UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(FARMACOLOGIA)

ALINE LIMA DE BARROS

"INFLUÊNCIA DA EXPOSIÇÃO PERINATAL AO INSETICIDA FIPRONIL: REPERCUSSÃO TARDIA EM PARÂMETROS REPRODUTIVOS MASCULINOS E FEMININOS, EM RATOS"

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Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Ciências Biológicas (Farmacologia).

Orientadora: Profa. Dra. Arielle Cristina Arena

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Dedicatória

Dedico este trabalho a minha família,

em especial ao meu marido Rodrigo e ao meu avô

Sebastião Candido de Lima (in memorian)

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Epígrafe

"A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê."

(Arthur Schopenhauer)

Sumário

Resumo

Fipronil é um inseticida amplamente utilizado na agricultura, medicina veterinária e saúde pública, e recentemente tem sido listado como um provável desregulador endócrino. Estudos demonstram que este composto pode afetar a função da tireoide, parâmetros reprodutivos e o sistema nervoso central. No entanto, a maioria dos estudos realizados com o fipronil avaliou sua toxicidade aguda, e poucos se referem aos seus possíveis efeitos reprodutivos, especialmente se a exposição ocorrer durante a gestação e lactação. Este estudo objetivou avaliar os efeitos resultantes da exposição perinatal ao fipronil e suas possíveis repercussões tardias sobre parâmetros reprodutivos masculinos e femininos, em ratos. Ratas prenhes foram expostas a três doses de fipronil (0,03; 0,3 ou 3 mg/kg) do dia gestacional 15 até o dia pós-natal 7, por gavage. Durante o tratamento, foram monitorados o consumo de água e ração e o peso corporal das ratas expostas. Após o desmame, foram coletados o sangue das mães para análises bioquímicas e órgãos para registro do peso. A prole masculina foi avaliada através dos seguintes parâmetros: peso corporal, distância anogenital e idades da separação prepucial e da descida testicular. Na vida adulta, peso de órgãos reprodutores, contagens, morfologia e motilidade espermática, histo-morfometria do testículo e epidídimo, contagem de células de Sertoli, comportamento e preferência sexual, fertilidade e expressão de receptores de andrógenos no testículo foram investigados. Nos filhotes do sexo feminino foram avaliados a distância anogenital, idade de instalação de puberdade, ciclo estral, dosagem hormonal, fertilidade, comportamento sexual e histologia de útero e ovário. O tratamento não alterou os parâmetros bioquímicos e os pesos da maioria dos órgãos analisados das mães expostas ao fipronil, com exceção do peso da hipófise, que apresentou uma redução no grupo exposto a menor dose. Nos machos, não houve diferenças no peso corporal, distância anogenital, idades da separação prepucial e descida testicular dos filhotes quando comparados ao grupo controle. A exposição perinatal ao fipronil também não alterou os pesos de órgãos ou a produção e

morfologia espermática dos machos na vida adulta. Além disso, não houve efeitos adversos relacionados ao tratamento no número de células de Sertoli por túbulo seminífero e histomorfometria testicular e epididimal, bem como nos padrões de expressão de receptor de andrógeno no testículo. Da mesma forma, não foram observadas alterações nos outros parâmetros analisados. No entanto, os animais expostos ao fipronil apresentaram uma alteração na motilidade espermática, com diminuição de espermatozoides móveis com trajeto progressivo e aumento de espermatozoides imóveis. Nos filhotes do sexo feminino, a exposição perinatal ao fipronil provocou atraso na abertura vaginal e primeiro estro na dose de 3 mg/kg e aumento na duração dos ciclos estrais no grupo de 0,3 mg/kg. Entretanto, os outros parâmetros avaliados não foram afetados. Concluiu-se que o fipronil, nestas condições experimentais, foi capaz de perturbar o desenvolvimento reprodutivo de fêmeas, sem afetar os parâmetros reprodutivos tardios. Em machos, os resultados demonstraram que a exposição perinatal ao fipronil tem efeitos a longo prazo sobre parâmetros espermáticos, e que o epidídimo pode ser um órgão-alvo. Novos estudos devem ser realizados para identificar os mecanismos tóxicos do fipronil sobre a função reprodutiva de machos e fêmeas.

