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Effects of tactile stimulation and underwater trauma on the behavior of protein-malnourished rats in the elevated plus-maze test

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Abstract

The objective of the present study was to evaluate the interaction of two kinds of external stimuli (long-term tactile stimulation and underwater trauma) on the exploratory behavior of malnourished rats in an elevated plus maze (EPM). The results showed that tactile stimulation partially recovered body weight deficits produced by malnutrition and decreased EPM exploration. Malnutrition increased EPM exploration while underwater trauma decreased it, thus reducing differences between well nourished and malnourished animals. These data show that only one of the two external stimuli (underwater trauma) interacted with diet condition, suggesting that the stress caused by trauma was efficient in decreasing higher EPM exploration, equalizing the response of malnourished animals to that of non-traumatized well-nourished animals. **Keywords:** protein malnutrition, environmental stimulation, underwater trauma, anxiety, elevated plus-maze.

Introduction

Protein or protein-calorie malnutrition imposed early in life is a well-known environmental factor that produces alterations in the brain. These alterations can comprise of morphological, neurochemical, neurophysiological and functional aspects of the developing brain (Almeida, Tonkiss, & Galler, 1996c; Dobbing, 1987; Morgane et al., 1978, 1993; Morgane, Austin-La France, Tonkiss, & Galler, 1992; Tonkiss, Galler, Morgane, Bronzino, & Austin-LaFrance, 1993). Furthermore, it is also well known that protein malnutrition during gestation and/or lactation can change the behavior of rats in some animal models of anxiety. Malnourished animals submitted to experimental models of anxiety such as light-dark transition (Brioni & Orsingher, 1988; Santucci, Daud, Almeida, & De Oliveira, 1994), elevated plus-maze (EPM) (Almeida, De Oliveira, & Graeff, 1991; Almeida et al., 1994; Almeira, Tonkiss, & Galler, 1996b; Moreira, De Araújo, De Oliveira, & Almeida, 1997; Riul, Carvalho, Almeida, & De Oliveira, 1998) and elevated T-maze (Almeida, Tonkiss, & Galler, 1996a) presented behavioral changes suggestive of lower anxiety and/or higher impulsiveness.

Previous studies (Almeida, Araújo, Moreira, Paiva, & De Oliveira, 1998; Françolin-Silva & Almeida, 2004) showed that anxiogenic or stressful procedures (short-term social isolation or immobilization) differentially affected the behavior of well-nourished and malnourished animals when tested in the EPM. These data suggest that the emotional changes produced by early protein malnutrition possibly interact with emotional changes induced by social isolation or immobilization applied just before the test, resulting in differences in exploratory behavior in the EPM. The exact way these two stressful situations (early malnutrition and isolation/immobilization) interact to produce behavioral alterations in the EPM is not known, but it has been suggested that stressful experiences early in life can interfere with the management of stressful experiences later in adulthood (Almeida et al., 1998; Cabib, Puglisi-Allegra, & D'Amato, 1993; Françolin-Silva & Almeida, 2004; Matthews & Robbins, 2003).

On the other hand, early environmental stimulation along with a protein-deficient diet can minimize the deleterious effects of malnutrition both on cerebral (Rocinholi, Oliveira, & Colafemina, 2001a; 2001b; and behavioral (De Oliveira & Almeida, 1985; Jaiswal, Upadhyay & Bhattacharya, 1990; Morgan & Winick, 1980) parameters. In addition, it has been shown that environmental stimulation, especially tactile stimulation, can also lead to reductions in anxiogenic responses in several animal models of anxiety (Ferre, Nunez, Garcia, Tobena, & Escorihuela, 1995; Meerlo, Horvath, Nagy, Bohus, & Koolhaas, 1999; Roy & Chapillon, 2002). Environmental stimulation has been suggested to reduce the effects of the so-called "functional isolation"

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produced by early malnutrition (Levitsky & Barnes, 1972). The concept of functional isolation refers to a situation of low energy caused by protein malnutrition where animals prefer to isolate themselves from the environment to save energy in order to survive. Functional isolation results in a reduction of social and environmental exploratory behaviors during critical periods of development.

Thus, the objective of the present study was to evaluate the effects of two kinds of external stimuli, one believed to be anxiogenic (underwater trauma) and the other believed to be anxiolytic (tactile stimulation), on the behavior of early protein-malnourished rats submitted to the EPM test.

