UNIVERSIDADE PAULISTA

MESTRADO EM PATOLOGIA AMBIENTAL E EXPERIMENTAL

A IVERMECTINA ADMINISTRADA NA IDADE JUVENIL PREJUDICA O COMPORTAMENTO SEXUALMENTE DIMÓRFICO DE RATOS EXPOSTOS OU NÃO AO ESTRESSE

Dissertação apresentada ao Programa de Pós-Graduação em Patologia Ambiental e Experimental da Universidade Paulista - UNIP, para obtenção do título de Mestre em Patologia Ambiental e Experimental.

FLAVIO RICARDO FERREIRA

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Orientadora: Prof[a] Dr[a]. Maria Martha Bernardi

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DEDICATÓRIA

Dedico nossa pesquisa a minha amada companheira, amiga e esposa Sinara G. C. Ferreira, sem ela e seu amor nada teria sido possível.

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RESUMO

Objetivos: Estudos prévios mostraram que as avermectinas, um grupo de antiparasitários, promovem efeitos sexualmente dimórficos em ratos. O objetivo deste trabalho foi investigar os efeitos sexualmente dimórficos da administração de ivermectina na idade juvenil em ratos expostos ou não ao estresse, em modelos animais de ansiedade, motores e exploratórios. **Métodos**: ratos Wistar machos e fêmeas com 29 e 44 dias de idade foram tratados com 0,2 ou 1,0 mg/kg de ivermectina. Aos 45 dias de idade metade destes ratos foram observados no campo aberto e labirinto em cruz elevada. A outra metade dos ratos foi submetida a 1 h de estresse pelo sistema *"Metro de Nova York"* e observada nos mesmos modelos comportamentais. Na sequência, o sangue foi coletado e os níveis plasmáticos de corticosterona avaliados. **Resultados**: Nos ratos não estressados do grupo controle e tratados com 0,2 mg/kg de ivermectina foi observado que fêmeas apresentaram maior atividade no campo aberto e no labirinto em cruz elevada que os machos, mas na dose de 1,0 mg/kg não foi observada diferença significativa entre os sexos nos dois comportamentos; somente após o tratamento com 1,0 mg/kg os níveis de corticosterona foram maiores em fêmeas do que em machos. A exposição ao estresse impediu a expressão do dimorfismo sexual em todos os grupos no labirinto em cruz elevado e nos níveis de corticosterona plasmática. **Conclusões**: a administração da maior dose de ivermectina no período juvenil prejudica o dimorfismo sexual no campo aberto e labirinto em cruz elevada. Atribuiu-se este dado ao aumento dos níveis de corticosterona nas fêmeas que levou a redução das respostas nos dois modelos comportamentais. O estresse modifica apenas o dimorfismo sexual no labirinto em cruz elevado, principalmente

por reduzir as respostas em fêmeas. Estes dados sugerem que fêmeas são mais sensíveis aos efeitos da ivermectina, particularmente em comportamentos ligados à ansiedade.

Palavras chaves: Avermectinas, estresse por contenção, campo aberto; labirinto em cruz elevada, corticosterona.

1. INTRODUÇÃO

Entende-se por dimorfismo sexual as características que diferem entre machos e fêmeas que envolvem aspectos físicos (tamanho pelagem e outras), comportamentais (agressividade, cortejo, exploração) e fisiológicas (hormônios, metabolismo e outras), sem levar em conta a morfologia dos órgãos sexuais. Estudo em animais de laboratório corroboram estas diferenças particularmente em estudos comportamentais. Por exemplo, ratas exploram mais um ambiente novo que machos(Hyde and Jerussi, 1983)(Quadagno et al., 1972) . Ratos apresentam respostas ligadas à ansiedade maiores que fêmeas no labirinto em cruz elevado (Palanza, 2001). Além disto, a resposta a drogas apresenta diferenças com relação ao sexo desde que é fato conhecido que as enzimas dos sistemas CYP são moduladas pelos hormônios gonadais de forma especifica. De fato, fêmeas biotransformam mais lentamente agentes químicos que machos(Florio, 2017).

As avermectinas e milbemicinas, muitas vezes também referidas como lactonas macrocíclicas, são os medicamentos antiparasitários mais vendidos no mundo. São amplamente utilizados na medicina veterinária para o tratamento de verminoses gastrointestinais e também para o controle de ectoparasitos, bem como na agricultura para o controle de infestações por pragas (Vercruysse et al., 2002), e na medicina humana para o tratamento de filariariose, e no tratamento e controle da oncocercose e da sarna(Õmura, 2008).

Uma série de estudos de nosso grupo de pesquisa mostrou que as avermectinas promovem prejuízos na esfera reprodutiva de animais de laboratório. No comportamento sexual de ratos machos a moxidectina reduziu o comportamento sexual, a ereção peniana e os níveis hipotalâmicos do ácido gama-aminobutírico (Rodrigues-Alves et al., 2008). A experiência sexual reverteu o prejuízo produzido

pela ivermectina no comportamento sexual de ratos machos (Bernardi et al., 2011), fato que não foi observado com a doramectina(Ferri et al., 2013). Em ratas, a ivermectina reduziu o comportamento sexual tanto em fêmeas no estro natural, como naquelas com estro induzido por hormônios (Moreira et al., 2014). Recentemente Moreira et al mostraram que a Ivermectina reduz o comportamento sexual e os níveis de testosterona em machos na idade adulta (artigo submetido).

Além disto, de Souza-Spinosa et al. (2002) evidenciaram efeitos ansiolíticos de ivermectina que foi atribuída aos seus efeitos no sistema Gabaérgico. Neste sentido, observou-se também que a doramectina, outra avermectina, também promove efeitos ansiolíticos e anticonvulsivantes (De Souza Spinosa et al., 2000). Todos esses estudos foram feitos em animais adultos. Sabendo-se que a ativação do comportamento sexual ocorre no período juvenil e que a Ivermectina é empregada em animais e crianças nessa fase da vida se torna importante investigar seus efeitos no dimorfismo sexual. Nesse trabalho escolheram-se modelos comportamentais que apresentam respostas dimórficas sexualmente claras como é o caso da atividade geral observada em campo aberto e das respostas comportamentais no labirinto em cruz elevado.

As doses empregadas (0,1mg/kg – 1,6mg/kg) estão dentro da faixa empregada para o tratamento de diferente doenças em ratos (Lankas and Gordon, 1989)

Além disso em estudos de nossos grupos verificamos que as doses de 0,2 e 1,0mg/kg de ivermectina produzem efeitos no âmbito reprodutivo. O período escolhido para o tratamento abrangeu o período juvenil dos animais e o intervalo entre os tratamentos foi escolhido como posologia para abranger o ciclo parasitário.

