HELOÍSA MARIA COTTA PIRES DE CARVALHO

COMPORTAMENTO DE RATOS MACHOS E FÊMEAS SUBMETIDOS A SESSÕES DE NADO FORÇADO CRÔNICO E A UM POSTERIOR TESTE NO LABIRINTO EM CRUZ ELEVADO

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Dissertação apresentada como parte dos requisitos para a obtenção do título de Mestre em Análise do Comportamento, ao Programa de Pós-Graduação em Análise do Comportamento da Universidade Estadual de Londrina.

Orientador: Prof. Dr. Célio Roberto Estanislau

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BANCA EXAMINADORA

Orientador: Prof. Dr. Célio Roberto Estanislau
UEL – Londrina - PR

Prof. Dr. Emerson José Venâncio
UEL – Londrina – PR

Prof. Dr. Carlos Eduardo Costa
UEL – Londrina – PR

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CARVALHO, H. M. C. P. **Comportamento de ratos machos e fêmeas submetidos a sessões de nado forçado crônico e a um posterior teste no labirinto em cruz elevado**. 2009. 51 f. Dissertação (Mestrado em Análise do Comportamento) – Universidade Estadual de Londrina, Londrina, 2009.

RESUMO

Nado forçado e labirinto em cruz elevado são modelos animais de depressão e ansiedade, respectivamente, amplamente utilizados. Sabe-se que existem conexões entre o estresse crônico e o desenvolvimento da depressão. Como as diferenças de gênero são aspectos importantes relacionados à ansiedade e depressão, o presente estudo objetivou avaliar possíveis diferenças de gênero ao longo de repetidas sessões de nado forçado e durante um teste no labirinto em cruz elevado. Ratos Wistar machos (n=36) e fêmeas (n=36) foram submetidos, por 14 dias, a um dos seguintes tratamentos: sessões de nado forçado, sessões de manuseio e permanência no biotério. No 15º dia, todos os animais foram testados no labirinto em cruz elevado por 10 minutos. O nado forçado crônico induziu comportamentos depressivos similarmente em machos e fêmeas, resultando em experiência de estresse para ambos os gêneros. Contrariamente ao que os ratos controle mostraram, machos submetidos ao nado forçado crônico foram menos ansiosos do que fêmeas. Além disso, o nado crônico produziu um efeito ansiogênico enquanto o manuseio produziu um efeito ansiolítico, independente do gênero. Esses resultados sugerem uma associação entre depressão e ansiedade e mostram que diferenças de gênero devem ser consideradas na avaliação e tratamento dessas doenças.

Palavras-chave: Gênero. Nado forçado. Labirinto em cruz elevado. Ansiedade. Depressão. Modelos animais. Ratos. Adrenal. Peso corporal.

CARVALHO, H. M. C. P. **Behavior of male and female rats submitted to chronic forced swim sessions and in a subsequent elevated plus-maze test**. 2009. 51 f. Dissertação (Master's Degree in Behavior Analysis) – Universidade Estadual de Londrina, Londrina, 2009.

ABSTRACT

Forced swimming and the elevated plus-maze are widely used animal models of depression and anxiety, respectively. There are connections between chronic stress and depression development. As gender differences constitute important features of anxiety and depression disorders, present work was aimed at evaluating possible gender differences along repeated forced swimming sessions and during an elevated plus-maze test. Male (n=36) and female (n=36) Wistar rats were submitted for 14 days to one of the following treatments: forced swimming sessions, handling control sessions or remained undisturbed. In the 15th day, animals were tested in the elevated plus-maze for ten minutes. Chronic forced swimming induced similar depressive-like behaviors in male and female rats resulting in stress experience for both genders. Contrary to what control rats showed, males submitted to forced swimming were less anxious than females. Moreover, chronic forced swim produced an anxiogenic effect whereas handling produced an anxiolytic effect irrespective of gender. Results suggest an association between depression and anxiety and show that gender differences should be considered in assessment and treatment of these disorders.

Keywords: Gender. Forced swimming. Elevated plus-maze. Anxiety. Depression. Animal models. Rats. Adrenal. Body weight.

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APRESENTAÇÃO

Os modelos animais representam um arranjo experimental desenvolvido para se estudar um determinado fenômeno em uma mesma espécie ou em espécies diferentes. Tipicamente, os modelos animais são arranjos que tentam mimetizar uma condição humana, o que inclui a psicopatologia. Neste caso, busca-se o desenvolvimento de síndromes, em animais, que se assemelhem àquelas dos humanos (Geyer & Markou, 1995). Outro propósito dos modelos animais é o de estudar de forma sistemática os efeitos de tratamentos terapêuticos potenciais, de forma que o propósito explícito é predizer a eficácia de um tratamento (Geyer & Markou, 1995).

Pesquisas com animais também são realizadas com o objetivo de auxiliar na elucidação dos mecanismos neurobiológicos e comportamentais dos transtornos mentais. Os estudos com modelos animais permitem uma melhor compreensão dos fatores envolvidos em determinado transtorno, além de permitir estudar a interação entre as múltiplas variáveis que podem estar envolvidas no mesmo. Considerando a característica multifatorial dos transtornos mentais, o estudo de variáveis utilizando modelos animais torna-se de extrema importância (Andreatini, 2002).

Dois modelos animais que têm sido freqüentemente utilizados para se avaliar transtornos como depressão e ansiedade são, respectivamente, o nado forçado (Fig. 1) e o labirinto em cruz elevado (Fig. 2) (Cryan, Markou & Lucki, 2002; Graeff, 1999). Esses modelos podem ser caminhos frutíferos para a compreensão daqueles transtornos, já que são sistemas experimentais validados para se estudar genética, neurobiologia e comportamento (Cryan & Holmes, 2005).

A validade de um modelo animal se refere a quanto o modelo é útil para avaliar o que ele se propõe. Critérios de validação de modelos animais são padrões gerais relevantes para a avaliação de qualquer modelo. Há dois critérios básicos que precisam ser satisfeitos para se avaliar qualquer modelo animal: confiabilidade e validade preditiva. A confiabilidade refere-se à consistência e estabilidade com a qual a variável de interesse é observada e a validade preditiva consiste na capacidade do modelo em prever drogas ou manipulações que poderiam ser efetivas também em humanos (Geyer & Markou, 1995).

Figura 1 – Procedimento do nado forçado.
Fonte: Cryan e Holmes (2005).

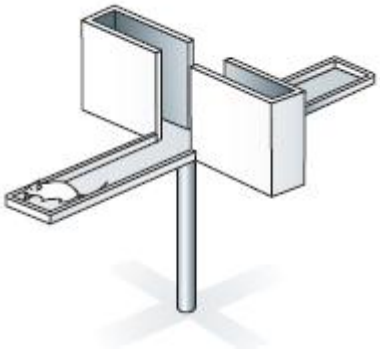

Figura 2 –Teste do labirinto em cruz elevado.
Fonte: Cryan e Holmes (2005).

O nado forçado apresenta alta confiabilidade (Cryan, Markou & Lucki, 2002), além de apresentar validade preditiva, já que este modelo permite a identificação de drogas com valor terapêutico potencial em humanos (Geyer & Markou, 1995). Já o labirinto em cruz elevado também apresenta confiabilidade e validade preditiva (Walf & Frye, 2007), pois drogas ansiolíticas que atuam no funcionamento do complexo dos receptores $GABA_A$ efetivas clinicamente aumentam de forma significativa a porcentagem de entradas e o tempo gasto nos braços abertos. Drogas ansiogênicas que atuam no mesmo complexo apresentam o efeito oposto (Pelow, Chopin, File & Briley, 1985).

Assim, modelos animais são particularmente úteis, principalmente, em situações nas quais o impacto do estresse não pode ser estudado em humanos, devido a questões éticas (Kalueff & Tuohimaa, 2004). Além disso, esses modelos são centrais para solucionar o desafio de prover um melhor entendimento à pesquisa sobre as bases das doenças mentais e, conseqüentemente, possibilitar o tratamento efetivo dessas doenças (Cryan & Holmes, 2005).

Este trabalho foi escrito de acordo com as normas da Revista Physiology and Behavior, que estão dispostas no Anexo A do presente trabalho. A Tabela 1 apresenta as medidas comportamentais avaliadas no labirinto em cruz elevado, bem como suas respectivas

interpretações. Os números contidos na referência desta tabela correspondem aos respectivos números da referência do artigo.

Tabela 1 – Medidas comportamentais avaliadas no labirinto em cruz elevado e suas respectivas interpretações.