Methods

Animals

A total of 99 male Wistar rats from the animal colony of the Ribeirão Preto Campus of the University of São Paulo were used. Each litter was culled to 6 male and 2 female pups on the day of birth. The dams and the pups were housed in transparent plastic cages (40 x 33 x 20 cm) and randomly assigned to a 6% or 16% protein diet ad libitum during the lactation phase (0 – 21 days). The two diets have been described in detail elsewhere (Almeida et al., 1994). Briefly, the protein-deficient diet contained approximately 8% casein (6% protein), a 5% salt mixture, a 1% vitamin mixture, 8% corn oil, 0.2% choline, and 77.8 % cornstarch. The regular protein diet contained approximately 20% casein (16% protein), 60.8% cornstarch and the same percentage of the other constituents as the protein-deficient diet. The two diets were supplemented with L-methionine (2.0 g/kg protein), since casein is deficient in this amino acid. Only male rats were used in this study. After weaning (21 days), the animals continued to receive the same diets until 49 days of age, when all animals started to receive a regular lab chow diet until the end of the experiment. The animals were maintained on a 12L:12D cycle (lights on at 6 A.M.) with room temperature kept at 23-25o C, and free access to water and food throughout the experiment. Behavioral tests were conducted during the light period (1 to 5 PM). The experiments were performed in compliance with the recommendations of the Brazilian Society of Neuroscience and Behavior (SBNeC), which are based on the US National Institutes of Health Guide for Care and Use of Laboratory Animals.

Apparatus

A perforated metal cage (17 x 23 x 19 cm) and a round polyethylene container with 20-liter capacity were used for the underwater trauma. After the trauma, animals were tested in the EPM, which consisted of two open arms (50x10 cm) and two enclosed arms (50 x 10 x 40 cm) with an open roof, arranged in such a way that the two open arms were opposite to each other. The maze was elevated to a height of 50 cm from the floor. The behavior in the EPM was recorded with a video camera connected to a monitor and to a video cassette recorder in an adjacent room.

Procedure

Tactile stimulation. Half the pups in both diet groups were exposed to daily 3-minute individual handlin g from birth to 49 days of age. The other half received no stimulation. The handling consisted of holding the animal in one hand and making cranio-sacral movements on the dorsal region with the thumb of the other hand.

Underwater trauma. At 70 days of age, the animals were exposed to the underwater trauma consisting of individually placing them in a perforated metal cage (17x23x19 cm), which was submerged in a round polyethylene container with a 20-liter capacity of water for a period of 30 seconds (Richter-Levin, 1998). The metal cage was then removed from the water and the rat was carefully dried with an absorbent cloth for a period of 60 seconds.

Elevated plus-maze. Soon after the drying procedure, the rat was placed in the center of the elevated plus-maze with its head directed toward one of the closed arms, and the behaviors of the animals were recorded in seven daily sessions of 5 minutes held at 24-hour intervals. The test sessions were recorded with a vertically mounted video camera (Sony-Tokyo, Japan) linked to a monitor and VCR in an adjacent room. The videotapes were analyzed by two independent experimenters, and the following behavioral categories were identified and recorded as previously described (Almeida, Garcia, & De Oliveira, 1993; Almeida et al., 1996b; Cole & Rodgers, 1994; Cruz, Frei, & Graeff, 1994; Rodgers & Cole, 19931; 1993b; Santucci et al., 1994): 1) the percentage of open-arm entries (an arm entry defined as the rat having all four paws inside an arm), 2) the duration of time spent in the open arms, 3) latency to first open-arm entry (latency to enter an open arm timed from the start of the test), 4) head-dipping (scanning over the sides of the maze toward the floor), 5) attempts to enter open arms (entering an open arm with only the forepaws and returning to the central platform or closed arm), 6) rearing (rising on the hind paws). The behavioral categories 'stretch-attend posture' and 'head-dips' have been previously described as risk-assessment behaviors (Almeida et al., 1994; Rodgers & Cole, 1993a; 1993b). The 'rearing' category was included as a classical measure of exploration in a novel environment (Almeida et al., 1994; Almeida et al., 1996b). The other behavioral categories have been behaviorally and pharmacologically validated for rats (Cruz et al., 1994; Pellow, Chopin, File, & Briley, 1985) and mice (Cole & Rodgers, 1994; Lister, 1987 Rodgers & Cole, 1993a; 1993b) as reliable measures of fear/anxiety.