A interação com variáveis ambientais pode exacerbar a toxicidade às avermectinas, dentre os quais se destaca o estresse. Considerando nossos achados de prejuízos sexuais e a ampla utilização das avermectinas sem preocupações em contextos estressores, no presente projeto foram avaliados os efeitos do estresse no dimorfismo sexual de ratos tratados com a ivermectina no período juvenil. Os comportamentos avaliados foram a atividade geral em campo aberto e o labirinto em cruz elevada. Mediu-se também os níveis de corticosterona plasmática.

2. OBJETIVOS

O objetivo deste trabalho foi investigar os efeitos sexualmente dimórficos da administração de ivermectina na idade juvenil em ratos expostos ou não ao estresse, em modelos animais de ansiedade, motores e exploratórios.

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ABSTRACT

Objectives: Previous studies have shown that avermectins, a group of antiparasitics, promote sexually dimorphic effects in rats. The objective of this study was to investigate the sexually dimorphic effects of ivermectin administration at juvenile age in rats exposed or not to stress in experimental animal, motor and exploratory models. **Methods:** Male and female Wistar rats at 29 and 44 days of age were treated with 0.2 or 1.0 mg / kg of ivermectin. At 45 days old, half of these rats were observed in the open field and elevated plus maze. The other half of the rats were subjected to 1 h of stress by the "New York Metro" system and observed in the same behavioral models. Following, blood was collected and plasma corticosterone levels were assessed. **Results:** In non-stressed rats from the control group and treated with 0.2 mg / kg of ivermectin, females showed greater activity in the open field and in the elevated plus maze than males, but at a dose of 1.0 mg / kg no significant difference was observed between the sexes in the two behaviors; Only after treatment with 1.0 mg / kg corticosterone levels were higher in females than in males. Exposure to stress prevented the expression of sexual dimorphism in all groups in the elevated plus maze and plasma corticosterone levels. **Conclusions:** administration of the highest dose of ivermectin in the juvenile period impairs sexual dimorphism in the open field and elevated plus maze. This data was attributed to the increase in corticosterone levels in females, which led to the reduction of responses in both behavioral models. Stress modifies only the sexual dimorphism in the elevated plus maze, mainly by reducing the responses in females. These data suggest that females are more sensitive to the effects of ivermectin, particularly on anxiety-related behaviors.

Keywords: Avermectins, containment stress, open field; elevated plus maze, corticosterone.

Highlights

Male and female rats were treated with ivermectin (IVM) in juvenile period.

Ivermectin increased exploration, anxiety and corticosterone levels only in females.

Stress disrupt sexual dimorphism in anxiety but not in exploration after ivermecatin.

Stress influenced sexual dimorphism after ivermectin depending on the behavioral model.

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SUMÁRIO

1. Introduction

Sexual dimorphism is defined as the characteristics that differ between males and females that involve physical aspects (coat size and others), behavioral (aggression, courtship, exploration) and physiological (hormones, metabolism and others), without considering the morphology of the Sexual organs. Studies in laboratory animals corroborate these differences particularly in behavioral studies. For example, female rats explore more a new environment rather than males (Hyde and Jerussi, 1983; Quadagno et al., 1972). Male Rats present anxiety-related responses greater than females rats in the elevated plus maze (Palanza, 2001). In addition, the response to drugs reveals differences between sexes since it is known that gonadal hormones specifically modulate the enzymes of CYP systems. In fact, females biotransform chemical agents more slowly than males (Florio, 2017).

Avermectins and milbemycins, often also referred to as macrocyclic lactones, are the bestselling antiparasitics drugs in the world. They are widely used in veterinary medicine for the treatment of gastrointestinal verminoses and also for the control of ectoparasites as well as in agriculture for the control of pest infestations (Vercruysse et al., 2002). In human medicine it is used for the treatment of filariariosis and on the treatment and control of onchocerciasis and scabies (Õmura, 2008).

A number of studies from our research group have shown that avermectins promote damage in the reproductive sphere of laboratory animals. In the sexual behavior of male rats, moxidectin reduced sexual behavior, penile erection and hypothalamic levels of gamma-aminobutyric acid (Rodrigues-Alves et al., 2008). The sexual experience reversed ivermectin-induced injury in male rats (Bernardi et al., 2011), a fact that was not observed with doramectin (Ferri et al., 2013). In rats,

ivermectin reduced sexual behavior both in females in natural estrus and in females with estrogen-induced hormones (Moreira et al., 2014).

Recently Moreira (2014) showed that Ivermectin reduces sexual behavior and testosterone levels in males in adulthood . In addition, Souza-Spinosa et al. (2002) evidenced the anxiolytic effects of ivermectin that was attributed to its effects on the GABAergic system. In this sense, it has also been observed that doramectin, another avermectin, also promotes anxiolytic and anticonvulsive effects (De Souza Spinosa et al., 2000). All of these studies were done on adult animals. Knowing that the activation of sexual behavior occurs in the juvenile period and that ivermectin is used in animals and children at this stage of life it becomes important to investigate its effects on sexual dimorphism. In this study, behavioral models were chosen that present sexually clear dimorphic responses such as the general activity observed in the open field and the behavioral responses in the elevated plus maze.

The doses used (0,2 and 1,0mg/kg) are within the range used to treat different diseases in rats (0.1mg / kg - 1.6mg / kg) (Lankas and Gordon, 1989). In addition, in our group studies, we found that doses of 0.2 and 1.0 mg/kg ivermectin produced reproductive effects. The treatment period covered the juvenile period of the animals and the interval between treatments was chosen as posology to encompass the parasitic cycle.

Interaction with environmental variables may exacerbate toxicity to avermectins, among which stress is highlighted. Considering our findings of sexual impairment and the widespread use of anxiety-free avermectins in stressful settings, the present study evaluated the effects of stress on the sexual dimorphism of rats treated with ivermectin in the juvenile period. The evaluated behaviors were the

general activity in open field and the elevated plus maze. Plasma corticosterone levels were also measured.

The objective of this study was to investigate the sexually dimorphic effects of ivermectin administration at juvenile age in rats exposed to stress or not, in animal models of anxiety, motor and exploratory.

2. Material and methods

2.1. Animals

Male and female Wistar rats 21 days old (male- 88.11± 3.29 g, female- 76.67 ±3.27 g) at the beginning of experiments were provided by the animal facility of Department of Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil).

