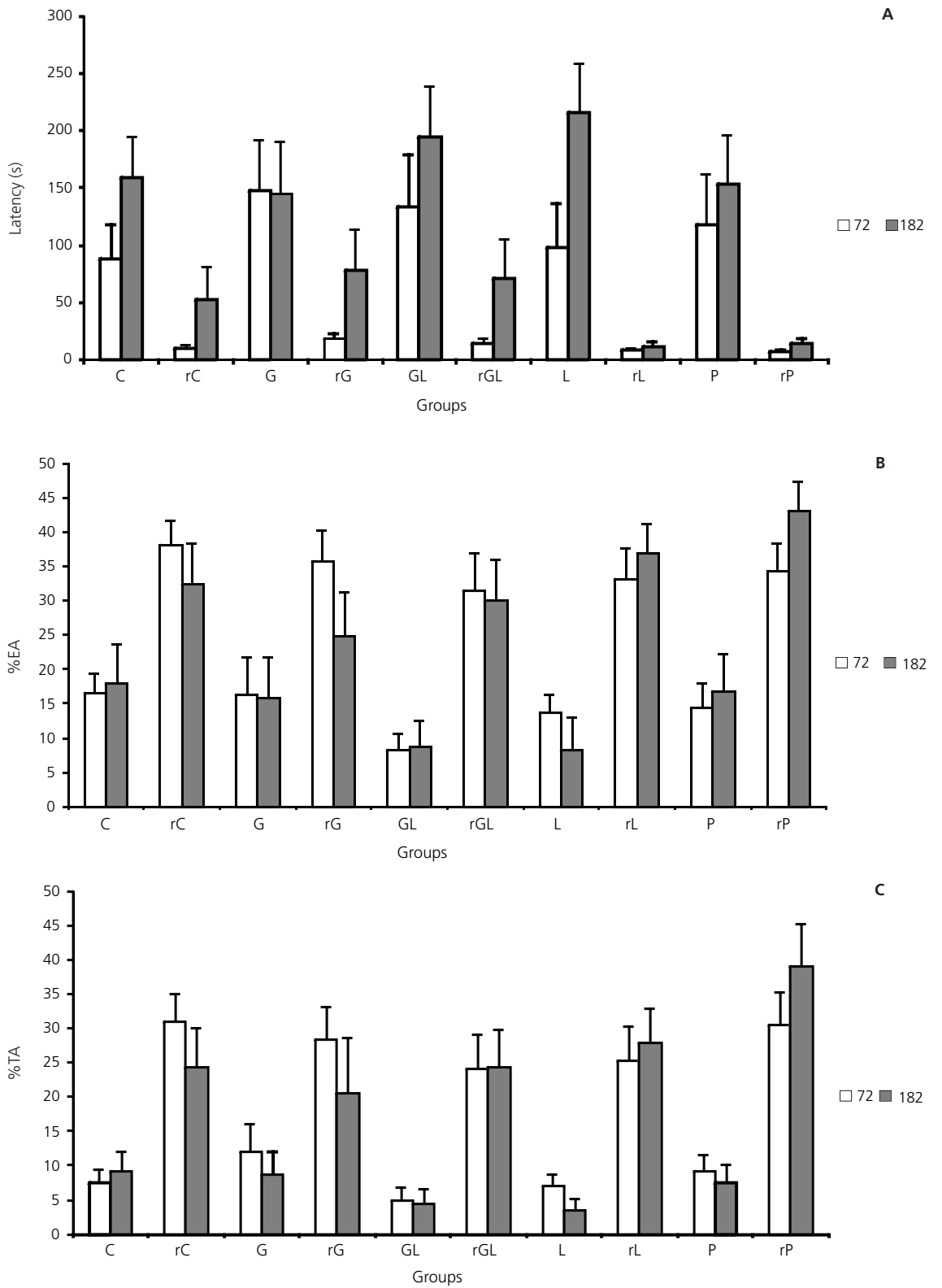
Figure 2. Latency of first entry (Part A), % of entry (Part B),% of time (Part C) and frequency of visits to the extremity (Part D) of the open arms of the Elevated Plus-Maze by animals at 72 and 182 days.