Data analysis

Body weight data are reported as means ± SEM and were analyzed by three-way (diet condition, stimulation and age) analysis of variance (ANOVA) with repeated measures based on age. Behavioral data are reported as means ± SEM and were analyzed by four-way ANOVA (diet, stimulation, trauma and session) with repeated measure based on session. When appropriate, post hoc comparisons were made using the Newman-Keuls test. The level of significance was set at .05.

Results

Body weight

During the lactation period, well-nourished (W) pups weighed more than malnourished (M) pups, as indicated by a significant effect of diet, $F(1, 13) = 98.51$, $p < .001$ (see Figure 1). Handling also produced higher body weight compared with non-handled animals, as indicated by a significant effect of stimulation, $F(1, 13) = 5.13$, $p < .05$. The increase in body weight during lactation also produced a significant effect of age, $F(3, 39) = 255.89$, $p < .001$. The higher increase in the weight of W pups compared with M pups produced a significant diet x age interaction, $F(3, 39) = 68.63$, $p < .01$, with post-hoc comparisons showing significant differences between the two diet conditions at 7, 14 and 21 days of age ($p < .05$).

After weaning, W pups continued to weigh more than M pups, as indicated by a significant effect of diet, $F(1, 95) = 975.59$, $p < .001$ (see Figure 1). Handling also produced higher body weight compared with non-handled animals as indicated by a significant effect of stimulation, $F(1, 95) = 11.27$; p<0, $p < .01$. The increase in body weight during lactation also produced a significant effect of age, $F(6, 570) = 3263,03$, $p < .001$. The higher increase in the weight of W rats compared with M rats produced a significant diet x age interaction, $F(6, 570) = 411.18$, $p < .001$, with post-hoc comparisons showing significant differences between the two diet conditions at all ages ($p < .05$). Finally, there was a significant effect of stimulation x age, $F(6, 570) = 3,25$, $p < .05$, showing that handled animals had higher body weights than non-handled ones at all ages ($p < .05$).
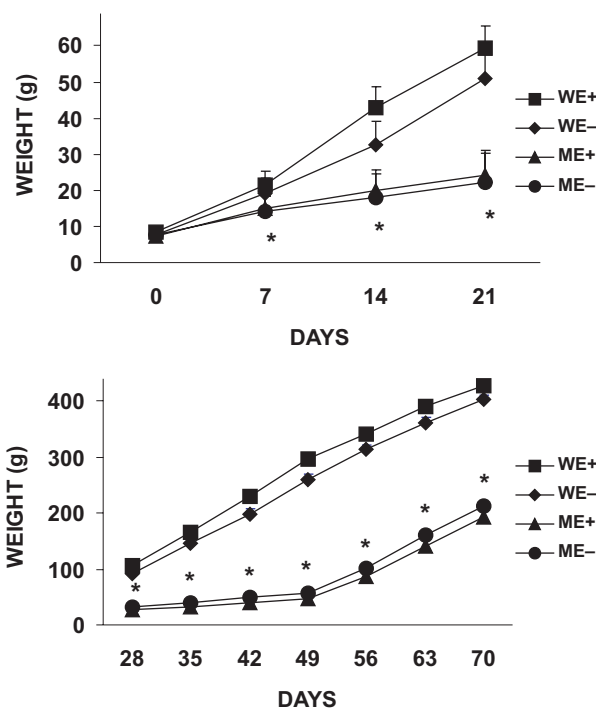

Figure 1. Body weight (mean ± SEM) of animals during lactation (top) and post-lactation (bottom) period (WE+ = well-nourished stimulated; WE- = well-nourished not stimulated; ME+ = malnourished stimulated and ME- = malnourished not stimulated). * p < .05 compared with W animals.