The animals were housed in polypropylene cages (40 × 50 × 20 cm) at a controlled temperature of (20 ± 21 °C) and humidity of (60 ± 5%) under a controlled light/dark schedule (12 h light/12 h dark), with lights on at 10:00 AM for at least 7 days before the beginning of experiments in the animal facilities of Paulista University - UNIP. Food (Nuvilab CR1, rodent diet, Quimtia Ind & Com Ltd, Curitiba, Paraná, Brazil) and filtered water were freely available throughout the study. All of the procedures were reviewed and approved by the Animal Care Committee CEUA – UNIP (permit nº 414/15) and were in accordance to the guidelines of NATIONAL RESEARCH COUNCIL- NRC(Committee, 2011).

2.2. Drugs

Ivermectin 1% (Ivomec injectable, Merial, Paulinia, São Paulo, Brazil) was dissolved in Tween 80 (1 drop/1 ml of 1% Ivomec) and administered subcutaneous at a dose of 0.2 or 1.0 mg/kg. Tween 80 was also administered in the same form as a

control solution (1 drop/1 ml of 0.9% NaCl). All of the solutions were prepared immediately before use and administered in a volume of 1 ml/kg body weight.

2.3. Treatments and groups

Forty-two male and forty-two female Wistar rats 29 days old were divided into six groups, two control male and female groups injected with the control solution (n=14/group); two experimental male and female groups injected with 0.2 mg/kg of ivermectin (n=14/group) and two experimental male and female groups injected with 1.0 mg/kg of ivermectin (n=14/group). At 44 days old these treatments were repeated and 24 h after treatments half of these rats were observed in the open field and the elevated plus maze. The other half of rats were submitted to 1 h of stress and also observed in the open field and the elevated plus maze. After the behavioral experiments the rats were rapidly decapitated and the blood collected from the trunk was maintained in temperature below -4º C until evaluation of the corticosterone levels.

2.4. Stress

The stress model of the “New York subway system” was described by Dhabhar and McEwen (1997) , whose laboratory is located in New York. It was named because it resembles the situation experienced by an individual boarding the subway during a time of great movement of users: restricted capacity to move the body and continuous shaking.

The apparatus to restrict movement consists of a laminated board (23.5 cm length) to which six polyvinyl chloride (PVC) pipes (7.2 cm diameter $\times$ 17.2 cm

length) are attached to restrict the movement of individuals. The pipes have front ventilation with small holes and at the back a hole for allowing passage of the tail. To induce the stress, the apparatus was placed on a mechanical shaker (Shaker Kline – Nova Ética, Model 108, Vargem Grande Paulista), set to 1 vibration/second. We exposed the rats to 1 hour of stress, during which the animals had no access water and food at 45 days old.

2.5. General activity in the open field

An open field (OF) was used to assess the effects of ivermectin on exploration and motility and constructed according to Broadhurst (Broadhurst, 1957). The OF apparatus was a white circular wooden arena with 1.20m of diameter. The floor of the arena was divided into three concentric circles that were divided into 19 straight segments, 48 cm above the floor. The apparatus was placed in a room with dim light (100 W). In the OF test, each animal was placed in the center of the arena and observed for 5 min. The animals in the control and experimental groups were alternately observed during the light phase of the light/dark cycle between 9:00 AM and 11:00 AM. The OF was cleaned with a 5% alcohol solution between sessions to remove any odors. We evaluated the total frequency of locomotion, the peripheral locomotion and duration of immobility. One unit of locomotion was defined as the animal entering one area of the arena floor with all four paws. Immobility was defined as the length of time (in seconds) during which the animal did not engage in any motor activity (i.e., the head, trunk, and limbs were still).

2.6. Elevated plus maze

Elevated plus maze (EPM) is an apparatus first conceived by the British psychologist Sheila Handley's group as a model to evaluate anxiety and it is one of

the most used for that purpose (Pellow et al., 1985). The EPM device used was made of wood and had two open arms (23.5 cm × 8 cm) and two enclosed arms of the same size with 20 cm high walls; the apparatus was elevated 80 cm above the ground, it was placed in a room with room lamp of 100 W. Basically, two strategies can be easily noticed: avoidance of the open arms while staying in the closed arm and escape from the open arm directly to the closed arm (Pellow et al., 1985). In the present study, the apparatus was used to assess anxiety, and the animals were assayed after being tested in the OF. The animals were allocated in the center of the maze, which was previously cleaned with 5% alcohol and observed during 3 min. Exploratory behavior was determined by the number of crosses in the center of the EPM. The time and entries in open arms and the risk assessment were employed to evaluate the anxiety-like behavior.

2.7. Corticosterone plasmatic levels

The blood, collected in conical tubes (15 ml) that contained 10% Ethylenediaminetetraacetic acid, was centrifuged, to plasma obtain. Plasma samples (1ml) from each animal were aliquoted in several Ependorf tubes for analyses (in duplicate) of corticosterone using commercial enzyme-linked immunosorbent assay kits according to the manufacturer's instructions. Corticosterone levels were determined using an Arbor Assays kit (catalog no. K014-H5, Ann Arbor, MI, USA). The results are expressed as ng/ml.

2.8. Experimental design

Forty-two male and forty-two female Wistar rats 29 days old were divided into six groups. The following groups were formed: control male and control female groups injected with the control solution (n=14/group); experimental male and female groups injected with 0.2 mg/kg of ivermectin (n=14/group); experimental male and female groups injected with 1.0 mg/kg of ivermectin (n=14/group). At 44 days old these treatments were repeated and 24 h after, half of these rats were observed in the OF and EPM; the other half of rats were submitted to 1 h of stress and also observed in the OF and EPM. In the sequence after the EPM, the rat trunk blood was collected to evaluate the corticosterone levels.

2.9. Statistical analysis

Homogeneity was verified using Bartlett's test. Normality was verified using the Kolmogorov-Smirnov test. Two-way analysis of variance (ANOVA) followed by Bonferroni's multiple-comparison test was used to compare the data. The results are expressed as the mean ± SEM. In all cases, the results were considered significant at $p < 0.05$.

3. Results

3.1. Effects of ivermectin treatment in male and female rats not exposed to stress

Fig.1 shows the general activity of male and female rats treated with 0.2 or 1.0 of ivermectin in the juvenile age observed in the OF at 45 days old.