Medida	Construto	Relação	Referência
Number of open arm entries	ansiedade	negativa	35, 36, 39
Time spent in the open arms	ansiedade	negativa	36, 39
Percentage of open arms entries	ansiedade	negativa	36,39
Number of entries in the open arm extremities	ansiedade	negativa	36, 39
Time spent in the open arms extremities[a]	ansiedade	negativa	39
Distance traveled into the open arms	ansiedade	negativa	36, 39
Time spent in the closed arms	ansiedade	positiva	36, 39
Closed arms entries	atividade motora; atividade motora horizontal	positiva	35, 36, 39
Time spent in the central square	capacidade de esperar / tomada de decisões; ansiedade	positiva	36, 39
Rearing frequency	atividade motora vertical; atividade motora e esquiva	positiva	35, 36
Rearing duration	atividade motora; atividade motora vertical	positiva	36, 39
Freezing frequency[b]	ansiedade	positiva	7
Freezing duration	ansiedade	positiva	7
Unprotected head out frequency[c]	ansiedade	negativa	35
Unprotected head out duration[d]	ansiedade	negativa	35
Protected head out frequency	ansiedade	positiva	35
Protected head out duration[d]	ansiedade	positiva	35
Protected stretched attend posture frequency	ansiedade	positiva	35
Unprotected stretched attend posture frequency	ansiedade	negativa	35
Protected flat back approach frequency[e]	ansiedade	positiva	36
Unprotected flat back approach frequency[e]	ansiedade	positiva	36
Grooming frequency	esquiva em situações de conflito; estresse/ansiedade	positiva	35, 37, 38
Grooming duration	esquiva em situações de conflito; estresse/ansiedade	positiva	37-39
Grooming latency	estresse/ansiedade	negativa	38
Grooming patterns transitions	estresse/ansiedade	positiva	36, 37
Unexpected transitions between grooming patterns (%)	estresse/ansiedade	positiva	37, 38
Interrupted grooming bouts (%)	estresse/ansiedade	positiva	37, 38
Forepaws/nose grooming frequency[f]	não é sensível ao estresse/ansiedade	-	38
Forepaws/nose grooming duration[f]	estresse/ansiedade	positiva	38
Head grooming frequency[g]	não é sensível ao estresse/ansiedade	-	38
Head grooming duration[g]	não é sensível ao estresse/ansiedade	-	38
Body/hind paws grooming frequency[h]	não é sensível ao estresse/ansiedade	-	38
Body/hind paws grooming duration[h]	não é sensível ao estresse/ansiedade	-	38
Tail/genital grooming frequency	estresse/ansiedade	negativa	38
Tail/genital grooming duration	estresse/ansiedade	negativa	38
Number of interruptions in grooming bouts	estresse/ansiedade	positiva	38

[a] esta medida corresponde ao *end-exploring* do artigo citado.

[b] freqüência de *freezing* não é avaliada na referência correspondente, portanto, foi adotada para esta medida a mesma interpretação dada à duração do *freezing*.

[c] *head out* corresponde ao *head-dipping* do artigo citado.

[d] durações de *head out* desprotegido e protegido não são avaliadas na referência correspondente, portanto, foram adotadas as mesmas interpretações dadas às respectivas freqüências de cada medida.

[e] *flat back approach* protegido e desprotegido foram analisados em conjunto na referência citada, por isso, a mesma interpretação foi utilizada em ambos.

[f] no artigo citado o *grooming* das patas dianteiras foi considerado como um padrão separado do nariz.

[g] este padrão foi unido ao *grooming* do nariz na referência correspondente.

[h] o *grooming* do corpo está separado do das patas traseiras no artigo citado.

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ARTIGO[1]

Behavior of male and female rats submitted to chronic forced swim sessions and in a subsequent elevated plus-maze test

Heloísa Maria Cotta Pires de Carvalho and Célio Estanislau

Laboratory of Experimental Psychology and Psychobiology, Londrina State University
Londrina, Paraná, 86051-990, Brazil

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1 INTRODUCTION

Forced swim and the elevated plus-maze tests are animal models which have been widely used as tools to study behavioral, physiological and genetic basis of depression and anxiety [1,2]. Forced swim is a depression animal model consisting of a two days protocol (test-retest) where a rat is exposed to an inescapable tank filled with water during 15 min on day 1 and 5 min on day 2. In this procedure, the main measure evaluated is floating duration. This behavior usually is developed in the first day and it is quickly resumed in the second day. This alteration on floating duration is typically reversed by antidepressant drugs. For this reason forced swim test has been used to assay depressive-like behaviors in rats and mice [1,3]. Variants of this procedure are commonly applied; one of them is to use forced swim as a chronic stressor [e.g. 4]. Some studies [4-6] demonstrate that in repeating forced swimming sessions, there are increases in floating duration similarly to what occurs on the two-day protocol.

The elevated plus-maze is a behavioral test used in assessing anxiety-like behavior in rodents. Briefly, it consists of two open arms crossed with two closed arms. The whole apparatus is elevated a certain distance from the ground. The animals are individually placed in the central square of the maze usually facing one of the closed arms during five minutes of free exploring. The test is based on the conflict between innate motivation to explore novel environments and a natural fear of open spaces [7]. Anxiolytic drugs increase the percentages of entries and time spent in the open arms while anxiogenic compounds decrease these measures [7]. Therefore, these are the two main parameters evaluated in this animal model of anxiety. Prior exposure to an inescapable stressor has the potential to influence the behavior of rats posteriorly tested in the elevated plus-maze [e.g. 8-10]. Behavioral change induced by exposition to chronic stressors as restraint [11-13], elevated open platform [10], forced swimming [14,15] and variable stress [11,12,16,17] have been investigated on the

elevated plus-maze. Such studies indicate that this model contributes to understanding of the interaction between chronic stress and behavioral processes associated to anxiety.

In humans, it is known that women are more vulnerable than men to mental diseases related to stress [18,19] and that stress is an important factor in susceptibility to depression and other behavioral disorders [20,21cited in 14]. Moreover, women present about twice greater prevalence of major depression and of anxiety disorders than men [22-24]. Anxiety may convey depression or vice versa and this comorbidity is frequently reported in humans [23,25,26]. On the other hand, a minority of animal studies have investigated this relationship between anxiety and depression [e.g.,14,15,27,28,29]. Also, not much animal studies have paid attention to gender differences related to each of those disorders [e.g. 30-33]. To our knowledge, there is no study which together evaluates gender differences in those animal models of anxiety and depression or during chronic forced swimming sessions. Thus, the present study purpose was to investigate if male and female rats present different depressive-like behaviors along chronic forced swimming sessions and if the exposition to this chronic stressor alters differently the anxiety-like behaviors of males and females during an elevated plus-maze test. “Ample evidence reveals a link of stress, particularly chronic stress, with depression development” [34]. For this reason the chronic forced swim was used in our study instead of a two-day protocol.

2 Materials and Methods

2.1 Subjects

Thirty six Wistar experimentally naïve male (weighing 286.9 ± 8.2 g) and thirty six female rats (weighting 239.3 ± 1.9 g) with nearly fifty days old were obtained from the Animal House of the Biological Sciences Center at the Londrina State University and housed in a *vivarium* in our laboratory. They were randomly housed in polypropylene cages (40 cm x 34 cm x 17 cm) in three divided by gender. All rats in a cage were submitted to the same treatment. Food and water were available *ad libitum* along the whole period (except during experiments). Room temperature was maintained between 21-25°C and it was established a 12:12h light/dark photoperiod (lights on at 0700h). The animals were kept undisturbed for a habituation period of 72 h before they have been submitted to the experimental treatments. The experiments reported in this paper were performed in

compliance with the recommendations of the Brazilian Society of Neuroscience and Behavior which, in turn, are based on the US National Institutes of Health Guide for Care and Use of Laboratory Animals. Additionally, experiments were developed with the approval from Londrina State University Ethics Committee for Animal Research (CEEA 66/07).

2.2 Forced Swimming

Rats from each gender were divided into three subgroups. Twelve rats were submitted to forced swimming (FSW) which was performed between 0800h and 1200h in black plastic cylinders (height: 50 cm, diameter: 22 cm) filled with tap water (25 ± 2°C) to a depth of 30 cm. Each rat was forced to swim individually for 15 min each day, for 14 consecutive days. The water was always changed and the cylinder was cleaned (ethanol 5 %) between sessions. The animals were dried with a cloth (10-20 s) after the sessions and returned to the cages. Twelve rats were submitted to the same procedure but in a cylinder without water as a handling control group (HAN). Twelve rats were kept at the *vivarium* for the same period and were later used as a control group (CTL). The time spent for the following behaviors was recorded: *floating* (complete immobility or smooth limb movements just enough to keep the nose/head above the water surface); *climbing* (vigorous movements with the forepaws above the water surface against the cylinder wall) and *diving* (whole body below the water surface). In addition, the *latency to start floating* was recorded. Behavioral recordings were performed by a trained observer with intra-rater concordance greater than 0.85.

2.3 Elevated Plus-Maze Test

In the day following the last session of forced swimming, all the rats were tested individually in a wooden elevated plus-maze. Briefly, the apparatus consisted of two opposite open arms (50 x 12 cm) and two opposite closed arms (50 x 12 x 40 cm). The arms were connected by a central square (12 x 12 cm). To avoid falls, open arms were provided with 1-cm high Plexiglass edges. The apparatus was 50 cm above the floor. It was cleaned with a 5%-ethanol solution and dried with paper towels between sessions. The experimental room was illuminated by a 60-W bulb placed 2.54 m above the maze. Sessions were video recorded by a camera placed 2.45 m above the apparatus. Each rat was gently placed in the

center of the maze facing one of the closed arms. Ten minutes later the rat was returned to its home cage. The elevated plus-maze session was separated in two session periods: 1-5 and 6-10. It was done because the grooming behavior is generally less frequent in 5-min sessions once other non-grooming behaviors can compete with them. Plus-maze sessions were performed between 1400h and 1800h. Observations were made from the TV set screen. Arm entries and time spent in each arm and in the central square were recorded. Whenever a rat entered with all four paws into an arm, an entry was recorded. The frequency and duration of the following behaviors were also measured: *head out* (sticking the head outside the maze border with at least one of the ears outside the border; as performed in the closed arms it correlates positively with anxiety while in the open arms it correlates negatively [35]. This behavior is somewhat similar to the head dipping described in this reference); *rearing* (rising on the hind limbs with forepaws moving into the air or touching the closed walls) and *freezing* (lack of movements apart from those from the vibrissae and those necessary for respiration). Additionally, the frequency of the following behaviors were recorded: *stretching attend posture* (posture in which the rat elongate the body forward and retracts to original position without rear paws locomotion; closed arm stretching correlates positively with anxiety while open arm stretching correlates negatively [35] and *flat back approach behavior* (behavior in which the rat stretches the whole body and moves forward). Head out, stretching attend postures and flat back approach behaviors were divided into regions on the plus-maze where they occurred: protected area (closed arms and central square) and unprotected area (open arms) [36].