Behavioral measures

The percentage of open-arm entries for all groups is illustrated in Figure 2. There was a significant effect of diet, $F(1, 91) = 10.14$, $p < .01$, indicating a higher percentage of open-arm entries for M than for W animals. There was also
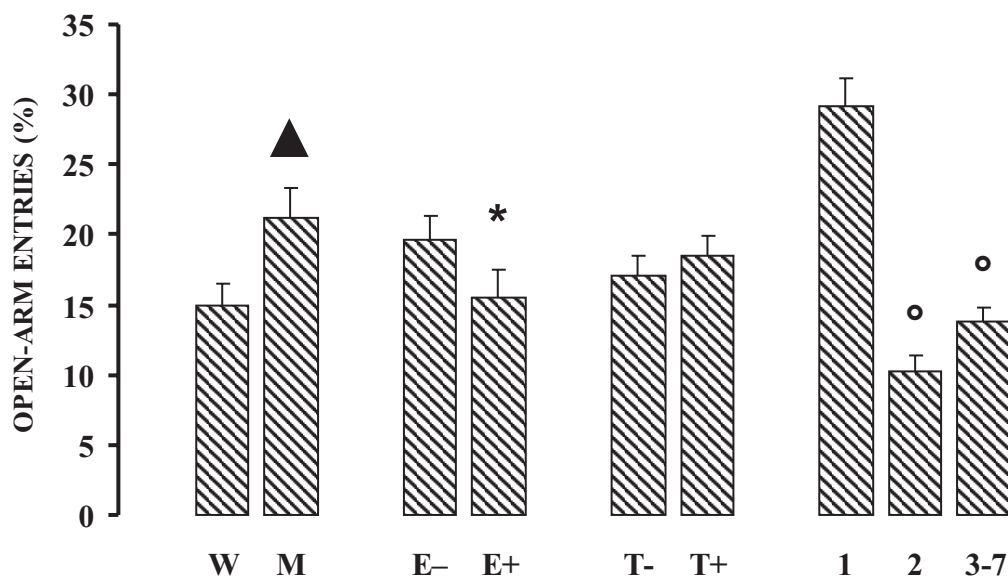

Figure 2. Percentage of open arm entries (mean ± SEM) for several experimental conditions (W = well-nourished; M = malnourished; E+ = stimulated; E- + not stimulated; T+ = traumatized; T- = not traumatized; 1 = session 1; 2 = session 2 and 3-7 = sessions 3 to 7). ▲ p < .05 compared with W animals; * p < .05 compared with E- animals and ● p < .05 compared with session 1.

a significant effect of chronic stimulation, $F(1, 91) = 4.99$, $p < .05$, indicating a lower percentage of open-arm entries for stimulated animals compared with non-stimulated animals. A significant effect of session was also found, $F(2, 182) = 64.28$, $p < .001$, indicating decreases in the percentage of open-arm entries across sessions. A post-hoc analysis showed a significantly lower number of open-arm entries in sessions 2-7 ($p < .005$) compared with the first exposure. Finally, there was a significant effect of session by trauma interaction, $F(2, 182) = 3.51$, $p < .05$, indicating a greater reduction of the percentage of open-arm entries in traumatized than in non-traumatized animals. The results for the time spent in the open-arms was similar to that of open-arm entries except for two aspects: a) there was a significant effect of trauma, $F(1, 91) = 4.68$, $p < .05$, indicating that traumatized animals spent less time in the open arms than non-traumatized animals; and b) there was a significant diet by trauma interaction, $F(1, 91) = 4.17$, $p < .05$, indicating that trauma significantly reduced the time spent in open-arms in M animals, with no effect on W animals. The reduction of the time spent in the open-arms by M animals was sufficient to equalize this time to that spent by non-traumatized W animals.

The latency to the first open-arm entry is illustrated in Figure 3. Latency was significantly lower for M than for W animals, as demonstrated by a significant effect of diet, $F(1, 91) = 7.96$, $p < .01$. The stimulated animals had a higher latency than non-stimulated animals, as demonstrated by a significant effect of stimulation, $F(1, 91) = 7.25$, $p < .01$. The traumatized animals had a lower latency than non-traumatized animals, as demonstrated by a significant effect of trauma, $F(1, 91) = 6.08$, $p < .05$. There was also a significant effect of session, $F(2, 182) = 60.90$, $p < .001$, with a post-hoc analysis indicating that the latency to the first open-arm entry increased across sessions ($p < .05$). Finally, there was a significant diet by trauma effect, $F(1, 91) = 6.04$, $p < .05$, with

post-hoc analysis showing that trauma significantly increased latency for M animals ($p < .05$), with no effect on W animals. The increase in latency in M animals was sufficient to equalize this latency to that of non-traumatized W animals.