Palavras-chave: Desreguladores endócrinos, fipronil, reprodução, ratos.

Abstract

Fipronil is an insecticide widely used in agriculture, veterinary medicine and public health, and recently has been listed as a possible endocrine disruptor. Studies demonstrate that this compound can affect the thyroid function, reproductive parameters and central nervous system. However, most of studies performed with fipronil evaluated the acute toxicity, and a few of them refer to its reproductive effects, especially if the exposure occurs during gestation and lactation. This study aimed to evaluate the effects resulting from perinatal exposure to fipronil and its possible late repercussion on male and female reproductive parameters, in rats. Pregnant rats received three doses of fipronil (0.03; 0.3 or 3 mg/kg) from gestational day 15 to postnatal day 7, by gavage. During the treatment, water and food consumptions as well as the body weight of females exposed were investigated. After weaning, we collected the blood of mothers for biochemical analysis and the organs to verify the weight. The male offspring was evaluated for body weight, anogenital distance and ages of preputial separation and testicular descent. At adult life, weight of reproductive organs, sperm counts, morphology and motility, testicular and epididymal histo-morphometry, sertoli cells count, sexual behavior and preference, fertility and patterns of expression of androgen receptor in the testis were evaluated. On female offspring was investigated anogenital distance, puberty onset, estrous cycle, hormonal levels, fertility, sexual behavior and histology of uterus and ovaries. The treatment did not alter the biochemical parameters and the weight of most of analyzed organs of mothers exposed to fipronil, however the weight of pituitary gland presented a reduction in the group exposed to a lower dose. On males, the perinatal exposure to fipronil did not affect the body weight, anogenital distance and puberty onset. Similarly, at adult life, fipronil did not alter the organ weights or the sperm production and morphology. Furthermore, there were no adverse effects related to treatment in the Sertoli cells number per seminiferous tubule, testicular and epididymal histo-morphometry and histopathology, as well as in the patterns

of expression of androgen receptor in the testis. In the same way, no alterations were observed in the other parameters analyzed. However, the animals exposed to fipronil presented an alteration on sperm motility, with a decrease in the motile spermand an increase in the immobile sperm. On females, fipronil exposure provoked a delay in the vaginal opening and first estrous at dose of 3 mg/kg and an increase in the duration of estrous cycle in the group that received 0.3 mg/kg. However, the other parameters investigated were not compromised. It can be concluded that the fipronil, in these experimental conditions, was able to disturb the female reproductive development, without affecting the late reproductive parameters. On males, the results demonstrated that the perinatal exposure to fipronil has long-term effects on sperm parameters, and the epididymis can be a target organ. Further studies should be conducted to identify the toxic mechanisms of fipronil on male and female reproductive function.

Keywords: Endocrine disruptors, fipronil, reproduction, rats.

Introdução

Nas últimas décadas tem sido observado um comprometimento reprodutivo, tanto masculino quanto feminino, e estudos demonstram que estas alterações, podem ser induzidas por substâncias que são capazes de mimetizar a ação de hormônios naturais do organismo, denominadas de desreguladores endócrinos (Diamanti-Kandarakis et al., 2009). Tal fato têm levado ao surgimento de várias investigações, cujo enfoque são os efeitos dos desreguladores endócrinos no sistema neuroendócrino e o impacto da exposição a estas substâncias durante o período perinatal sobre o desenvolvimento e vida adulta reprodutiva do indivíduo exposto (Dickerson et al., 2011). Durante este período, o feto é bastante sensível à ação hormonal e uma alteração na diferenciação sexual do cérebro, que é dependente da ação hormonal, pode comprometer a reprodução (Schwarz & McCarthy, 2008). Assim, nos próximos itens serão descritos a importância do processo de diferenciação sexual do cérebro para a função reprodutiva e como estes desreguladores endócrinos podem interferir neste evento.