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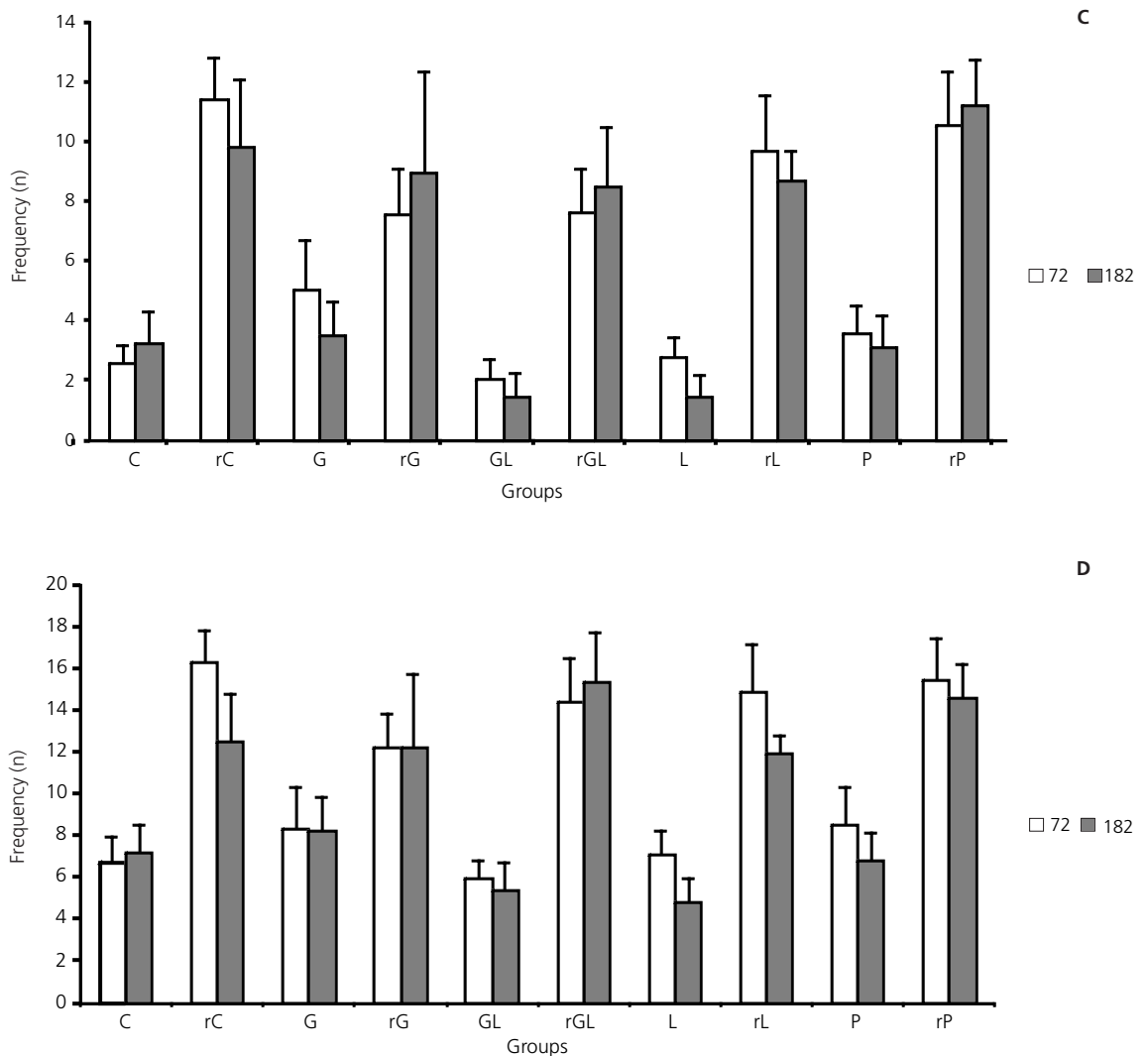
Note: C: Control; G: Carbohydrate; GL: Carbohydrate and Lipid; L: Lipid; rC: Control Restriction; rG: Carbohydrate Restriction; rGL: Carbohydrate and Lipid Restriction; rL: Lipid Restriction; rP: Protein Restriction.
Parts A, B, C and D: rC, rG, rGL, rL, rP>C, G, GL, L, P; Part A: 182>72; Part D: 72>182, by the Newman-Keuls Test ($p<0.05$).
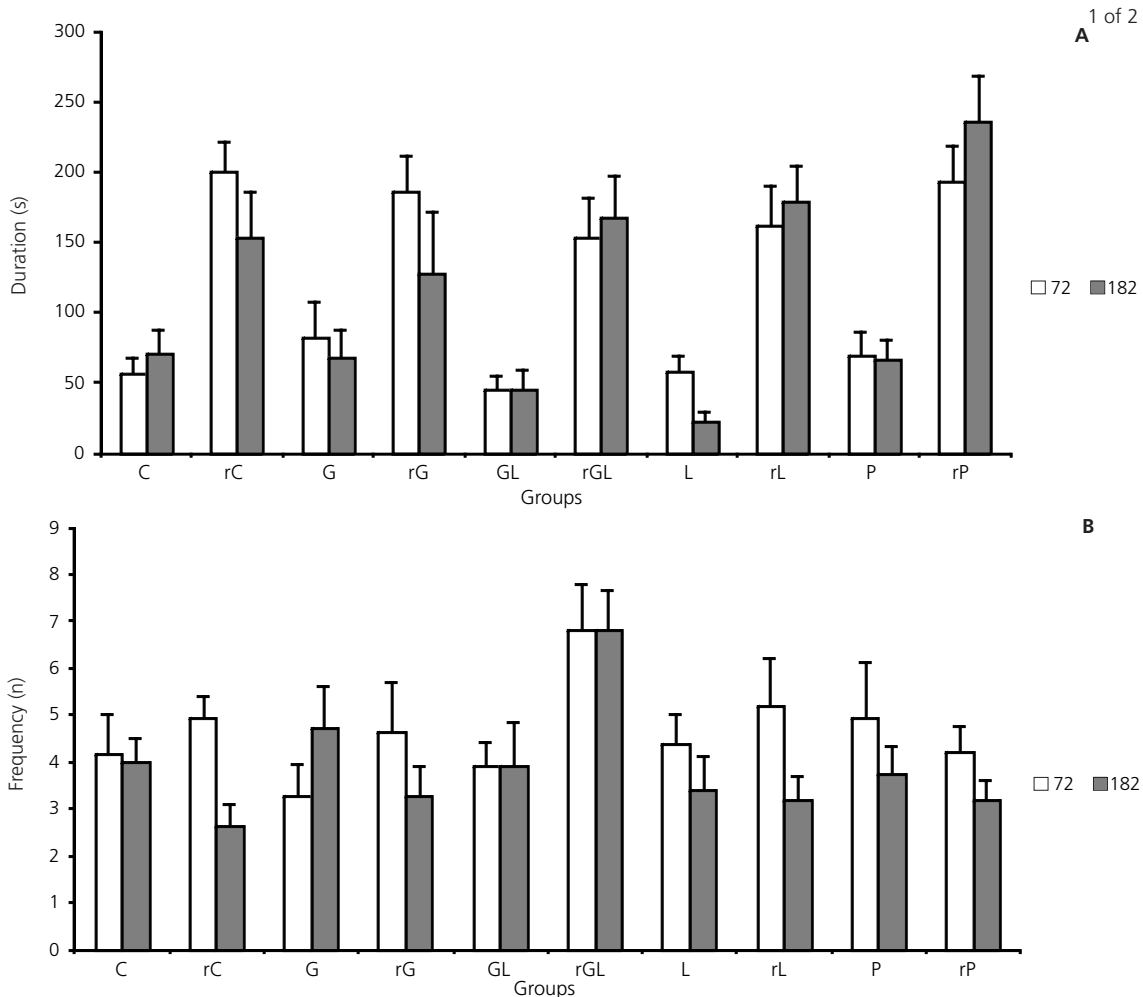
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Figure 3. Duration in the central area (Part A), protected head dipping (Part B), unprotected (Part C) and total (Part D) in the Elevated Plus-Maze by animals at 72 and 182 days.