Head-dips are illustrated in Figure 4. The number of head-dips was higher for M than for W animals, as demonstrated by a significant effect of diet, $F(1, 91) = 8.71$, $p < .01$. The stimulated animals had a lower frequency of head-dips when compared with non-stimulated animals, as demonstrated by a significant effect of diet, $F(1, 91) = 4.23$, $p < .01$. The traumatized animals had a lower frequency of head-dips when compared with non- traumatized animals, as demonstrated by a significant effect of trauma, $F(1, 91) = 23.44$, $p < .001$. There was also a significant effect of session, $F(2, 182) = 45.69$, $p < .001$, with a post-hoc analysis indicating that the frequency of head-dips decreased across sessions ($p < .05$). Finally, there was a significant diet by trauma effect, $F(1, 91) = 8.43$, $p < .01$, with a post-hoc analysis showing that trauma significantly decreased latency for M animals ($p < .05$), with no effect on W animals. This decrease in latency for M animals was sufficient to equalize this latency to that of non-traumatized W animals.

The frequency of attempts to enter the open arms was less in traumatized animals when compared with non-traumatized ones, as demonstrated by a significant effect of trauma, $F(1, 91) = 22.48$, $p < .001$, and a significant effect of session, $F(2, 182) = 19,61$, $p < .001$, with a post-hoc analysis showing an increase in frequency across sessions ($p < .05$).

Finally, the frequency of rearings was lower in traumatized animals when compared with non-traumatized ones, as demonstrated by a significant effect of trauma, $F(1, 91) = 45.58$, $p < .001$. There was also a significant effect of session, $F(2, 182) = 29.79$, $p < .001$, with a post-hoc analysis showing increases in frequency across sessions ($p < .05$).
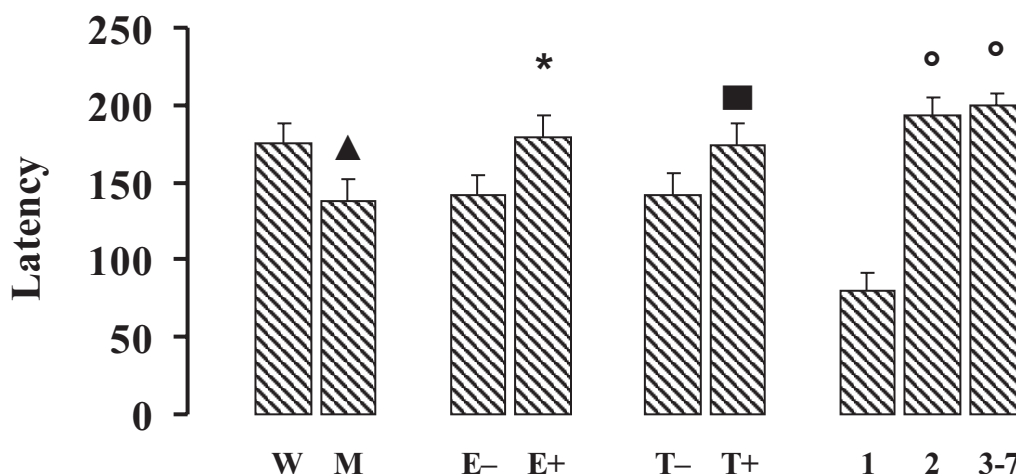

Figure 3. Latency to the first open-arm entry (mean ± SEM) for several experimental conditions (W = well-nourished; M = malnourished; E+ = stimulated; E- + not stimulated; T+ = traumatized; T- = not traumatized; 1 = session 1; 2 = session 2 and 3-7 = sessions 3 to 7). ▲ $p < .05$ compared with W animals; * $p < .05$ compared with E- animals; ■ $p < .05$ compared with T- and ● $p < .05$ compared with session 1.
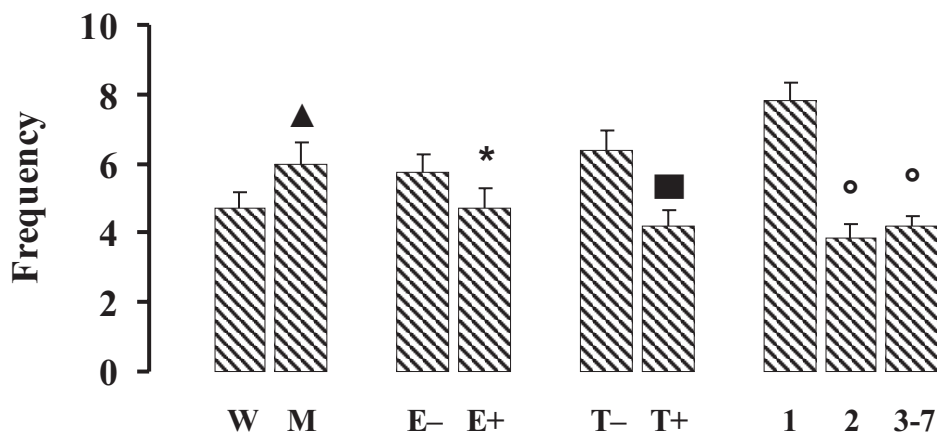
Figure 4. Frequency of head-dips (mean ± SEM) under several experimental conditions (W = well-nourished; M = malnourished; E+ = stimulated; E- + not stimulated; T+ = traumatized; T- = not traumatized; 1 = session 1; 2 = session 2 and 3-7 = sessions 3 to 7). ▲ p < .05 compared with W animals; * p < .05 compared with E- animals; ■ p < .05 compared with T- and ● p < .05 s compared with session 1.