1. Diferenciação Sexual Hipotalâmica

No início do desenvolvimento, tanto as gônadas como o cérebro são órgãos bipotentes, os quais são diferenciados em macho ou fêmea por uma variedade de sinais hormonais (Schwarz & McCarthy, 2008). Em machos (XY), o gene SRY localizado no cromossomo Y conduz o desenvolvimento dos testículos (Koopman et at., 1990), enquanto em fêmeas (XX), na ausência do gene SRY, a gônada bipotente torna-se um ovário (Sinclair et al., 1990). Durante os últimos dias de gestação em roedores, e, logo no segundo trimestre em primatas, os testículos desenvolvem e começam a produzir quantidades significativas de testosterona (Weisz & Ward, 1980; Rhoda et al., 1984), induzindo neste momento, a formação dos caracteres sexuais secundárias, incluindo o epidídimo, canal deferente, e órgãos genitais masculinos (Jost, 1947). Além da diferenciação gonadal, a

testosterona é convertida a estradiol, o qual induz mudanças em várias regiões do hipotálamo, o que irá determinar a diferenciação sexual do cérebro e garantir que o sexo gonadal seja o mesmo que o sexo do cérebro (Schwarz & McCarthy, 2008).

O hipotálamo, localizado na base do cérebro, é um importante centro integrador de informações que garante a homeostase do organismo, coordenação de funções viscerais e iniciação de comportamentos, como a reprodução. Este centro serve como uma grande interface entre o sistema nervoso central e o resto do corpo (Gore, 2010). Em mamíferos, o hipotálamo antes do período crítico de diferenciação, está organizado intrinsecamente do tipo feminino, determinando na vida adulta, o comportamento sexual típico de fêmea e um padrão de secreção cíclico de gonadotrofinas. Nos machos, o hipotálamo precisa ser masculinizado para que ocorra o comportamento sexual tipicamente masculino e apareça o padrão tônico de secreção de gonadotrofinas (MacLusky & Naftolin, 1981). Deste modo, dois processos distintos determinam a diferenciação do sistema nervoso central (SNC) em machos: a defeminização e a masculinização. A defeminização é a perda da capacidade de um adulto em responder aos efeitos do estradiol e progesterona, ou seja, ausência de comportamento feminino (lordose), e a masculinização, é a capacidade de apresentar comportamento de monta (McEwen, 1978).

O desenvolvimento e diferenciação do cérebro envolvem uma série de eventos que começam durante a gestação e continua nas primeiras horas de vida pós-natal em roedores (Negri-Cesi et al., 2001). Os hormônios esteroidais são fundamentais para a diferenciação sexual do cérebro durante o desenvolvimento inicial. Como já foi dito anteriormente, em ratos machos, o gene SRY (cromossomo Y) conduz o desenvolvimento dos testículos, os quais começam a produzir quantidades significativas de testosterona durante os últimos dias de gestação (DG) 18-19 (Weisz & Ward, 1980; Ward & Weisz, 1984), e novamente durante as primeiras horas após o parto (Corbier et al., 1978; Konkle & McCarthy, 2011),

provocando masculinização e defiminização em regiões do hipotálamo. Estas ações garantem o comportamento copulatório típico do sexo masculino e os padrões de secreção de gonadotrofinas (Figura 1).

A masculinização do hipotálamo é dependente de testosterona, porém, esse processo é decorrente da sua metabolização, por ação da enzima citocromo P450 aromatase, originando o estrógeno no SNC (Rhoda et al., 1984; Erskine et al., 1988). Após a aromatização, o estrógeno liga-se a dois subtipos de receptores de estrógeno, o α e β, sendo que, o receptor de estrógeno α está primeiramente envolvido na masculinização, enquanto o receptor de estrógeno β tem uma maior função na defeminização do comportamento sexual (Kudwa et al., 2006). Neste período, também conhecido como o período crítico de diferenciação sexual hipotalâmica, o cérebro é particularmente sensível à exposição hormonal, e pode ser definido como uma janela restrita de desenvolvimento, caracterizada por uma sensibilidade aumentada a um estímulo ambiental (Schwarz & McCarthy, 2008).