Concerning the total locomotion (fig.1A) the two way ANOVA did not show interaction between sex and treatments ($F_{2, 36} = 0.82$, $p = 0.45$), the sex influenced the results ($F_{1, 36} = 23.3$, $p < 0.0001$) but not the treatments ($F_{2, 36} = 2.15$, $P = 0.13$). The Bonferroni test indicates an increased total locomotion in female of control and 0.2 mg/kg ivermectin relative to control and 0.2 mg/kg ivermectin groups of males, respectively. No differences were observed between male and female treated with 1.0 mg/kg of ivermectin.

In peripheral locomotion (fig.1B) the two way ANOVA did not show interaction between sex and treatments ($F_{2, 36} = 0.68$, $p = 0.52$), the sex influenced the results ($F_{1, 36} = 6.91$, $p < 0.01$) but not the treatments ($F_{2, 36} = 2.67$, $P = 0.08$). The Bonferroni test indicates an increased peripheral locomotion in female of control group relative to control males. No differences were observed between male and female treated with both ivermectin doses.

Relative to immobility (fig.1C) no interaction between sex and treatments ($F_{2, 36} = 2.03$, $p = 0.15$) was observed; the sex influenced the results ($F_{1, 36} = 8.42$, $p < 0.006$) but not the treatments ($F_{2, 36} = 1.45$, $P = 0.25$). The Bonferroni test indicates a decreased immobility in female of control group relative to control males. No differences were observed between male and female treated with both ivermectin doses.

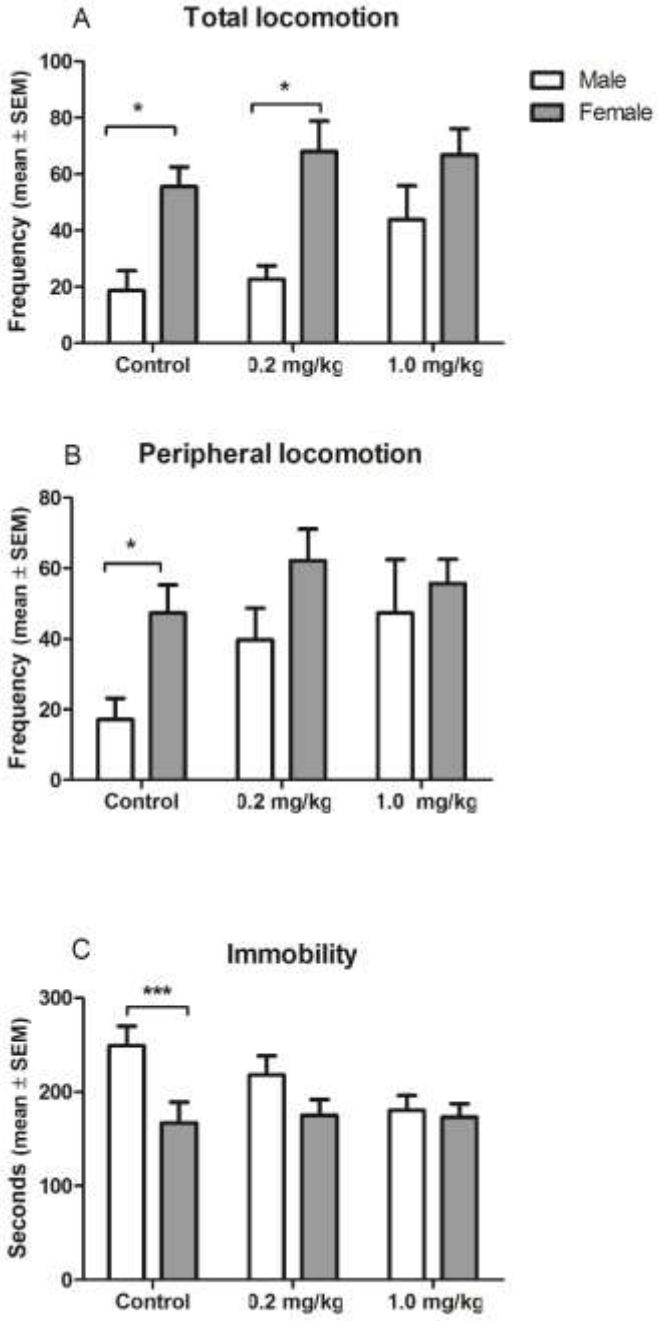

Fig.1. General Activity of male and female rats treated with 0.2 or 1.0 of ivermectin in the juvenile age observed in the open field at 45 days old. A- Total locomotion frequency; B-peripheral locomotion frequency; C- immobility duration (sec). Data are presented as means ± SEM. N= 7/group. Two way ANOVA followed by the Bonferroni test. *p< 0.05, ***p< 0.0001, relative to the male rat of the same treatment.

Fig.2 illustrates the effects of ivermectin treatment in male and female rats observed in the EPM at 45 days of old.

Relative to time in open arms (fig.2A), no interaction between sex and treatments ($F_{2, 36} = 1.08$. $p = 0.35$) and between treatments ($F_{2, 36} = 0.17$, $p = 0.84$) were observed but sex influenced the results ($F_{1, 36} = 4.51$, $p = 0.04$). Female rats of control and 0.2 mg/kg of ivermectin remained more time in the open arms than males of the same treatments. No differences were detected between male and female rats treated with 1.0 mg/kg of ivermectin.

Concerning the number of entries in the open arms (fig.2C) and the number of crosses (fig.2D), no differences were observed between sex and treatments without interactions between factors (entries in open arms- interaction –$F_{2, 36} = 2.85$, $p = 0.07$; treatments- $F_{2, 36} = 1.10$, $p = 0.32$; sex-$F_{1, 36} = 0.01$, $p = 0.93$; number of crosses- . interaction –$F_{2,36} = 1.15$, $p = 0.33$; treatments- $F_{2,36} = 0.91$, $p = 0.42$; sex-$F_{1,36} = 3.3$, $p = 0.07$).

Relative to risk assessment (fig.2 D) differences were observed between treatments ($F_{2, 36} = 4.76$, $p = 0.03$) but not relative to sex ($F_{1, 36} = 0.03$, $p = 0.97$) without interaction between factors ($F_{2, 36} = 2.33$, $p = 0.11$). Female rats of control group showed increased risk assessment than male of control group. No differences were observed between male and female treated with both ivermectin doses.

Fig.3 illustrates the effects of ivermectin treatment on plasmatic corticosterone levels of male and female rats at 45 days of old. No interaction was observed between sex and treatments ($F_{2/30} = 0.55$, $p = 0.5$); the sex ($F_{1, 30} = 7.35$, $p = 0.01$); the treatments ($F_{1/30} = 3.87$, $p = 0.03$) influenced the results. The post hoc test indicates an increased corticosterone plasmatic level in female rats treated with 1.0 of ivermectin relative to male treated with the same dose. No differences were

observed between male and female of control and treated with 0.2 mg/kg of ivermectin dose groups.