Discussion

The present results showed that early postnatal protein malnutrition significantly reduced the body weight of the animals as reported in previous studies (Fukuda, Françolin-Silva, & Almeida, 2002; Françolin-Silva & Almeida, 2004). However, it was also demonstrated that chronic tactile stimulation produced an increase in the body weight of both W and M animals. It is possible that chronic tactile stimulation changed the eating behavior of dams and/or pups during lactation and of pups after weaning, resulting in increased food ingestion resulting in higher weight gain. Increases in brain weight, DNA and RNA levels after chronic tactile stimulation have also been previously reported by Lima, De-Oliveira, & Almeida, 1999, suggesting that environmental stimulation can be an advantageous external stimulus for weight gain.

Early malnourished animals increased behaviors believed to indicate lower anxiety in the EPM, a result consistent with previously reported data (Almeida et al., 1991; 1994). This increase in the exploration of the maze was not due to alterations in locomotor activity produced by malnutrition, since there were no differences between W and M animals in the number of closed arm entries, which is a measure indicative of locomotion.

Re-exposure to the EPM produced an increase in anxiety as indicated by significant reductions of exploration across sessions, a result consistent with previously reported studies on rats (Almeida et al., 1993) and mice (Espejo, 1997). This significant reduction in maze exploration has been interpreted as a different kind of fear after the first exposure to the maze. It is suggested that the anxiety generated by the first exposure changes to phobia in subsequent exposures (File & Zangrossi Jr, 1993). Since the present study showed there was no difference due to diet by session interactions, we conclude that if a different form of fear develops across sessions, it is not influenced by early malnutrition.

Chronic tactile stimulation decreased exploration in the maze, an indicator of increased anxiety. This result contrasts with reports showing that acute environmental stimulation has an anxiolytic effect (Vallee et al. 1997). However, our use of chronic environmental stimulation in this study may explain the divergences. Thus, chronic stimulation appears to be anxiogenic rather than anxiolytic, as measured in the EPM, a classical animal model of anxiety. In fact, the results showing that chronic tactile stimulation increased weight gain lend support to such an interpretation. It has been demonstrated that chronic stress increases weight gain in rats (Renata-Marquez et al., 2003) and mice (Moles et al., 2006). Thus, both the increased weight gain and the increased anxiety reported in the present study could be the result of stress produced by a regularchronic tactile stimulation procedure. Other studies have also demonstrated that stress produces an activation of the hypothalamus-pituitary-adrenal axis, raising the levels of corticosterone (Renata-Marquez et al., 2003).

In the same way, the procedure of acute underwater trauma also reduced maze exploration indicating an anxiogenic effect, a result that is consistent with previously reported data showing that underwater trauma reduces the exploration of open arms in the EPM (Moles et al., 2006). However, a significant diet by underwater trauma interaction showed that trauma contributed to reducing differences in the open arm exploration between W and M animals. In other words, trauma contributed to bringing the behaviors of M animals to levels similar to those of non-traumatized W animals. In this respect, acute underwater trauma was an effective external stimulus to reduce the anxiolysis produced by early protein malnutrition in this classical animal model of anxiety.

Thus, of the two external stimuli used in the present study, only underwater trauma significantly interacted with diet condition, suggesting that this acute stressor preferentially affects M animals, leading to a decrease in the higher plus-

maze exploration and thus abolishing diet-related differences. This higher susceptibility to respond to acute underwater trauma in M animals compared with W animals deserves future investigation in order to determine whether this responsiveness can be extended to other environmental stressors.

Acknowledgment

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