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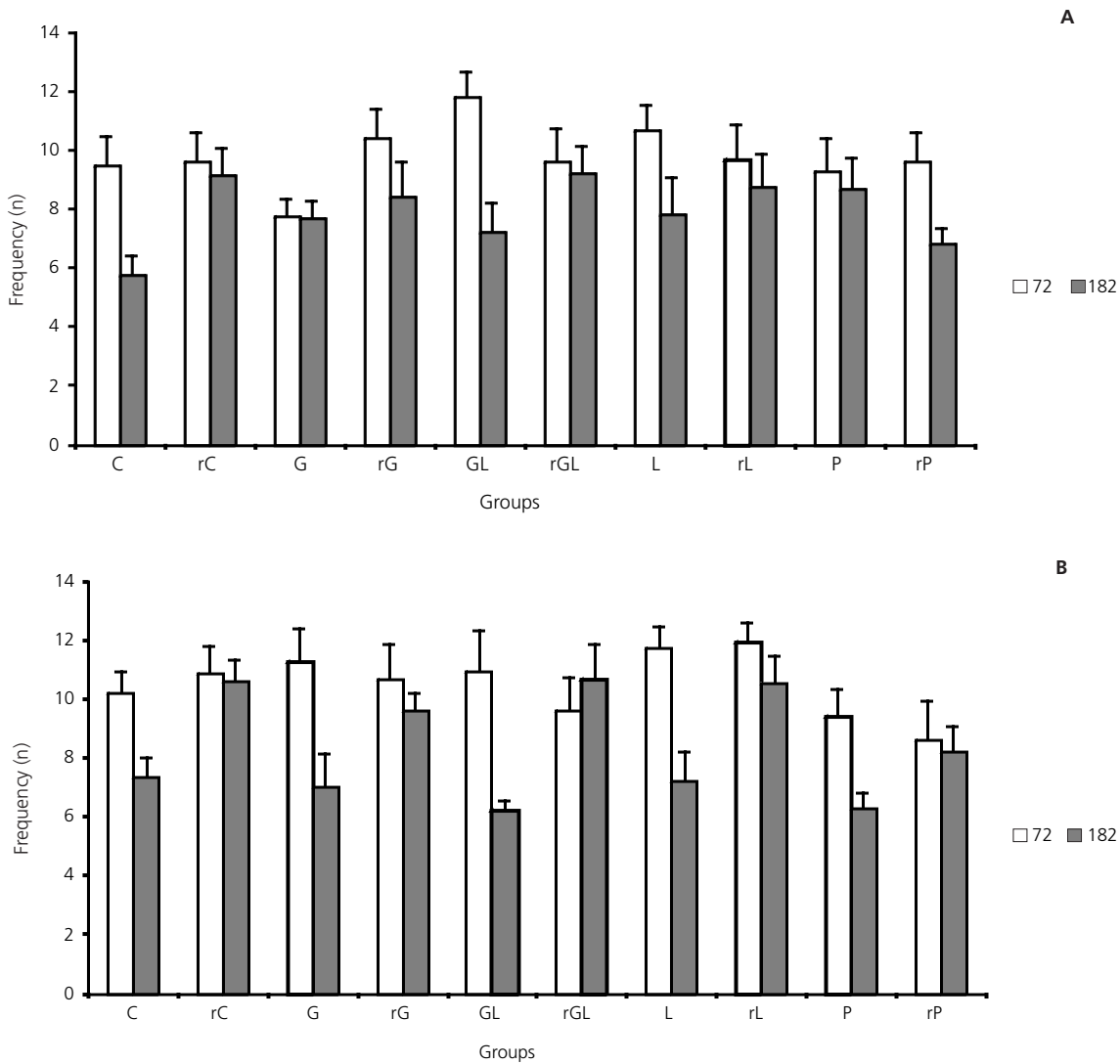
Note: C: Control; G: Carbohydrate; GL: Carbohydrate and Lipid; L: Lipid; rC: Control Restriction; rG: Carbohydrate Restriction; rGL: Carbohydrate and Lipid Restriction; rL: Lipid Restriction; rP: Protein Restriction.