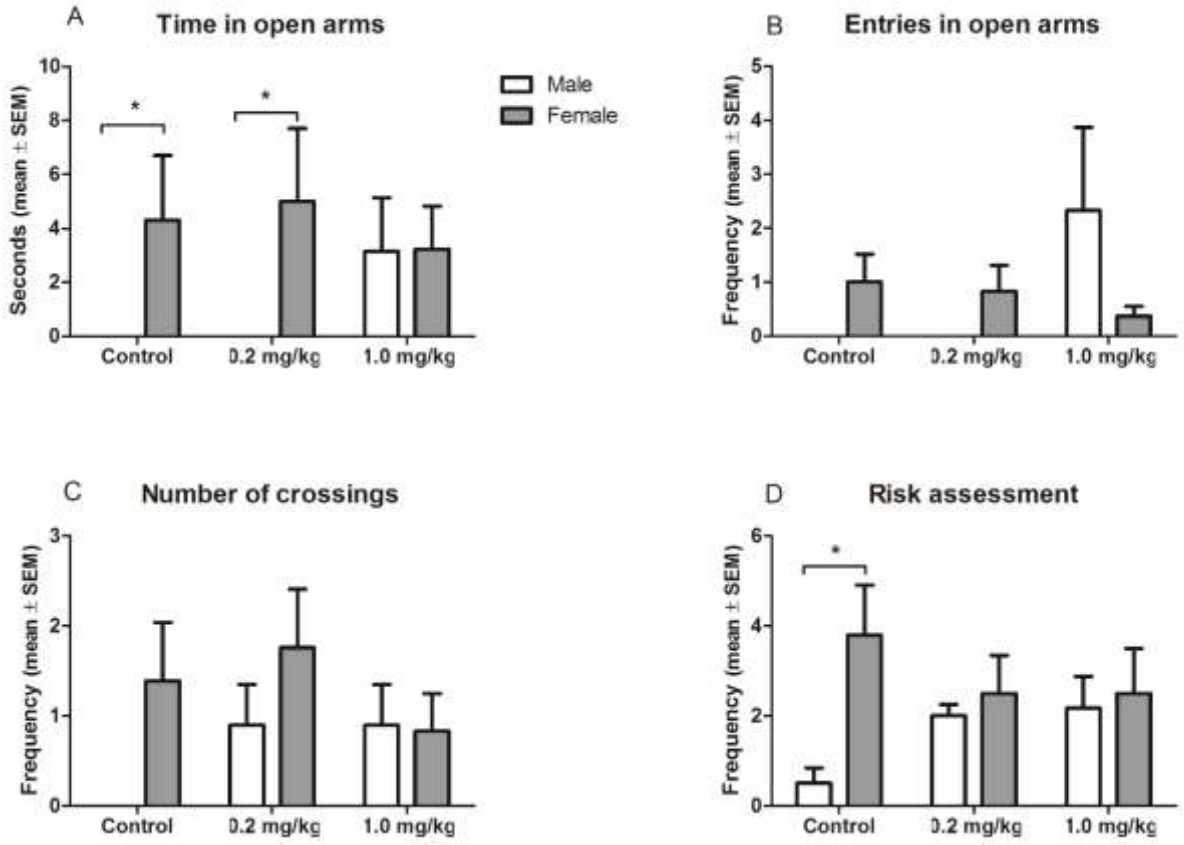

Fig.2. Elevated plus maze behavior of male and female rats treated with 0.2 or 1.0 of ivermectin in the juvenile age observed at 45 days old. A- Time in open arms; B- number of entries in the open arms; C- number of crossings the center of the elevated plus maze; D- number of risk assessment. Data are presented as means ± SEM. N= 7/group. Two way ANOVA followed by the Bonferroni test. *p< 0.05 relative to the male rat of the same treatment.

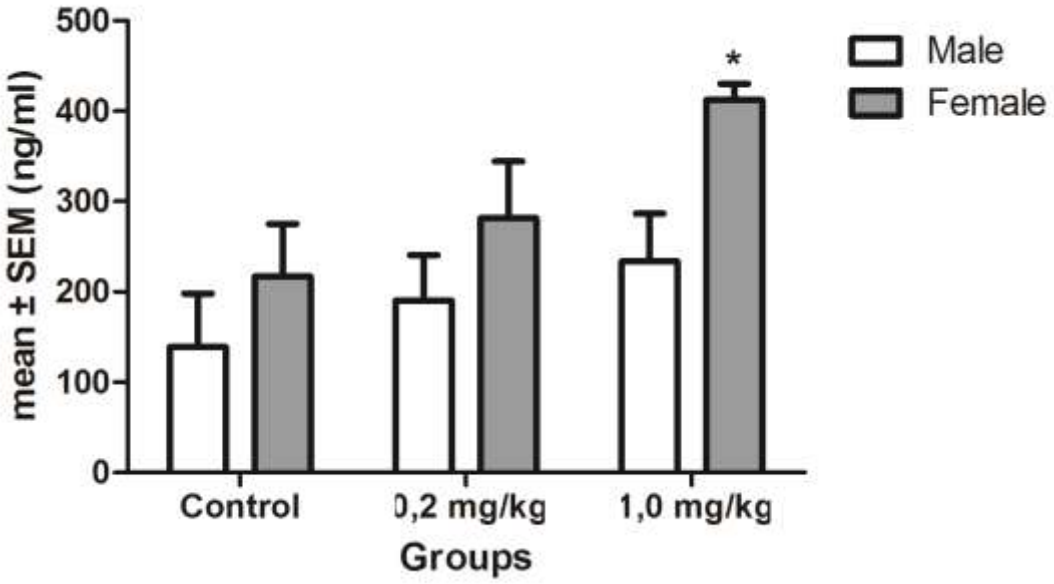

Fig. 3. Corticosterone plasmatic levels of male and female rats treated with 0.2 or 1.0 of ivermectin in the juvenile age evaluated at 45 days old. Data are presented as means ± SEM. N= 7/group. Two way ANOVA followed by the Bonferroni test. *p< 0.05 relative to the male rat of the same treatment.

3.2. Effects of ivermectin treatment in male and female rats exposed to stress

Fig.4 shows the general activity of male and female rats exposed to stress treated with 0.2 or 1.0 of ivermectin in the juvenile age observed in the OF at 45 days old.