Grooming behavior was studied through an adapted protocol [37]. Grooming bouts were divided into five patterns, i.e.: paw licking and nose/face wash; head wash; body grooming and leg licking; tail/genitals grooming and no grooming. These patterns frequently proceed in a cephalocaudal progression [38] which corresponds to expected transitions in this study. Unexpected transitions did not follow this progression. The total frequency and total duration of grooming behavior and the latency to start grooming were also measured. In addition, the percentage of unexpected transitions between grooming patterns, the total number of transitions (the sum of expected and unexpected transitions), the frequency and duration of each grooming pattern, frequency of the interruptions (interruptions longer than 5 s determine separate grooming episodes) and percentage of interrupted bouts were recorded.

Immediately after the elevated plus-maze test, it was determined by vaginal smears cytology the estrous cycle phase of rats from the three female groups. Such procedure

was always done by the same investigator. It was found that there were similar numbers of rats in each cycle phase among the groups. Therefore, no further analyses were performed in respect to estrous cycle phase.

2.4 Adrenal and Body Weights

Adrenal glands were removed from 6 rats per group, dissected from fat and weighted. The body weight from each animal was measured in the 1st, 5th, 10th and 14th forced swimming days after the sessions.

2.5 Data Analysis

Forced swimming data were analyzed through two-way analyses of variance (ANOVA) for repeated measures, with the gender as one factor (two levels: male and female) and session as the repeated measure factor (fourteen sessions). Elevated plus-maze data were analyzed through three-way ANOVAs for repeated measures, with the factors: gender (two levels: male and female), chronic treatment (three levels: FSW, HAN and CTL) and elevated plus-maze session period as the repeated measure (two levels: first five minutes and last five minutes). Data from plus-maze grooming latency, percentages of unexpected grooming transitions and interrupted grooming bouts were analyzed through two-way ANOVA, with the factors: gender (two levels: male and female) and chronic treatment (three levels: FSW, HAN and CTL). Adrenal weight data comparisons among the groups were performed through a two-way ANOVA with the factors: gender (two levels: male and female) and chronic treatment (three levels: FSW, HAN and CTL). Body weight data comparisons among the groups were performed through three-way ANOVAs for repeated measures, with the factors: gender (two levels: male and female), chronic treatment (three levels: FSW, HAN and CTL) and days of experiment as the repeated measure (three levels: fifth, tenth and fourteenth days). Whenever appropriate, *post hoc* Fisher LSD test was performed. The significance level was set at $p < 0.05$.

3 RESULTS

3.1 Forced Swimming

The following measures changed along the repeated forced swimming sessions: floating latency [$F(13,286) = 19.086$, $p<0.001$], climbing [$F(13,286) = 16.081$, $p<0.001$] and floating durations [$F(13,286) = 2.727$, $p<0.01$]. Post hoc comparisons showed that female floating latencies were longer in the first session than in the following sessions. Such decrease in floating latency was shown similarly by males, however starting only in the third session. In the following sessions, the male climbing duration was shorter than in the first session. Similar decrease was shown by females, however since the fourth session. As compared to the first session, males floated longer in the fourth session and in most of the remaining sessions. Females however, floated for a longer time only in the fourth, ninth and tenth sessions than in the first session. In each one of the previously mentioned behavioral measures, no differences between male and female rats were found when the same day scores were compared (Figure 1). Nearly all male and female rats dived a few times in the first three swimming sessions. Later this behavior was rather rare (since this behavior was rare and no sex difference was found, its results are not shown).

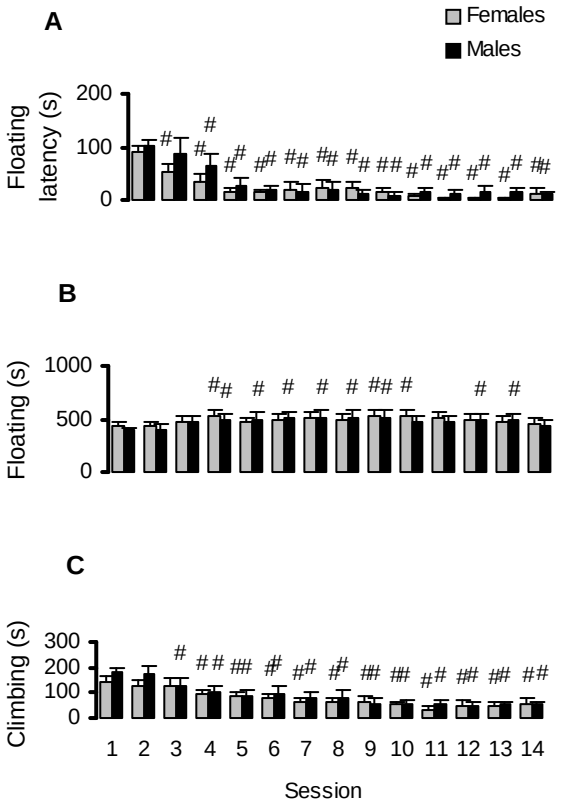

Figura 1 – Behavioral outcomes of male and female rats along forced swimming sessions. (A) Floating latency, (B) floating duration and (C) climbing duration. All data are presented as means ± S.E.M. #, $p<0.05$ as compared to their first forced swimming session (Fisher LSD).

3.2 Adrenal and Body Weights

The adrenal weight is shown at Figure 2. ANOVA revealed a main effect of gender [$F(1,30) = 26.262$, $p<0.001$]. No interaction was found. Post hoc comparisons showed that adrenal weights from all the female groups were heavier than that of their respective male groups.

The change in the body weight is shown at Figure 2. ANOVA revealed a main effect of gender [$F(1,66) = 24.813$, $p<0.001$] and day [$F(2,132) = 23.805$, $p<0.001$] and significant chronic treatment per day [$F(4,132) = 4.017$, $p<0.01$] and gender per day [$F(2,132) = 24.698$, $p<0.001$] interactions. Post hoc comparisons showed that the change in the body weight of CTL and HAN females were smaller than that of their male groups in all days of experiment. Yet, FSW females had body weight loss while FSW males had body weight gain until the tenth swimming day. In the fourteenth day, body weights from both genders were similar to that of their respective tenth day. Additionally, comparisons with CTL groups

showed that FSW females had smaller body weights in the tenth and fourteenth days while FSW males had larger body weight only in fifth day, in the following days it was similar. Further, the body weight from CTL females did not modify along the days. Body weight from HAN females increased in fourteenth day and that of FSW females decreased in tenth day and it was maintained in fourteen day. CTL and HAN male body weights increased along the all days. Body weight from FSW males increased in tenth day and it was maintained in the fourteenth day.

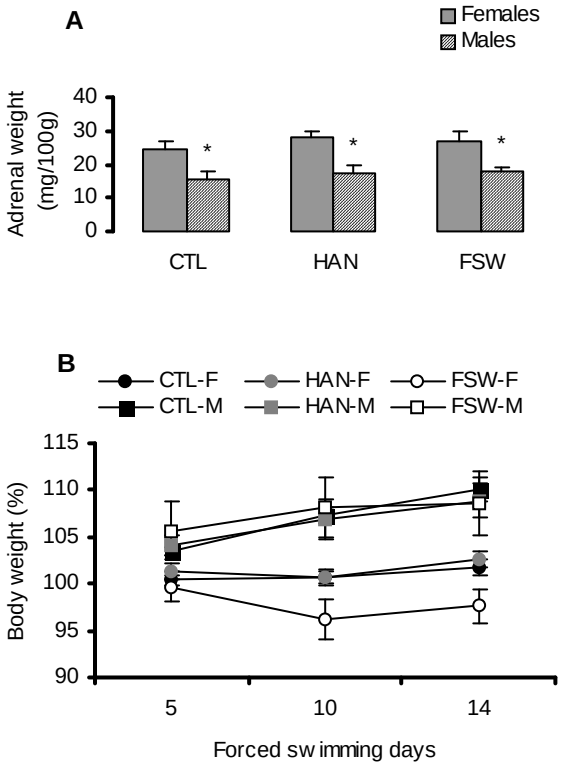

Figura 2 – Adrenal and body weights of male and female rats. (A) Adrenal weight to body weight ratio (mg/100g) and (B) percentage of body weight change (reference: first day) through the fifth, tenth and fourteenth forced swimming days. All data are presented as means ± S.E.M. CTL, control; HAN, handling; FSW, chronic forced swimming. *, $p<0.05$ as compared to females (Fisher LSD).

3.3 Elevated Plus-Maze

3.3.1 Spatiotemporal Measures: Open Arms

Open arm spatiotemporal measures in the present work are considered negatively related to anxiety [36,39-41]. Regarding the time spent in the open arms ANOVA

showed main effects of the factors: chronic treatment [$F(2,66) = 8.049$, $p<0.001$] and session period [$F(1,66) = 26.126$, $p<0.001$]. Additionally, significant chronic treatment per session period [$F(2,66) = 6.176$, $p<0.01$] and chronic treatment per gender per session period [$F(2,66) = 3.841$, $p<0.05$] interactions were found. Post hoc comparisons revealed that FSW females spent shorter time in the open arms than their correspondent male group in the 6-10 session period. Within the 1-5 session period, HAN males and females spent longer time in the open arms than their correspondent CTL groups. Such effect extended to the 6-10 session period only for females. In addition, FSW males stayed shorter time in these arms in the 1-5 and longer in the 6-10 session period as compared to their CTL groups. Furthermore, time spent in the open arms decreased from 1-5 to 6-10 session period for all the groups, except for FSW males and females (Figure 3).

The number of open arm entries is shown at Figure 3. ANOVA revealed main effects of the factors: chronic treatment [$F(2,66) = 6.844$, $p<0.01$] and session period [$F(1,66) = 104.676$, $p<0.001$] and a significant chronic treatment per session period interaction [$F(2,66) = 4.525$, $p<0.05$]. Post hoc comparisons showed that within HAN groups, females entered in the open arms more times than males in the 6-10 session period. In addition, HAN males and females entered in these arms more than their respective CTL groups during the 1-5 session period. Females had such effect extended to the 6-10 session period. During the 1-5 session period, FSW females entered in the open arms less than their correspondent CTL group. Furthermore, for all the groups the open arm entries decreased from 1-5 to 6-10 session period, except for FSW males.