Além da testosterona ou estradiol, outras diferenças no cérebro entre os sexos, podem ser atribuídas à alfa-fetoproteína (AFP), uma glicoproteína plasmática produzida em grandes quantidades durante a vida fetal pelas células da endoderme do saco vitelino, pelos hepatócitos e, em menor quantidade, pelo trato gastrointestinal. Esta proteína é capaz de se ligar com elevada afinidade ao estrógeno em ratos e camundongos, protegendo o cérebro fetal feminino da exposição a estrógenos circulantes maternos. A síntese de AFP diminui acentuadamente logo após o nascimento e apenas quantidades traços são detectadas em adultos (Bakker et al., 2006; Bakker & Baum, 2008).

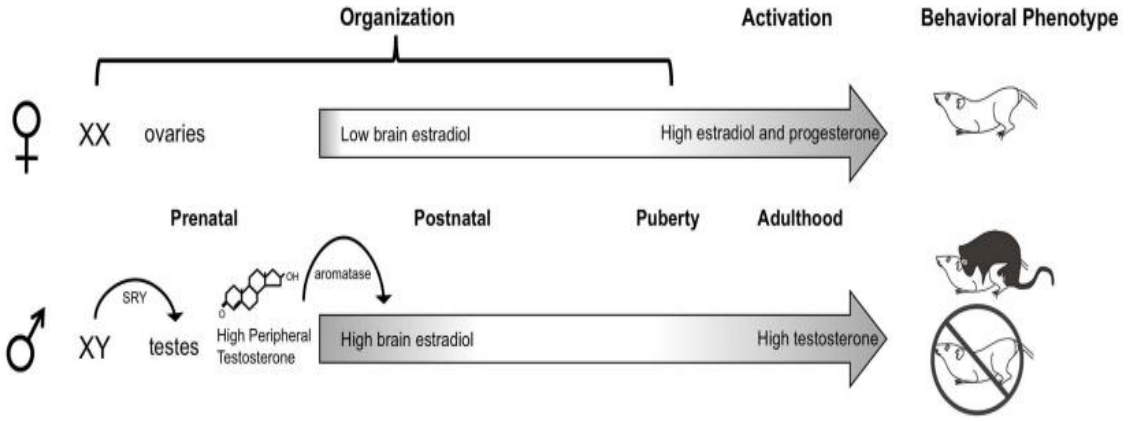

Figura 1- Organização neonatal do cérebro e comportamento (Lenz et al., 2012).

Neste sentido, existem inúmeras diferenças sexuais estruturais e funcionais em todo o cérebro de mamíferos, em particular no hipotálamo e em estruturas circundantes (Simerly, 2002; De Vries, 2004; Bonthuis et al., 2010), que garantem o dimorfismo sexual fisiológico e comportamental. Estas regiões podem diferir quanto ao tamanho ou volume (por exemplo, o núcleo dimórfico sexual da área pré-óptica – SDN-POA que é maior no sexo masculino e o núcleo periventricular anteroventral - AVPV que é maior no sexo feminino) e até mesmo quanto à expressão de receptores de estrógenos, que estão intrinsecamente relacionados com a masculinização e defeminização destas regiões (Schwarz & McCarthy, 2008). Machos recém-nascidos apresentam 2 a 3 vezes mais receptores de estrógenos no hipotálamo do que as fêmeas, fato esperado devido a hipótese de aromatização, que baseia-se na conversão da testosterona em estradiol pela ação da enzima aromatase (McCarthy, 2008).