Concerning the total locomotion (fig.4A) the two way ANOVA did not show interaction between sex and treatments ($F_{2, 36} = 0.60$, $p = 0.56$), the sex influenced the results ($F_{1, 36} = 5.22$, $p = 0.03$) but not the treatments ($F_{2, 36} = 0.76$, $P = 0.48$). The Bonferroni test indicates an increased total locomotion in female of control and 0.2 mg/kg ivermectin relative to control and 0.2 mg/kg ivermectin groups of males, respectively. No differences were observed between male and female treated with 1.0 mg/kg of ivermectin.

In peripheral locomotion (fig.4B) the two way ANOVA did not show interaction between sex and treatments ($F_{2, 36} = 1.01$, $p = 0.38$); the sex influenced the results ($F_{1, 36} = 7.11$, $p = 0.01$) but not the treatments ($F_{2, 36} = 1.92$, $P = 0.16$). The Bonferroni test indicates an increased peripheral locomotion in female of control and 0.2 mg/kg ivermectin relative to control and 0.2 mg/kg ivermectin groups of males, respectively. No differences were observed between male and female treated with 1.0 mg/kg of ivermectin.

Relative to immobility (fig.4C) no interaction between sex and treatments ($F_{2, 36} = 0.52$, $p = 0.60$) was observed; the sex ($F_{1, 36} = 0.78$ $p = 0.89$) and treatments ($F_{2, 36} = 0.11$, $p = 0.38$) did not influenced the results.

Fig.5 illustrates the effects of ivermectin treatment in male and female rats exposed to stress and observed in the EPM at 45 days old.

Relative to time in open arms (fig.5A), an interaction was observed between sex and treatments ($F_{2, 36} = 4.22$, $p = 0.02$), but not between treatments ($F_{2, 36} = 0.64$, $p = 0.43$) and sex ($F_{1, 36} = 0.08$, $p = 0.92$

Concerning the number of entries in the open arms (fig.5B) no differences were observed between sex and treatments without interactions between factors (interaction – $F_{2, 36} = 2.32$, $p = 0.14$; treatments- $F_{2, 36} = 0.29$, $p = 0.75$; sex-$F_{1, 36} = 0.29$, $p = 0.75$).

Relative to the number of crossing (fig.5C), an interaction between factors were observed ($F_{2, 36} = 4.21$, $p = 0.02$) but not between treatments ($F_{2, 36} = 2.25$, $p = 0.14$) and sex-($F_{1, 36} = 0.96$, $p = 0.39$). The Bonferroni test indicates that female of control groups showed increased crossings relative to control males.

Relative to risk assessment (fig.5 D) no interaction between sex and treatments (F 2, 36 = 0.50, p =0.61, treatments (F2, 36 = 1.22, p = 0.28) and sex (F1, 36 = 0.31, p = 0.73) were observed.

Fig.6 illustrates the effects of ivermectin treatment on plasmatic corticosterone levels of male and female rats exposed to stress at 45 days old. No interaction between sex and treatments (F2/38 = 0.55, p = 0.5), sex (F1, 38 = 0.15, p = 0.70) and treatments (F2/38 = 2.23, p = 0.12) were observed.

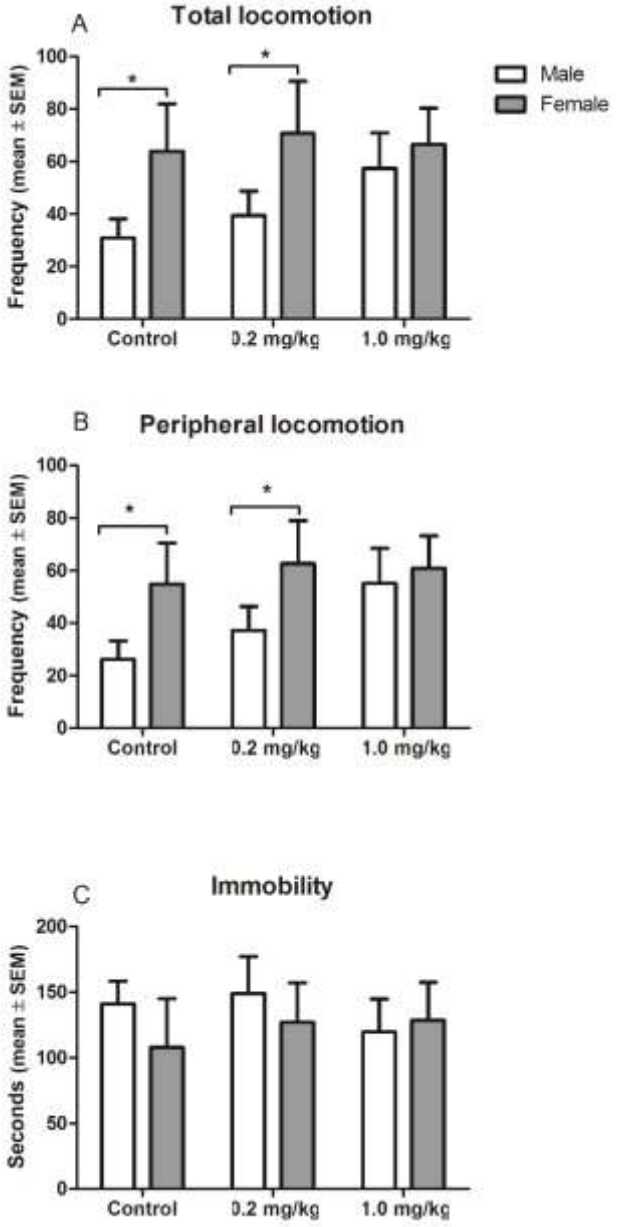

Fig.4. General Activity of male and female rats submitted to stress, treated with 0.2 or 1.0 of ivermectin in the juvenile age observed in the open field at 45 days old. A- Total locomotion frequency; B-peripheral locomotion frequency; C- immobility duration (sec). Data are presented as means ± SEM. N= 7/group. Two way ANOVA followed by the Bonferroni test. *p< 0.05 relative to the male rat of the same treatment.