The distance traveled into the open arms is shown at Figure 3. ANOVA revealed main effects of chronic treatment [$F(2,66) = 8.443$, $p<0.001$] and session period [$F(1,66) = 94.209$, $p<0.001$] and a significant chronic treatment per session period interaction [$F(2,66) = 9.528$, $p<0.001$]. Post hoc comparisons showed that HAN females traveled more into the open arms than their correspondent male group in the 6-10 session period. Additionally, it was found that HAN males and females traveled more into these arms and that FSW males and females traveled less compared with their respective CTL groups during the 1-5 session period. Furthermore, the distance traveled into the open arms decreased from 1-5 to 6-10 session period for all the groups, except for FSW males.

The percentage of open arm entries is shown at Figure 3. ANOVA showed main effects of chronic treatment [$F(2,66) = 5.888$, $p<0.01$] and session period [$F(1,66) = 23.074$, $p<0.001$]. No interaction was found. Post hoc comparisons revealed that percentage

of open arm entries decreased from 1-5 to 6-10 session period only for CTL males and females and for HAN males.

Regarding the time spent in the open arm extremities, ANOVA showed main effects of chronic treatment [$F(2,66) = 5.506$, $p<0.01$], session period [$F(1,66) = 19.101$, $p<0.001$] and a significant chronic treatment per session period interaction [$F(2,66) = 9.065$, $p<0.001$]. Post hoc comparisons revealed that HAN females stayed longer in these extremities than males in the 6-10 session period. Also, it was found that HAN males and females spent longer time in the open arm extremities than their respective CTL groups in the 1-5 session period. Such effect was extended to the 6-10 session period only for females. In the 1-5 session period, FSW males spent shorter time in these extremities than their respective CTL group. Furthermore, the time spent in the open arm extremities decreased from 1-5 to 6-10 session period only in CTL and HAN male groups (Figure 3).

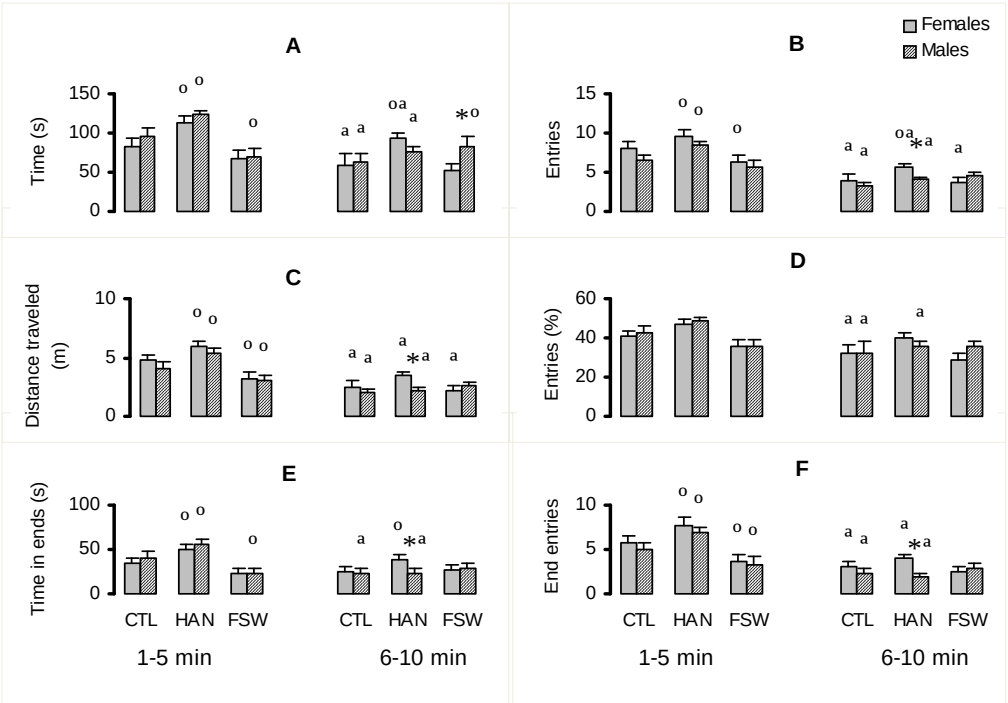

Figura 3 – Open arms spatiotemporal measures in the elevated plus-maze of male and female rats in the first and in the second 5-min session period. (A) Time spent, (B) number of entries, (C) distance traveled, (D) percentage of entries, (E) time spent in the ends and (F) number of entries in the ends. All data are presented as means ± S.E.M. CTL, control; HAN, handling; FSW, chronic forced swimming. *, $p<0.05$ as compared to females of the same group and session period; º, $p<0.05$ as compared to the CTL of the same gender in the same session period; ª, $p<0.05$ as compared to their 1-5 session period (Fisher LSD).

The number of entries in the open arm extremities is shown at Figure 3. ANOVA revealed main effects of chronic treatment [$F(2,66) = 6.791$, $p<0.01$] and session period [$F(1,66) = 80.218$, $p < 0.001$] and a significant chronic treatment per session period interaction [$F(2,66) = 12.292$, $p < 0.001$]. Post hoc comparisons showed that HAN females entered in the open arm extremities more than males in the 6-10 session period. It was found that HAN males and females entered more in the open arm extremities and that FSW males and females entered less than their respective CTL groups during the 1-5 session period. Furthermore, only FSW males and females did not decrease entries in these extremities from 1-5 to 6-10 session period.

3.3.2 Spatiotemporal Measures: Closed Arms and Central Square

The time spent in the closed arms is considered positively related to anxiety [36,39]. ANOVA revealed main effects of chronic treatment [$F(2,66) = 4.034$, $p<0.05$] and session period [$F(1,66) = 22.840$, $p<0.001$], significant chronic treatment per session period [$F(2,66) = 10.847$, $p<0.001$] and chronic treatment per gender per session period [$F(2,66) = 4.863$, $p<0.05$] interactions. Post hoc comparisons showed that HAN females spent shorter time in the closed arms than their correspondent male group in the 6-10 session period. In the same period, FSW females spent longer time in the closed arms than their correspondent male group. Also, it was found that HAN males spent shorter time while FSW males and females spent longer time in the closed arms than their respective CTL groups in 1-5 session period. During the 6-10 session period, HAN females and FSW males spent shorter time in the closed arms than their correspondent CTL groups. From 1-5 to 6-10 session period, CTL males and females increased the time spent in the closed arms such as HAN males. On the other hand, FSW males showed a decrease in such measure (Figure 4).

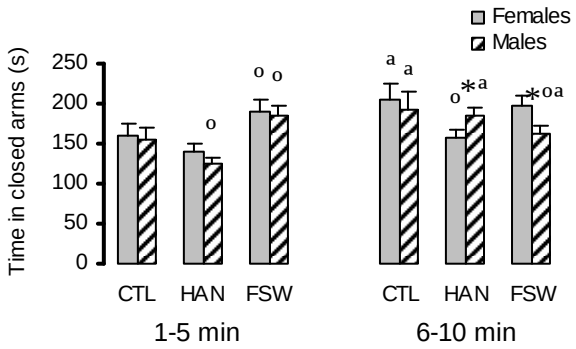

Figura 4 – Time spent by males and females in the closed arms in the first and in the second 5-min session period in an elevated plus-maze test. All data are presented as means ± S.E.M. CTL, control; HAN, handling; FSW, chronic forced swimming. *, $p<0.05$ as compared to females of the same group and session period; º, $p<0.05$ as compared to the CTL of the same gender in the same session period; ª, $p<0.05$ as compared to their 1-5 session period (Fisher LSD).

Closed arms entries are frequently considered a measure of motor activity [35,39] or more specifically horizontal motor activity [36]. ANOVA indicated main effects of gender [$F(1,66) = 5.033$, $p<0.05$] and session period [$F(1,66) = 70.666$, $p<0.001$]. No interaction was found. Post hoc comparisons revealed that CTL and HAN females entered in the closed arms more than their correspondent male groups in the 1-5 session period. Such effect extended to the 6-10 session period only in CTL group. In both session periods, it was found that FSW males entered in these arms more than their respective CTL groups. A similar effect was shown by HAN males, but only in the 6-10 session period. Moreover, the closed arm entries decreased from 1-5 to 6-10 session period for all the groups (Figure 5).

The time spent in the central square indicates waiting capacity and decision making [39] and is positively related to anxiety [36]. ANOVA revealed a significant chronic treatment per session period interaction [$F(2,66) = 5.500$, $p<0.01$]. No main effects were found. Post hoc comparisons showed that HAN and FSW females spent longer time in the central square than their CTL group in the 6-10 session period. Furthermore, the time spent in the central square decreased from 1-5 to 6-10 session period only in the female CTL group (Figure 5).

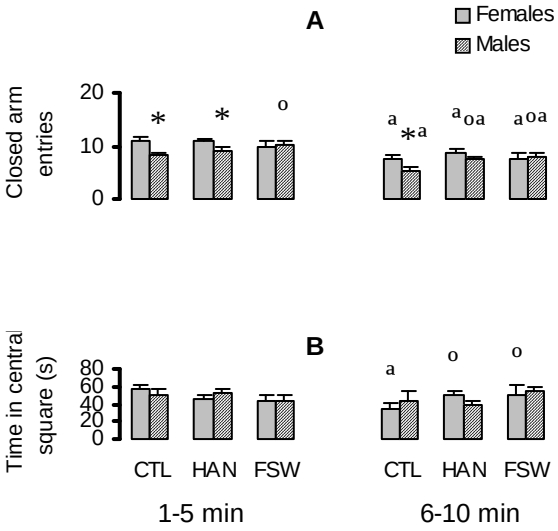

Figura 5 – Number of closed arms entries and time spent in the central square of male and female rats in the first and in the second 5-min session period in an elevated plus-maze test. (A) Entries and (B) time. All data are presented as means ± S.E.M. CTL, control; HAN, handling; FSW, chronic forced swimming. *, $p<0.05$ as compared to females of the same group and session period; º, $p<0.05$ as compared to the CTL of the same gender in the same session period; ª, $p<0.05$ as compared to their 1-5 session period (Fisher LSD).