O estradiol induz mudanças permanentes em diferentes regiões do cérebro durante o período crítico de desenvolvimento, através de mecanismos distintos (Figura 2). Este hormônio induz diferenças sexuais volumétricas nos núcleos hipotalâmicos através da morte celular induzida, diferença sexual na morfologia celular (aumentando as espinhas dendríticas dos neurônios), induz diferença sexual da área pré-optica através da

prostaglandina E_2, alteração da função neurotransmissora no hipotálamo mediobasal e também mudanças celulares no núcleo arqueado através da neurotransmissão GABAérgica (aumentando a complexidade dos astrócitos nesta região em machos). Além destes eventos relacionados à masculinização do hipotálamo, o estradiol também está envolvido na defeminização hipotalâmica em machos (Schwarz & McCarthy, 2008). Desta forma, substâncias capazes de suprimir, atrasar ou retardar o pico de testosterona neonatal ou ainda alterar a expressão de receptores e enzimas envolvidas na diferenciação sexual hipotalâmica, podem alterar o processo de masculinização e defeminização do hipotálamo (Gore, 2010).

Neste contexto, a exposição a substâncias químicas sintéticas e de origem natural hormonalmente ativas, denominadas desreguladores endócrinos e até mesmo fatores estressantes, são capazes de interferir na diferenciação sexual do cérebro (Gerecke et al., 2012).

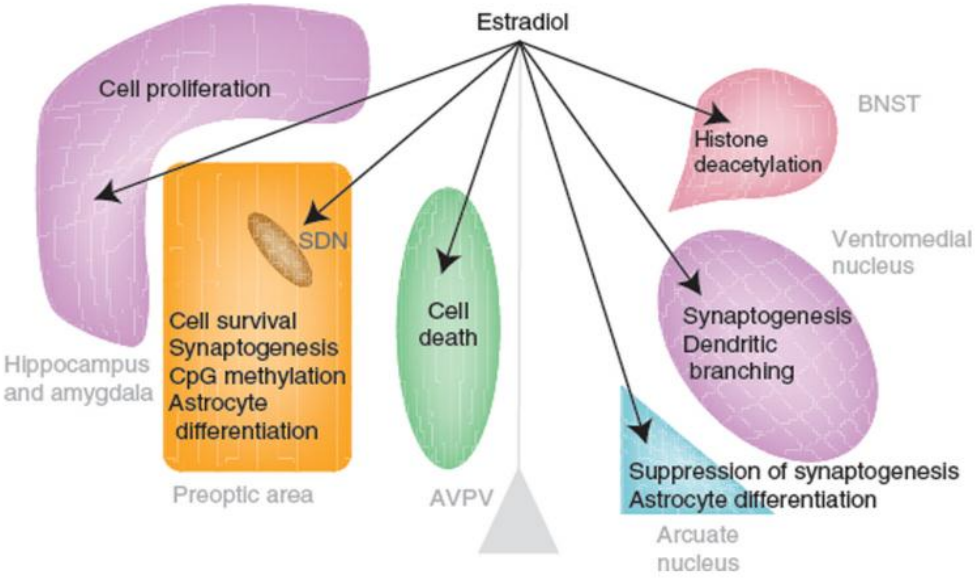

Figura 2 - Vários mecanismos de diferenciação induzido pelo estradiol (McCarthy & Arnold, 2011).

2. Desreguladores Endócrinos

Ao longo dos últimos 50 anos, dados epidemiológicos revelaram um aumento significativo na incidência e prevalência de uma série de efeitos adversos na saúde humana, tais como, alterações no processo de desenvolvimento e crescimento, disfunções neurológicas e do sistema imune, redução da fertilidade e do início de algumas doenças, como diabetes, obesidade, e alguns tipos de câncer (mama, ovário, testículo e próstata) (WHO, 2012). Uma possível explicação para o aumento na incidência dessas doenças consiste em uma crescente exposição dos trabalhadores e da população em geral a contaminantes, que podem exercer efeitos adversos devido à sua ação como desreguladores endócrinos (DEs) (Iavicoli et al., 2013).