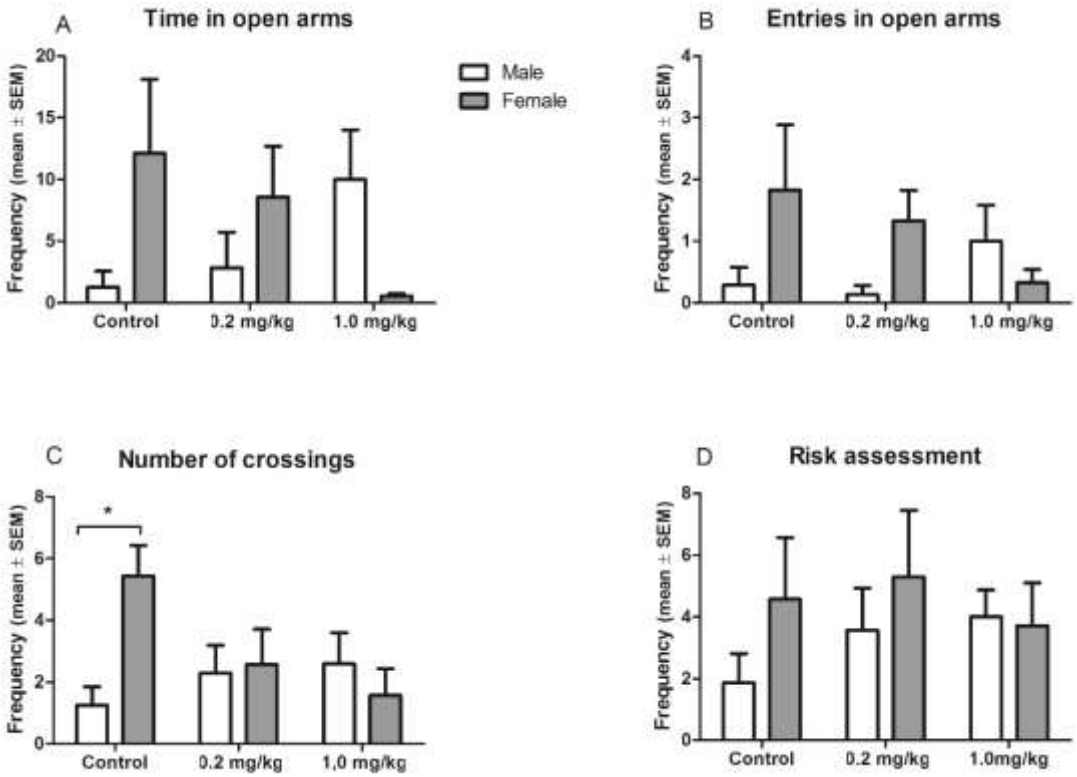

Fig.5. Elevated plus maze behavior of male and female rats exposed to stress and treated with 0.2 or 1.0 of ivermectin in the juvenile age observed at 45 days old. A- Time in open arms; B-number of entries in the open arms; C- number of crossings the center of the elevated plus maze; D- number of risk assessment. Data are presented as means ± SEM. N= 7/group. Two way ANOVA followed by the Bonferroni test. *p< 0.05 relative to the male rat of the same treatment.

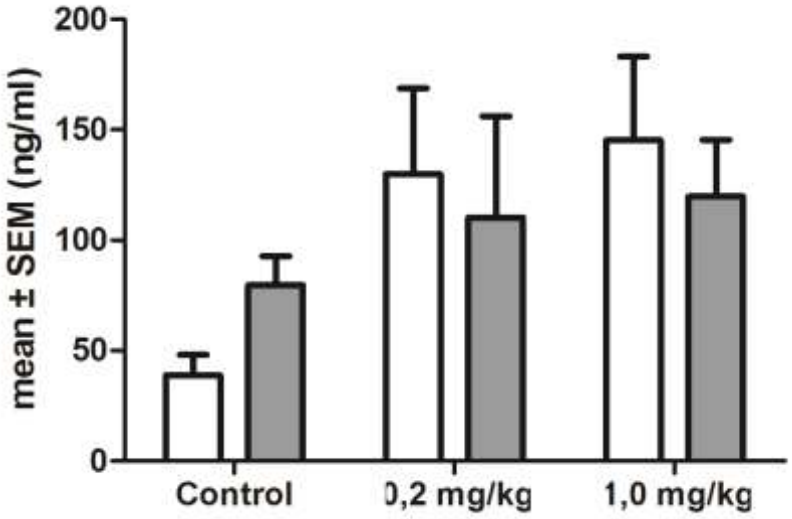

Fig.6. Corticosterone plasmatic levels of male and female rats submitted to stress and treated with 0.2 or 1.0 of ivermectin in the juvenile age evaluated at 45 days old. Data are presented as means ± SEM. N= 7/group. Two way ANOVA.

4. Discussion

Several studies revealed that sexual differences exist in exploration, fear and anxiety-related behavior, observed in different behavioral test situations. Male rats generally exhibit more fear-related behaviors than females, as reported in the open field test (Blizard et al., 1975), elevated plus maze (Kokras et al., 2012), light dark test (An et al., 2011) and forced swimming test (Kokras et al., 2012).

Our results corroborated with these data because a sexual dimorphic behavior of male and female of control groups was observed at 45 days old where female rats explore more the open field than males. In fact, the total and peripheral locomotion is higher in female than in males of control groups with reduced immobility in females.

In the open-field besides rat's tendency to explore the new environments, as measured by ambulation (Walsh and Cummins, 1976), they are more active and remain longer in the periphery of the apparatus in comparison to the central areas,

interpreted as being determined by thigmotaxis (Valle, 1970). Rats show aversion to open areas by a natural defensive response in which rats remain close to vertical surfaces, thereby shielding themselves from predators(Crawford and Masterson, 1982).

Despite peripheral locomotion could take as an anxiety-related parameter, this study evaluated only its frequency and not the time in the peripheral area at the open field. Time in open field peripheral area reflects more accurately the rat's aversion to open spaces because it shows how much the animal prefers to be by the open field walls. Therefore, we suggest that the increased peripheral locomotion could be considered as a vertical exploratory activity, which occurs preferentially near the open field wall. These differences were attenuated in rats treated with 0.2 mg/kg of ivermectin (no differences in immobility) and disappear after 1.0 mg/kg.

De Souza Spinosa et al.(2002) reported that in the open field, 1.0 mg/kg ivermectin decreased locomotion frequency of male rats at 15 and 60 min of observation, rearing behavior showed a biphasic effect at 15 and 30 min and duration of immobility was increased. We did not show differences between male rats of control and 1.0 mg/kg in the open field parameters. These contradictory data could be attributed to the time of observations and the route of ivermectin employed. We observed the effects of ivermectin 24 h after administration because at this time a decreased testosterone levels in male rats was observed, suggesting a hormonal effect related to sexual aspects. Ivermectin reduced the sexual behavior in female rats in natural estrus (physiological condition) in normal cycling rat or induced in intact female rats with estradiol valerate (Moreira et al., 2014) 24 h after treatment, reinforcing the hormonal effects of this drug. Concerning the route of ivermectin administration was employed because this is the clinical route of administration.