3.3.3 Ethological Measures

The rearing behavior reflects motor activity [35,39] or more specifically vertical motor activity [36]. Additionally, rearing frequency is also related to displacement response in conflict situations [35]. ANOVA performed on rearing frequency showed a session period main effect [$F(1,66) = 6.424$, $p<0.05$] and a significant chronic treatment per gender interaction [$F(2,66) = 8.215$, $p<0.001$]. Post hoc comparisons revealed that HAN females reared more than their correspondent male group in the 1-5 session period. In the whole session, FSW females reared less than their correspondent male group. Also in the whole session FSW males reared more and FSW females reared less than their correspondent CTL groups. Additionally, HAN males reared more than their CTL group only in the 6-10 session period (Figure 6).

The rearing duration is shown at Figure 6. ANOVA revealed a main effect of gender [$F(1,66) = 4.376$, $p<0.05$] and significant chronic treatment per gender [$F(2,66) = 4.007$, $p<0.05$] and chronic treatment per session period [$F(2,66) = 5.153$, $p<0.01$] interactions. Post hoc comparisons showed that in the whole session FSW males reared longer than their correspondent female group and than their correspondent CTL group. HAN males

reared longer than their CTL group, in the 6-10 session period, and also showed an increase in the rearing duration from 1-5 to 6-10 session period.

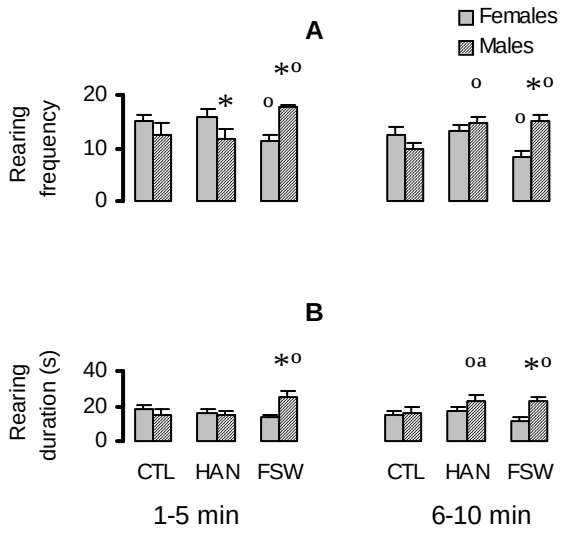

Figura 6 – Rearing behavior of male and female rats in the first and in the second 5-min session period in an elevated plus-maze test. (A) Frequency and (B) duration. All data are presented as means ± S.E.M. CTL, control; HAN, handling; FSW, chronic forced swimming. *, $p<0.05$ as compared to females of the same group and session period; º, $p<0.05$ as compared to the CTL of the same gender in the same session period; ª, $p<0.05$ as compared to their 1-5 session period (Fisher LSD).

Freezing behavior is considered to reflect anxiety [7]. ANOVA performed on freezing frequency showed a main effect of session period [$F(1,66) = 14.215$, $p<0.001$]. No interaction was found. Post hoc comparisons revealed that, in the 6-10 session period, freezing frequency from FSW females was greater than that of their correspondent male group; HAN males and females froze less than their respective CTL groups as well as FSW males. Furthermore, freezing frequency increased from 1-5 to 6-10 session period only in CTL males and females (Table 1).

Freezing duration also showed a session period main effect [$F(1,66) = 5.776$, $p<0.05$]. No interaction was found. Post hoc comparisons showed that FSW females froze longer than their correspondent male group in the whole session. In the 6-10 session period, CTL females froze shorter than their correspondent male group. Also in the same period, HAN and FSW males froze shorter than their CTL group. Additionally, it was found that FSW females froze longer than their CTL group in the whole session. Moreover, freezing

duration increased from 1-5 to 6-10 session period only in CTL males and FSW females (Table 1).

Table 1 – Anxiety-related behaviors in the elevated plus-maze of male and female rats of control group, handled group and group submitted to forced swimming in the first and second session periods. Data are expressed as mean ± S.E.M. P < 0.05. *gender difference [o]group control difference [a]session period difference

	Control				Handled				Forced swimming			
	1-5		6-10		1-5		6-10		1-5		6-10	
Measures	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males
Freezing	0.25 ± 0.18	0.17 ± 0.11	1.00 ± 0.43[a]	1.33 ± 0.73[a]	0.00 ± 0.00	0.08 ± 0.08	0.17 ± 0.11[o]	0.50 ± 0.34[o]	0.33 ± 0.26	0.08 ± 0.08	1.00 ± 0.46	0.17 ± 0.11*[o]
Freezing (s)	0.27 ± 0.19	0.33 ± 0.26	1.19 ± 0.48	6.34 ± 3.44*[a]	0.00 ± 0.00	0.04 ± 0.04	0.08 ± 0.06	0.51 ± 0.37[o]	9.23 ± 9.08[o]	0.05 ± 0.05*	13.4 ± 12.7[oa]	0.08 ± 0.06*[o]
Protected head out frequency	5.75 ± 0.62	5.17 ± 1.25	2.08 ± 0.50[a]	2.33 ± 0.58[a]	4.17 ± 0.68	6.17 ± 0.81	2.25 ± 0.49	2.67 ± 0.74[a]	4.33 ± 0.72	4.92 ± 0.79	3.00 ± 0.70	4.17 ± 0.64
Protected flat back approach	1.25 ± 0.43	2.17 ± 0.81	0.58 ± 0.23	0.75 ± 0.45[a]	1.42 ± 0.47	1.33 ± 0.54	0.25 ± 0.13[a]	0.25 ± 0.18	1.83 ± 0.81	2.42 ± 0.86	1.00 ± 0.44	1.17 ± 0.52[a]
Unprotected flat back approach	1.42 ± 0.51	1.25 ± 0.49	0.50 ± 0.23[a]	0.67 ± 0.31	1.50 ± 0.34	1.25 ± 0.39	1.00 ± 0.39	0.50 ± 0.23	1.67 ± 0.26	1.17 ± 0.37	1.08 ± 0.38	0.92 ± 0.34
Forepaws/nose groom. (s)	16.3 ± 4.91	14.8 ± 3.51	25.8 ± 5.97[a]	24.0 ± 3.10[a]	12.4 ± 2.68	9.67 ± 1.88	13.9 ± 3.22[o]	13.1 ± 2.59[o]	13.9 ± 2.00	14.0 ± 1.98	19.1 ± 3.39	14.6 ± 2.48[o]
Body/hind paws groom.	0.67 ± 0.51	0.25 ± 0.18	1.00 ± 0.39	1.67 ± 0.59[a]	0.00 ± 0.00	0.25 ± 0.18	0.17 ± 0.11[o]	0.42 ± 0.19[o]	0.08 ± 0.08	0.08 ± 0.08	0.58 ± 0.36	0.33 ± 0.19[o]
Body/hind paws groom. (s)	2.29 ± 1.84	0.60 ± 0.41	9.88 ± 5.91[a]	13.4 ± 5.30[a]	0.00 ± 0.00	0.44 ± 0.36	0.17 ± 0.11[o]	1.93 ± 0.95[o]	0.10 ± 0.10	0.12 ± 0.12	3.28 ± 2.34	2.86 ± 1.88[o]
Genital/tail groom.	0.25 ± 0.18	0.00 ± 0.00	0.67 ± 0.36	0.92 ± 0.29[a]	0.08 ± 0.08	0.00 ± 0.00	0.25 ± 0.18	0.25 ± 0.13[o]	0.08 ± 0.08	0.00 ± 0.00	0.67 ± 0.28[a]	0.17 ± 0.11[o]
Genital/tail groom. (s)	0.87 ± 0.64	0.00 ± 0.00	3.27 ± 1.94	4.29 ± 1.41[a]	0.12 ± 0.12	0.00 ± 0.00	1.02 ± 0.82	0.76 ± 0.42[o]	0.27 ± 0.27	0.00 ± 0.00	2.80 ± 1.38	1.04 ± 0.70[o]

When head out occurs in the protected area, the anxiety level is higher and when it occurs in the unprotected area, the anxiety level is lower [35]. ANOVA performed on unprotected head out frequency showed a chronic treatment [$F(2,66) = 6.548$, $p<0.01$] and a session period [$F(1,66) = 93.529$, $p<0.001$] main effects and a significant chronic treatment per session period interaction [$F(2,66) = 6.944$, $p<0.01$]. Post hoc comparisons revealed that in the 6-10 session period, FSW females showed smaller unprotected head out frequency than their correspondent male group. Additionally, HAN males showed higher levels of this measure than their CTL group in the 1-5 session period. In the same period, FSW males and females showed lower levels than their respective CTL groups. Furthermore, unprotected head out frequency decreased from 1-5 to 6-10 session period for all the groups, except FSW males (Figure 7).

The unprotected head out duration is shown at Figure 7. ANOVA revealed chronic treatment [$F(2,66) = 4.361$, $p<0.05$] and session period [$F(1,66) = 37.149$, $p<0.001$] main effects and a significant chronic treatment per session period interaction [$F(2,66) = 3.881$, $p<0.05$]. Post hoc comparisons showed that unprotected head out duration from FSW females was shorter than that of their correspondent male group in the 6-10 session period. Also, FSW males and females showed shorter unprotected head out duration than their respective CTL groups in the 1-5 session period. Moreover, all the groups showed decreases in unprotected head out duration from 1-5 to 6-10 session period, except FSW males.