O termo DEs é utilizado para classificar substâncias capazes de mimetizar a ação de hormônios naturais, que em geral, podem atuar através da ligação a receptores hormonais, interação com enzimas que sintetizam ou metabolizam hormônios, interferir na liberação hipotalâmica-hipofisária de hormônios e/ou alterar a transdução de sinais (Kavlock et al., 1996; US EPA, 2007). Entretanto, existem substâncias que apresentam um efeito mais amplo e são denominadas de desreguladores neuroendócrinos (DNs), que são definidos como substâncias capazes de atuar como agonista/antagonistas ou alterando a síntese e/ou o metabolismo dos neuropeptídeos, neurotransmissores ou neuro-hormônios, ou alterando diversos processos fisiológicos, comportamentais ou hormonais que afetam a capacidade de um animal de se reproduzir, desenvolver e crescer ou lidar com estresse ou desafios (Waye & Trudeau, 2011). Como estes compostos estão presentes tanto na natureza quanto nos alimentos, a exposição humana aos DEs e DNs pode ser por via oral, inalatória ou cutânea (Yoon et al., 2014).

O FDA (U.S Food and Drug Administration) lista mais de 1000 substâncias químicas atualmente no comércio, com propriedades confirmadas ou suspeitas de

desregulação endócrina (FDA, 2010). Este grupo de moléculas identificadas como DEs é altamente heterogêneo e inclui produtos químicos sintéticos utilizados como solventes industriais, lubrificantes e seus subprodutos (bifenilos policlorados - PCB), bifenilos polibromados (PBB), dioxinas, plásticos (bisfenol A - BPA), plastificantes (ftalatos), pesticidas (metoxicloro, clorpirifós, diclorodifeniltricloroetano - DDT), fungicidas (vinclozolin) e agentes farmacêuticos (dietilestilbestrol - DES) (Diamanti-Kandarakis et al., 2009). Além de produtos sintéticos, alguns produtos químicos naturais produzidos por plantas e fungos também são DEs, os fitoestrógenos, como a genisteína, coumestrol ou isoflavonas presentes na soja, cuja exposição pode ocorrer em altas doses, através de alimentos ou suplementos (Safe, 1995). Neste sentido, um estudo relatou que concentrações urinárias do fitoestrógeno genisteína e daidzeína foram cerca de 500 vezes maior em bebês alimentados com formulações contendo soja em comparação com aqueles alimentados com leite de vaca (Cao et al., 2009). Desta forma, o potencial para a perturbação do sistema endócrino por fitoestrógenos precisa ser considerado.

Um grande desafio na investigação de substâncias suspeitas de DEs consiste na dificuldade de sua identificação, já que essas substâncias muitas vezes não apresentam semelhanças estruturais entre si, o que dificulta prever se um composto pode ou não exercer ações de desregulação no sistema endócrino. No entanto, alguns DEs, como as dioxinas, PCBs, PBB muitas vezes contêm um grupo halogênio substituído de cloro e bromo. Muitos ainda apresentam uma fração fenólica, que pode imitar os hormônios esteroides naturais, possibilitando desta forma a interação dos DEs com receptores esteroidais, como agonistas ou antagonistas deste receptor (Figura 3) (Diamanti-Kandarakis et al., 2009).