In rats, sex differences in behavior measured in the open field and elevated plus-maze appear to be age-related, occurring arising around day 60 of age (Imhof et al., 1993; Johnston and File, 1991;Masur et al., 1980). However, treatment at 29 and 44 days of age with ivermectin reduced the sexual dimorphism, mainly in the high dose, by decrease the exploration of female rats in the open field and the time in open arms. These ages are previously reported as the critical periods of the brain sexual activation (Wilson and Davies, 2007). Thus, it is possible that ivermectin exposure during these periods, by affecting hormonal milieu of male and female rats, affected the sexual behavioral responses particularly in females.

Another hypothesis to explain the reduced sexual dimorphism here observed is the involvement of the GABAergic system on the regulation of the hypothalamic – pituitary-adrenal axis (HPA) or −gonadal (HPG) axis (Cullinan et al., 2008). Activation or blockade of the GABA-A receptors during early life induces brain and behavioral abnormalities in adulthood, and may alter physiological phenotypes in a sex-dependent manner in mice (Salari and Amani, 2017). Thus, it is possible that ivermectin acting as agonists at GABA-A receptors and stimulating GABA release (Dawson et al., 2000) could affect the hormone regulating sexual activation during puberty.

In the elevated plus maze the sexual dimorphism occurs. Female rats of control and 0.2 mg/kg of ivermectin groups show a reduced aversion for the open arms in the elevated plus maze compared to male rats. Moreover, females of control group tended to perform more number of risk assessment indicating a higher overall level of activity in this test. These findings are in agreement with previous report which suggested that female Wistar rats were less anxious than male Wistar rats based on their performance in an elevated plus maze test (Imhof et al., 1993).

According this statement female of control group had increased risk assessment. Measures of risk assessment (primarily, stretched-attend postures) have proved extremely valuable in identifying anxiolytic-like actions of drugs not detected by other parameters in this test (Fernandes et al., 1999).

After treatment with 1.0 mg/kg dose, this sexual dimorphism disappears in both, open field and elevated plus maze. This lack of dimorphism could be explained by the increased female plasmatic corticosterone levels relative to male. It is known that activation of the hypothalamus-pituitary-adrenal (HPA) axis is the prototypical response to stress in all vertebrates and reduces exploration in the open field (Prut and Belzung, 2003) and the time in open arms in elevated plus maze (Rodgers and Dalvi, 1997).

In this respect, the ivermectin recommended doses for the treatment of various diseases in rats range from 0.1 to 1.6 mg / kg and the 1.0 mg / kg dose would not be expected to induce toxicity in females (Soll, 1989). However, female rats are probably more sensitive to the effects of drugs because of their metabolic characteristics. It is a known that female hormones (estrogens and progesterone) reduce the activity of CYP enzymes that metabolize drugs (Kamataki et al., 1983). Thus, we propose that the absence of sexual dimorphism observed in both, the open field and the elevated plus maze was a consequence of ivermectin toxicity in females.

Presently, stress exposure had few effects on sexual dimorphism of male and female rats observed in the open field. In fact, control and 0.2 mg/kg of ivermectin groups showed a clear dimorphic behavior in total and peripheral locomotion while no differences were observed between male and female rats treated with 1.0 mg/kg. Only the sexual dimorphism in immobility behavior of control

male and female disappear. Thus, our model of stress did not affect the sexual dimorphism effects of ivermectin effects in the open field test.

In the elevated plus maze, a great variability was observed in female responses relative to males but, no differences were observed in all parameters between sexes, except in the number of crosses in the control group. Female control rats crossed more the center of the elevated plus maze than male rats meaning increased plus maze arms exploration.

Concerning the corticosterone levels of stressed rats, no differences were observed between sexes of all groups. Thus, in the elevated plus maze and corticosterone levels, stress reduced the sexual dimorphism. In the open field, stress did not modify the effects of ivermectin as here described in rats no stressed.

Additional comparisons between the corticosterone levels of control groups exposed or not to stress, did not show significant differences between these groups. This is an unexpected result.

It was found that the adult-like ACTH stress response, from the pituitary, develops during the later stages of adolescence (PND46 to PND59), while the corticosterone response from the adrenal gland changes earlier between the PND30 to PND40 (Foilb et al., 2011). These results indicate that shifts in hormonal stress responses occur throughout the juvenile age and that each gland along this neuroendocrine axis displays a unique developmental trajectory (Romeo et al., 2016). In our study, ivermectin was administered at 29 days old followed by the second dose at 44 days old. The restraint stress was applied in beginning of puberty of rats, when the ACTH stress response from the pituitary was developed. In addition, gender is one major variable related to differential vulnerability to stress. Thus, it is possible that treatment during early the juvenile period desensitized rats to

restraint stress. However, this statement did not explain the lack of increases of corticosterone in control rats.

Another explanation about the lack of effects on corticosterone levels in juvenile controls and treated rats could be explained by the time of plasma collection. Maximal concentrations of corticosterone were reached between 30 and 60 min after restraint stress in adult Sprague-Dawley rats and we collected the blood between 80-90 minutes after stress. Thus it is possible that at this moment the levels of corticosterone were decreased and no differences could be observed. This time effects of restraint stress was previously reported by Martí et al. (2001).

Another question that remains to explain in this last experiment: why the sexual dimorphism after ivermectin treatment was not affected by the exposure to stress in the open field and in the elevated plus maze sexual dimorphic response disappear? Despite speculative, because in the open field the levels of corticosterone were low at the moment of observation, no effects of were observed; only the ivermectin effect remained. In the elevated plus maze, previous stress could be induced anxiety explaining the reduced sexual dimorphism.

5. Conclusions

A lack of sexual dimorphism was observed in the open field and elevated plus maze tests after the ivermectin high dose administered during the juvenile period. We attribute this effect by a disruption in female rats to the high levels of corticosterone. The 0.2 mg/kg did not affect the sexual dimorphism in both behavioral tests. Restraint stress was unable to alter the effects on sexual dimorphism of both ivermectin doses in the open field behavior but in the elevated plus maze the sexual behavioral dimorphism was impaired in controls and experimental groups. Moreover, no differences on sexual dimorphism were observed in the corticosterone plasmatic

levels of male and female of all groups treated or not with ivermectin exposed to stress. These data suggest that female rats were more sensible to ivermectin, particularly related to anxiety behavior and not to a new environment.

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