Parts A, B, C and D: rC, rG, rGL, rL, rP> C, G, GL, L, P; Part B: 72>182, by the Newman-Keuls Test (*p*<0.05).

Food restrictions for all groups increased the length of stay in the central area ($F_{(9.210)}=13.90$, *p*<0.0001) (Figure 3A), the frequency of protected head dipping ($F_{(9.210)}=2.83$, *p*<0.005) (Figure 3B), unprotected head dipping ($F_{(9.209)}=10.83$, *p*<0.0001) (Figure 3C) and total head dipping ($F_{(9.209)}=9.21$, *p*<0.0001) (Figure 3D). The 72-day-old animals showed a higher frequency of protected head dipping ($F_{(1.210)}=5.13$, *p*<0.05) (Figure 3B) and false entries in the open arms ($F_{(1.207)}=17.18$, *p*<0.0001) than the animals with 182 days of life (Figure 4A).

Regarding rearing, animals at 72 days were more frequently rearing than those at 182 days of life ($F_{(1.210)}=26.43$, *p*<0.0001). There was also an interaction effect between diet and age ($F_{(9.210)}=2.23$, *p*<0.05), with the animals in the GL group that exhibited a higher rearing frequency at 182 than at 72 days of life (Figure 4B).

Figure 4. Frequency of false entries (Part A) and rearing (Part B) in the Elevated Plus-Maze by animals aged 72 and 182 days.

Note: C: Control; G: Carbohydrate; GL: Carbohydrate and Lipid; L: Lipid; rC: Control Restriction; rG: Carbohydrate Restriction; rGL: Carbohydrate and Lipid Restriction; rL: Lipid Restriction; rP: Protein Restriction.
Parts A and B: 72>182, by the Newman-Keuls Test (p<0.05).