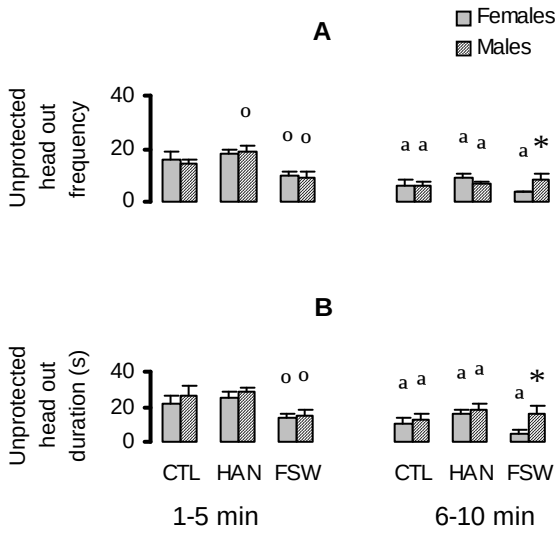

Figura 7 – Unprotected head out behavior of male and female rats in the first and in the second 5-min session period in an elevated plus-maze test. (A) Frequency and (B) duration. All data are presented as means ± S.E.M. CTL, control; HAN, handling; FSW, chronic forced swimming. *, $p<0.05$ as compared to females of the same group and session period; º, $p<0.05$ as compared to the CTL of the same gender in the same session period; ª, $p<0.05$ as compared to their 1-5 session period (Fisher LSD).

Protected head out frequency showed only a main effect of session period [F(1,66) = 30.325, p<0.001] (Table 1). ANOVA performed on protected head out duration revealed a significant chronic treatment per session period interaction [F(2,66) = 3.738, p<0.05]. No main effect was found. Post hoc comparisons revealed that FSW males showed longer duration than their CTL group in the 6-10 session period. Furthermore, CTL males showed decrease whereas FSW males showed increase in this measure from 1-5 to 6-10 session period (Figure 8).

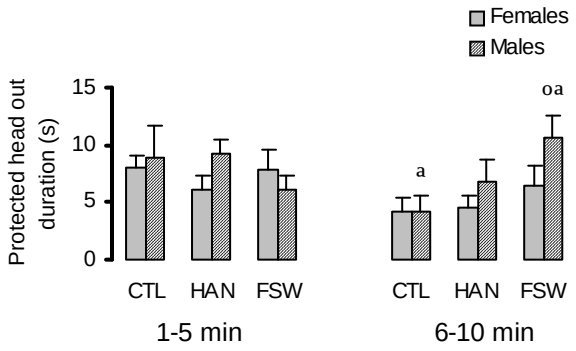

Figura 8 – Duration of protected head out of male and female rats in the first and in the second 5-min session period in an elevated plus-maze test. All data are presented as means ± S.E.M. CTL, control; HAN, handling; FSW, chronic forced swimming. º, p<0.05 as compared to the CTL of the same gender in the same session period; ª, p<0.05 as compared to their 1-5 session period (Fisher LSD).

Stretching attend posture was also differentiated in protected and unprotected areas, thus, the same interpretation for the head out is valid in this analysis. ANOVA revealed a main effect of session period [F(1,66) = 12.680, p<0.001] and a significant interaction between chronic treatment and gender [F(2,66) = 3.416, p<0.05] on protected stretching attend posture frequency. Post hoc comparisons showed that protected stretching attend posture frequency from HAN females was smaller than that of their respective CTL group in the 1-5 session period. Besides, this measure decreased from 1-5 to 6-10 session period only in FSW females (Figure 9).

ANOVA performed on unprotected stretching attend posture frequency showed a main effect of session period [F(1,66) = 6.881, p<0.05]. No interaction was found. Post hoc comparisons revealed that exhibitions of unprotected stretching attend posture from CTL and HAN females were more frequent than that of their respective male groups in the 1-

5 session period. Further, this measure decreased from 1-5 to 6-10 session period only for CTL and HAN females (Figure 9).

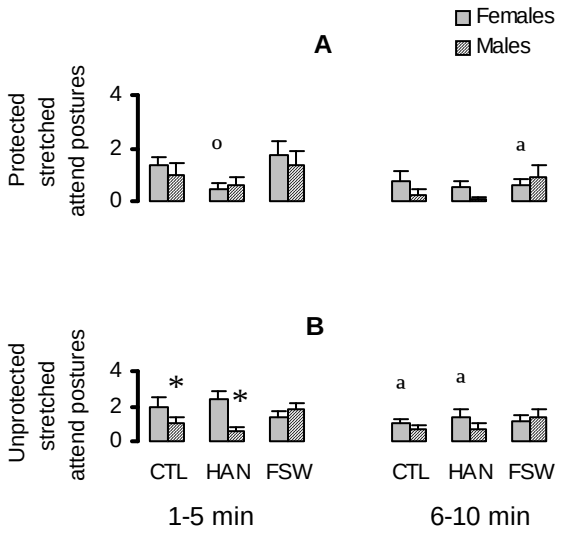

Figura 9 – Frequency of protected and unprotected stretched attend postures of male and female rats in the first and in the second 5-min session period in an elevated plus-maze test. (A) Protected and (B) unprotected. All data are presented as means ± S.E.M. CTL, control; HAN, handling; FSW, chronic forced swimming. *, $p<0.05$ as compared to females of the same group and session period; ^{o}, $p<0.05$ as compared to the CTL of the same gender in the same session period; ^{a}, $p<0.05$ as compared to their 1-5 session period (Fisher LSD).

The protected flat back approach behavior is positively related to anxiety [36]. ANOVA showed only main effects of session period on protected [$F(1,66) = 20.580$, $p<0.001$] and unprotected [$F(1,66) = 13.013$, $p<0.001$] flat back approach behaviors (Table 1).

The grooming duration is positively related to stress and anxiety [38] and is also used as an index of displacement in conflict situations [35,39]. ANOVA revealed a chronic treatment [$F(2,66) = 7.561$, $p<0.01$] and a session period [$F(1,66) = 18.393$, $p<0.001$] main effects and a significant chronic treatment per session period interaction [$F(2,66) = 4.333$, $p<0.05$] on grooming duration. Post hoc comparisons showed that HAN males and females groomed for a shorter time than their respective CTL groups as well as FSW males in the 6-10 session period. In addition, grooming duration increased from 1-5 to 6-10 session period only in CTL males and females (Figure 10).

Grooming pattern transitions, number of interruptions in grooming bouts and percentages of unexpected transitions and interrupted bouts are positively related to stress

and anxiety [37,38]. ANOVA performed on grooming pattern transitions showed a main effect of chronic treatment [$F(2,66) = 3.613$, $p<0.05$] and a significant chronic treatment per gender per session period interaction [$F(2,66) = 3.420$, $p<0.05$]. Post hoc comparisons revealed that grooming pattern transitions from CTL females were larger than that of their correspondent male group in the 1-5 session period. In the same session period, it was found that HAN females had lower scores than their CTL group in this measure. In the 6-10 session period, HAN and FSW males had lower transitions than their CTL group. Furthermore, grooming pattern transitions increased from 1-5 to 6-10 session period only in CTL males (Figure 10).

ANOVA performed on the number of interruptions in grooming bouts showed an effect of chronic treatment [$F(2,66) = 3.395$, $p<0.05$] and session period [$F(1,66) = 5.960$, $p<0.05$] and a significant interaction between chronic treatment and gender [$F(2,66) = 3.513$, $p<0.05$]. Post hoc comparisons revealed that the interruption frequencies from CTL females were smaller than that of their male group in the 6-10 session period. Also in the same period, the interruption frequencies from HAN and FSW males were smaller than that of their CTL group. Further, the interruption frequencies increased from 1-5 to 6-10 session period only in CTL males (Figure 10).

Percentage of unexpected transitions between grooming patterns is shown at Figure 10. ANOVA revealed a main effect of chronic treatment [$F(2,66) = 4.216$, $p<0.05$]. No interaction was found. Post hoc comparisons showed that percentages of unexpected transitions from HAN and FSW females were smaller than that of their CTL group in the whole session.

On percentage of interrupted grooming bouts, ANOVA showed a main effect of gender [$F(1,66) = 5.853$, $p<0.05$] and a significant interaction between chronic treatment and gender [$F(2,66) = 4.280$, $p<0.05$]. Post hoc comparisons revealed that in the whole session percentage of interrupted bouts from CTL females was smaller than that of their male group. Further, HAN and FSW males had lower score in this measure than their CTL group (Figure 10).

The forepaws/nose grooming duration is positively related to stress and anxiety [38]. ANOVA revealed an effect of chronic treatment [$F(2,66) = 5.149$, $p<0.01$] and session period [$F(1,66) = 7.200$, $p<0.01$]. No interaction was found. Post hoc comparisons showed that forepaws/nose grooming durations from HAN males and females and from FSW males were shorter than that of their CTL groups during the 6-10 session period. Additionally,

forepaws/nose grooming durations increased from 1-5 to 6-10 session period only in CTL males and females (Table 1).