DISCUSSION

Data consistency on the shortening of the first entry latency and the increase in the % of entries, % of time and frequency of visits to the extremities of the open arms, head dipping and time in the central area of the EPM, demonstrate that the animals that had undergone moderate and chronic food restriction (from birth to the age of 72 or 182 days) were less anxious and/or more impulsive than those who received an *ad libitum* diet.

These data suggest that moderate chronic food restriction, as well as moderate food restriction for a short period (3 weeks [25]) or fasting for a single night [27], which is an insufficient time to cause malnutrition, also demonstrated an anxiogenic effect, *i.e.*, the same effect on anxiety behavior caused by severe malnutrition [29,30].

Another explanation for these results is that an adaptive behavior to the limited food setting occurred in animals under food restriction, leading them to enhance exploration of the maze, in an attempt to find food, even if the maze's open arms are aversive.

In fact, the animals in food restriction ate all the food in a few hours, and at the time of the test they were deprived of food and the greater exploration of the open arms could mean the search for food; however, it was expected that the animals would also enter more in the closed arms and this happened only for the rG, rGL and rP groups.

On the other hand, in an experiment with severe restriction for 2 months [24] there was no effect on the anxiety test using the EPM. In contrast, other trials using moderate restriction that started 2 weeks before mating until the 20th day of gestation and the test performed with the pups in adult life [28], for 11 days [26], severe restriction for 5 days during pregnancy [30] demonstrated an anxiogenic effect.

One of the mechanisms that can explain alterations in the anxiety levels is the hypothesis of monoamines and glucocorticoids imbalance in the central nervous system. Literature data showed that anxiety symptoms decreased after treatment for weight loss (dietary restriction) that reduced serotonin and dopamine concentrations [31] and increased corticosterone in rats [32].

Food restriction can induce an activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis, which is one of the main pathophysiological changes observed in patients suffering from mood and anxiety disorders and, since the hypothalamus is involved in the regulation of the HPA axis activity, it is believed that dysregulation of monoamines neurotransmission in the hypothalamus is involved in anxiety [31].

The lack of treatment effect among animals that were offered different types of diets *ad libitum* and among animals offered different types of diet in restricted quantities indicates that nutrients (protein, carbohydrate and/or lipid) did not interfere in the behavior of the animals assessed in the EPM, despite data in the literature showing that sucrose and lipids altered responses to stress on the HPA axis [33].

Despite the animals in groups P and especially those in group G showing less weight gain after 72 or 182 days treatment, the behavior in EPM was similar to animals in groups C, GL and L. These data match those in the literature when animals were treated with a diet rich in sucrose [5,6], rich in protein [20] and rich in lipids [7,9,10,16] and also showed no change when tested in the EPM. However, the diet rich in lipids had an anxiolytic [13] and anxiogenic effect [11,12,14,15,17]. The diet rich in carbohydrates and lipids (cafeteria or western diet) also caused an anxiogenic effect [18,19].

A hypothesis to explain the lower weight gain for animals in groups P, G and rG is the existence of a mechanism in the central nervous system that controls weight gain, reducing the intake of carbohydrates (more intense) and proteins (less intense) (data not shown) actually more important than the caloric content of the diets and sufficient to cause changes in the animals' body weight.

The greater latency for the first entry into the open arms and the reduced frequency of entry into the closed arms, false entries and visits to the end of the open arms, rearing as well as protected head dipping by the animals at 182 days, show that adults are more anxious and move around less than young adults. These data are consistent with those in the literature [34]. It is likely that structural changes, as well as the production of neurotransmitters and hormones, that occur during development, are responsible for the increased anxiety with aging.

Great care is needed when recommending dietary restriction, even if moderate, especially for individuals during the growth and development phases while we are not sure of the beneficial and adverse effects of this feeding practice. Further studies are needed to assess the impact of macronutrients in the behavior and health, in general.

CONCLUSION

Food restriction caused a reduction in anxiety and/or an increase in impulsivity; aging resulted in an enhancement of anxiety and/or a reduction in impulsivity, and macronutrients did not affect animals' behavior in the EPM. Further studies are needed to better understand the mechanisms involved in the consumption of macronutrients, production of neurotransmitters, alterations in the HPA axis and in anxious behavior.

ACNOWLEDGEMENTS

CONTRIBUTORS

TR RIUL contributed of the conception and design, analysis and interpretation of data, review and final approval of the article and SS ALMEIDA contributed of the conception and design, review and final approval of the article.

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