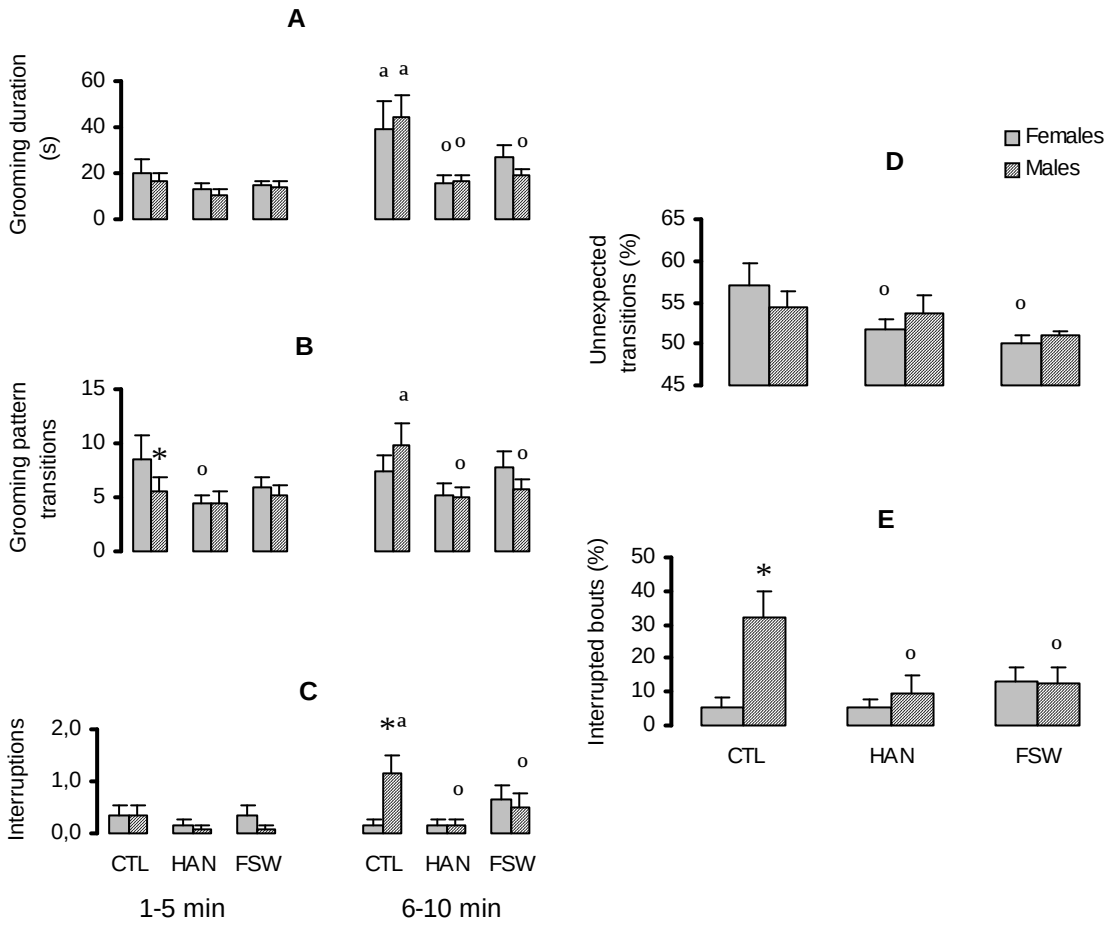

Figura 10 – Grooming measures of male and female rats in the fist and in the second 5-min session period in an elevated plus-maze test. (A) Duration, (B) pattern transitions, (C) interruptions, (D) percentage of unexpected transitions and (E) percentage of interrupted bouts. All data are presented as means ± S.E.M. CTL, control; HAN, handling; FSW, chronic forced swimming. *, $p<0.05$ as compared to females of the same group and session period; ^{o}, $p<0.05$ as compared to the CTL of the same gender in the same session period; ^{a}, $p<0.05$ as compared to their 1-5 session period (Fisher LSD).

The body/hind paws grooming frequency and duration are not related to stress and anxiety [38]. ANOVA showed an effect of chronic treatment [$F(2,66) = 5.340$, $p<0.01$] and session period [$F(1,66) = 9.797$, $p<0.01$] on body/hind paws grooming frequency. No interaction was found. Post hoc comparisons revealed that body/hind paws grooming frequencies from HAN males and females and from FSW males were smaller than that of

their CTL groups in the 6-10 session period. Further, body/hind paws grooming frequency increased from 1-5 to 6-10 session period only in CTL males (Table 1).

The body/hind paws grooming duration is shown at Table 1. ANOVA revealed an effect of chronic treatment [$F(2,66) = 5.838$, $p<0.01$] and session period [$F(1,66) = 11.132$, $p<0.01$] and a significant interaction between chronic treatment and session period [$F(2,66) = 4.120$, $p<0.05$]. Post hoc comparisons showed that body/hind paws grooming durations from HAN males and females and from FSW males were shorter than that of their CTL groups in the 6-10 session period. Moreover, body/hind paws grooming durations increased from 1-5 to 6-10 session period only for CTL males and females.

Tail/genital grooming pattern frequency and its duration are negatively related to anxiety [38]. ANOVA performed on tail/genital grooming frequency showed an effect of chronic treatment [$F(2,66) = 3.275$, $p<0.05$] and session period [$F(1,66) = 15.184$, $p<0.001$]. No interaction was found. Post hoc comparisons revealed that tail/genital grooming frequencies from HAN and FSW males were smaller than that of their CTL group in the 6-10 session period. Additionally, tail/genital grooming frequencies increased from 1-5 to 6-10 session period only in CTL males and FSW females (Table 1).

The tail/genital grooming duration is shown at Table 1. ANOVA revealed an effect of chronic treatment [$F(2,66) = 3.727$, $p<0.05$] and session period [$F(1,66) = 14.148$, $p<0.001$]. No interaction was found. Post hoc comparisons showed that tail/genital grooming durations from HAN and FSW males were shorter than that of their CTL group in the 6-10 session period. Additionally, tail/genital grooming duration increased from 1-5 to 6-10 session period only for CTL males.

Grooming latency and frequency, fore paws/nose grooming frequency and head grooming (frequency and duration) did not show neither main effects nor significant interactions (data not shown).

4 DISCUSSION

Forced swimming is one of the most widely used animal models of depression and has been considered as a valid test in rodents for investigating a drug antidepressant potential [3]. In the present study, females showed decreases in floating latency and in climbing duration since the second and the fourth sessions, respectively. Males similarly showed such decreases, but since the third session for both measures. Also, both

genders showed increases in floating duration since the fourth session. Decreases in floating latency and in climbing duration and increases in floating duration are interpreted as increased behavioral despair [42]. Regarding studies which employed chronic forced swimming, our work was the first to evaluate floating latency and climbing duration for such a long period. According to other studies, similar increases in floating duration occur with C57BL/6J male mice along fourteen daily sessions [4], Swiss Webster male mice along three sessions [6] and Sprague-Dawley male rats in the fifteenth session after fourteen sessions of forced swimming [5]. Therefore our results demonstrated that these three measures evaluated (floating latency, floating duration and climbing duration) were consonant with the floating duration results of studies early cited. All these results indicate the occurrence of depressive-like behaviors.

Some studies reporting gender differences in a test-retest forced swimming procedure indicate opposite results. Two studies using Wistar rats [31,43] report female score higher in floating and lower in climbing as compared with males in the retest session. Other studies using Wistar [30] and Long-Evans [44] rats report an opposite gender differences. In the present study, no gender differences in climbing or floating behaviors were found along 14 sessions of forced swimming. Such result indicates that as well as two-day protocol the chronic forced swimming induced depressive-like behaviors in rats however of similar way in male and female rats.

Exposure to chronic stressors has been reported to decrease body weight gain or even lead to weight loss [45]. However, stress can increase food intake leading to body weight gain depending on the stressor severity [46]. Our study found gender differences concerning the body weight change along the experimental days in all the groups. CTL females did not modify their body weights while CTL males had body weight gain. HAN females only increased their body weight in fourteenth day whereas HAN males had body weight gain in all days. FSW females decreased their body weights in tenth day and this decrease was maintained in the fourteenth day. FSW males increased their body weights in the tenth day and this increase was maintained in the fourteenth day. Result of FSW female body weight loss is in accordance with other study [47] in which aged male and female Fischer rats were submitted to 21 days of chronic restraint stress. Nevertheless, in the present work the body weight gains found for males are opposite to the above study results.

In comparison with their CTL groups, FSW females showed smaller body weights in the 10^{th} and 14^{th} day while males showed larger body weight only in the 5^{th} day. Female results are in accordance with other study [47]. Male body weight gain observed in 5^{th} day could be explained by stress-induced increase in food intake, which has been previously

reported in male mice exposed to psychosocial stress [48]. Remaining results of males are in accordance with studies which show that body weight is not affected by chronic forced swimming [5], restraint [49] or electric foot shocks [50]. Concluding, body weight data indicate that both genders experienced stress as exposed to chronic forced swimming. However, males habituated more rapidly to this situation of stress than females.

Adrenal weight is among the commonly used indexes to assess rat stress experience [51,52]. Our study showed that females had heavier adrenals than males. This finding corroborates a prior report [53]. Though such study does not show direct comparison between male and female adrenal weights, sex difference is visually evident in a figure of the cited research.

Increases in adrenal weights are reported to result from chronic stressors such as social defeat [52], unpredictable mild stress [51,54], restraint [53], immersion in cold water, electric foot shocks and immobilization [50]. However, in the present study chronic forced swimming did not result in adrenal weight change. This result agrees with other study in which chronic restrain stress and chronic variable stress did not affect male adrenal weights [49]. Thus, chronic forced swimming procedure applied in the present study was not a stressor severe enough to change adrenal weights. Other studies demonstrate that only severe or long-lasting stress procedures cause adrenal weight increase [49,55].

In the first 5-min period of the plus-maze session, males previously submitted to forced swimming were more anxious than their control group. The following behavioral measures support such view: time spent in the open arms, time spent in the closed arms, entries and time spent in the open arms extremities, distance traveled into these arms and unprotected head out. Open arms measures are negatively related to anxiety, including the time spent in the open arms which is one of the main anxiety-related measures because it is increased by anxiolytic drugs and decreased by anxiogenic drugs [7]. Additionally, time in the closed arms and unprotected head out behavior are positively and negatively related to anxiety respectively. The anxiogenic effect found in the present study agrees with other studies results in which are described the effects of chronic forced swim [14,15,29], of chronic immobilization stress [11,12] and of chronic variable stress [56].

In the second 5-min period of the plus-maze session, the same male rats showed an opposite profile regarding some anxiety measures. They were less anxious than their control group according to the time spent in the open arms, time spent in the closed arms, freezing frequency and duration, grooming duration, grooming pattern transitions, grooming bout interruptions and forepaws/nose grooming duration. Additionally, the

percentage of interrupted grooming bouts in the whole session also indicated lower anxiety. Such anxiolytic-like effect possibly occurred due to an increase in general motor activity shown by closed arms entries measure. Probably the increase in motor activity led to an increase in open arm exploration. In accordance to the 1-5 session period result, protected head out duration showed that males remained more anxious than their control group also in the 6-10 session period. In this case, it is likely that this measure has shown a pure effect of anxiety because it is highly related to anxiety. In conclusion, these results indicate that chronic forced swimming produced an increase in the male level of anxiety in the 1-5 session period. Only protected head out duration showed the same effect in the 6-10 session period.

Many measures (entries in the open arms and their extremities, distance traveled into these arms, time spent in the closed arms, freezing duration and unprotected head out frequency and duration) indicate an anxiogenic effect shown by females submitted to forced swimming as compared with their control group in the 1-5 session period. In freezing duration such effect extended to the second 5-min session period. Nevertheless, according to the percentage of unexpected grooming pattern transitions as evaluated in the whole session, chronic forced swimming led to an anxiolytic effect on females. Another study reports an anxiogenic effect in mice exposed to chronic psychoemotional influences (intermale confrontations and daily transfer to new litter previously used for fights during one month) [57] whereas other reports an anxiolytic effect in rats submitted to chronic variable stress [56]. These opposite results evidence the necessity of more studies evaluating female behaviors to contribute with data to elucidate the discrepancy observed.

According to the time spent in the open arms, male and female handled rats showed decreased anxiety in the first 5-min session period. Only females showed the same effect also in the second 5-min session period. Most of the other anxiety-related spatiotemporal measures strengthen this view, being only the exception the percentage of entries in the open arms. The number of entries in the open arm extremities and the distance traveled into these arms did not confirm the result in the 6-10 session period only. Some other anxiety-related behaviors also support an anxiolytic handling effect in the second session period for both genders (freezing frequency, grooming duration, forepaws/nose grooming duration and grooming bout interruptions). Handling anxiolytic properties are in agreement with previous studies [58,59]. Only the tail/genital grooming frequency and duration suggest an opposite interpretation according to which handled males were more anxious than their control group in the 6-10 session period. So, most of anxiety measures agree with previous studies which report anxiolytic effects of handling.

Furthermore, handled groups showed gender differences in unprotected stretched attend postures during the first 5-min session period and during the second period they showed gender differences in all the anxiety-related spatiotemporal measures except time spent and percentage of entries in the open arms. Cited measures indicate that males were more anxious than females. For control groups, grooming pattern transitions suggest that females were more anxious than males in the first 5-min session period. However, in the same period, unprotected stretched attend postures indicates the opposite. This gender difference also was found in the 6-10 session period on freezing duration and grooming bout interruptions. Moreover, the percentage of interrupted grooming bouts showed this same effect in the whole session. Regarding rats submitted to forced swimming, females were more anxious than their male group in the 1-5 (according to freezing duration) and in the 6-10 session period (time spent in the open and closed arms, unprotected head out and freezing frequency and duration). This result is opposite to other study [56] reporting gender differences in Wistar rats submitted to chronic variable stress. Therefore, these findings indicate that forced swimming inverted the anxiety of males in comparison with females whereas control and handled males showed higher anxiety than females.

The main measure of motor activity in the elevated plus-maze is the number of closed arms entries [35,36,39]. Our study found that control females showed more locomotion than males in both session periods. There was no effect of handling in male and female groups in the 1-5 session period. In the 6-10 session period, handled males showed higher motor activity than their control group as well as males submitted to forced swimming in the whole session. Other studies [12,14,15,29] do not show effect of chronic stress on closed arm entries in males. Females did not show effect of chronic forced swimming. This result is in agreement with other study in which chronic stress by either immobilization or unpredictable stress was applied [9]. Thus, a possible explanation for the increase in motor activity of males submitted to handling or chronic forced swimming in the 6-10 session period could be due to handling effect because both groups were exposed to that manipulation. Additionally, it can be asserted that the decrease found on anxiety-related spatiotemporal measures due to chronic forced swimming could not be explained by means of decreased general motor activity, what gives more reliability to the results of anxiety.

Other measure related to motor activity [35,39], or more specifically vertical motor activity [36], is rearing behavior. Our study found a gender difference in handled groups: females showed more vertical activity than males in the 1-5 session period. On the other hand, females submitted to forced swimming showed less vertical activity than their

male group and than their control group in the whole session. Moreover, males submitted to forced swimming presented more vertical activity than their control group in both session periods as well as HAN males in the 6-10 session period. Control groups showed no gender differences in this behavior. However, other study reports larger rearing frequency in adult control females as compared to males [33]. Thus, these findings show that forced swimming produced a decrease in vertical activity on females and an increase on males. The result from males in the 6-10 session period is in accordance with and could be interpreted of the same way than those of closed arms entries.

In regard to time spent in the central square, it reflects waiting capacity and decision making in conflict situations [36,39] or anxiety [36]. No chronic treatment effect or gender difference was found in this measure during the 1-5 session period. According to other study, females submitted to chronic psychoemotional influences spend shorter time in the maze center [57]. During the second 5-min session period females submitted to handling or to forced swimming spent more time in the central square than their control group. Such results indicate that these rats had higher anxiety level and that they did not habituate to avoidance/approach conflict.

Grooming behavior has been used as a stress/anxiety measure [37,38] or as an index of displacement behavior in conflict situations [35,39]. The traditional measures evaluated in grooming behavior as frequency and duration are positively related to stress/anxiety and displacement whereas grooming latency is negatively related to stress/anxiety. In our study, these measures did not show differences neither due to gender nor due to chronic treatment other than that on grooming duration. According to this measure, males submitted to forced swimming had lower levels of stress/anxiety than their control group in the second 5-min session period. This result contrasts with other study [38] in which grooming duration and frequency increased while grooming latency decreased in male Wistar rats restricted to a well illuminated box (a situation of high stress/anxiety) as compared to a group restricted to a dark box (one situation of low stress/anxiety).

Other grooming measures also are used to evaluate stress/anxiety: percentages of unexpected transitions and interrupted grooming bouts, number of grooming pattern transitions and number of grooming activity interruptions. All these measures are positively related to stress/anxiety and are based in the behavioral alteration in the grooming microstructure [37,38]. Our study found that males submitted to forced swimming were less stressed/anxious than control group in all these measures except the percentage of unexpected transitions. Differently, other study found that C57BL/6 male mice showed higher stress

levels than their control group after exposure to novelty (elevated plus-maze) and social stress (social encounter) according to the percentages of interrupted bouts and unexpected transitions [37]. Other study reports more grooming pattern transitions and more grooming activity interruptions in rats restricted in a well illuminated box as compared with rats restricted in a dark box, situations of high and low stress/anxiety respectively [38].

Furthermore, grooming behavior is composed of several patterns which are differently affected by stress: stressed rats show more rostral grooming and less genital/tail grooming [38]. Intermediary patterns as head, body and hind paws grooming are not sensible to stress [38]. Concerning some measures (forepaws/nose grooming frequency, head grooming frequency and duration), no chronic treatment effect was found, result which agree with that of cited study [38]. The body/hind paws grooming frequency and duration which, according to other study [38], are not sensible to stress, also did not show differences in our study in the 1-5 session period. However, the same measure was sensible to stress on males in the 6-10 session period. Grooming patterns which are affected by stress such as forepaws/nose grooming duration and tail/genital grooming frequency and duration did not show results in the 1-5 session period. In the 6-10, results were opposite, thus, males submitted to forced swimming presented low stress/anxiety according to forepaws/nose grooming duration and high stress/anxiety according to tail/genital grooming frequency and duration. Other study [38] report longer forepaws/nose grooming duration and lower tail/genital grooming frequency and duration in light-exposed rats compared with dark-exposed rats, situations of high and low stress/anxiety respectively.

Thus, regarding all grooming measures in our study, it can be asserted that absence of results in the 1-5 session period could possibly be due to competitive activities. In the 6-10 session period – as there was not an habituation to the experimental setting in males submitted to forced swimming, indicated by most of the anxiety-related measures – this competition was maintained. Control groups showed this habituation and present an increase in grooming behavior from one session period to the other. Maybe a longer elevated plus-maze session would lead to habituation and would reveal a more robust grooming behavior in stressed groups.

The divergences observed between our grooming results and that of other studies [37,38] could be due to procedural differences. For example, in our study grooming analysis was done during the elevated plus-maze test instead of in a separate place (actimeter) after exposure to plus-maze [37]. Moreover, some grooming patterns were jointed in our study due to difficulty to record each one separately what not was done in the others [37,38].

Further, it was used a chronic stressor in our work instead of an acute stressor [37,38]. Other possible explanation for the discrepant results could be the stressor exposure time which was fifteen minutes in our research instead of five minutes [37,38].

In summary, the present study indicates that chronic forced swim induced similar depressive-like behaviors in male and female rats resulting in stress experience for both genders with males habituating more rapidly to the situation of stress than females. Despite both genders had experienced stress, chronic forced swim was not sufficiently severe or long-lasting to change the adrenal weight. Additionally, elevated plus-maze test revealed that males of control and handled groups were more anxious and males submitted to forced swimming were less anxious than females, what show that the forced swim inverted the gender difference. Moreover, the chronic forced swimming produced an anxiogenic effect and the handling produced an anxiolytic effect in both genders. These findings suggest which there is an association between depression and anxiety, what is in accordance with comorbidity clinical studies [60]. In addition, gender differences are implicated in this association what suggest a need to plan specific treatments which could attend the specific demand of each gender.

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ANEXOS

ANEXO A

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