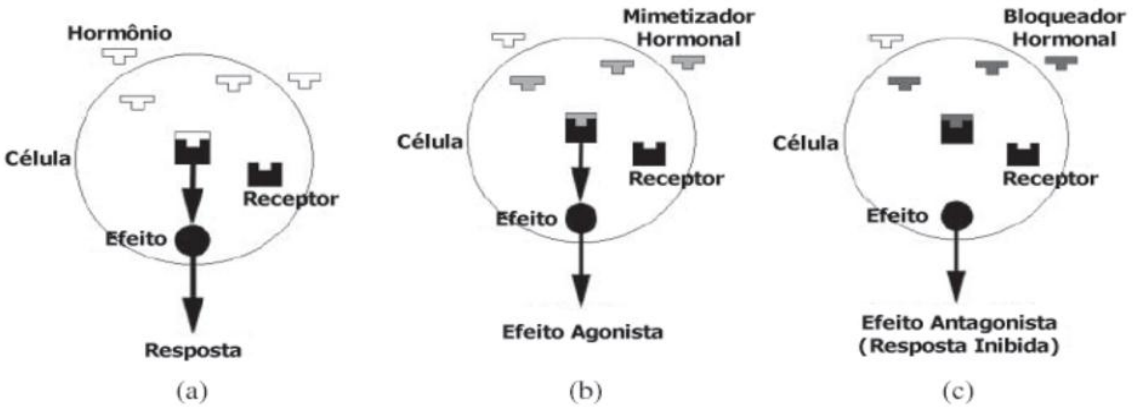

Figura 3 - Disfunções endócrinas: a) resposta natural; b) efeito agonista; c) efeito antagonista (Ghiselli & Jardim, 2007).

Na década de 1990, cientistas propuseram a "hipótese de baixa dose", que postulava que DEs teriam efeitos em baixas dosagens, especialmente na reprodução e desenvolvimento. Desde então, pesquisas em todo mundo se concentraram em abordar esta hipótese, e alguns cientistas acreditam que esta pesquisa avançou de tal forma que este conceito não deve ser mais considerado uma "hipótese" (Vandenberg, 2014). Durante décadas, os hormônios têm sido conhecidos por produzir alterações marcantes na morfologia do tecido, fisiologia e comportamento em doses extremamente baixas. Nos últimos anos, fortes evidências sugerem que os DEs, da mesma forma que os hormônios endógenos, podem atuar em doses baixas (Diamanti-Kandarakis et al., 2009; Zoeller et al., 2012). Neste sentido, estudos cada vez mais tentam mimetizar as concentrações reais a que o homem está exposto, como as concentrações encontradas nos alimentos, produtos de higiene pessoal, detergentes domésticos, embalagens de alimentos, dentre outras fontes (Vandenberg, 2014).

Estudos em animais e humanos evidenciam que os DEs afetam a reprodução masculina e feminina, a função da tireoide, e o controle do balanço energético. Estas substâncias também podem aumentar o risco de câncer de mama ou de próstata, bem como

o risco de desenvolvimento de síndrome metabólica (Diamanti-Kandarakis et al., 2009). Como o organismo em desenvolvimento é altamente dependente de hormônios sexuais esteroidais e da tireoide para sua maturação, o feto e a criança são muito sensíveis a qualquer alteração do seu ambiente hormonal. Desta forma, uma atenção maior deve ser tomada em relação à exposição a estes compostos DEs nestes períodos considerados críticos de desenvolvimento, já que mesmo a exposição sendo no início da vida, a maioria dos efeitos relatados em estudos só foi observada tardiamente, na vida adulta (Fudvoye, 2014).

3. Desreguladores Endócrinos e Diferenciação Sexual Hipotalâmica

A exposição a DEs durante períodos críticos de desenvolvimento, particularmente durante a gestação e infância, estão constantemente ligados a prejuízo na homeostase, processos endócrinos e neurobiológicos na vida adulta (Dickerson & Gore, 2007). Em meio a preocupações de que as exposições crônicas a baixas doses de DEs poderia contribuir para um declínio na fertilidade em seres humanos (Diamanti-Kandarakis et al., 2009), um recente interesse se voltou para elucidar os mecanismos pelo qual a exposição a essas substâncias durante o período crítico poderia afetar o sistema neuroendócrino e reprodutivo (Dickerson et al., 2011).

Como já foi descrito anteriormente, a diferenciação sexual do cérebro é altamente dependente de hormônios esteroides, sendo desta forma susceptível a ação de DEs durante o período crítico de desenvolvimento. Os mecanismos de diferenciação sexual do cérebro são complexos, e tentar entender a desregulação endócrina neste processo é bastante difícil. Entretanto, já foi constatado que administrações pós-natais a filhotes de rato (durante os dois primeiros dias de vida) com bisfenol A, ou genisteína (fitoestrógeno), não afetou o volume total do núcleo dimorfico sexual do hipotálamo (SDN), no entanto, um aumento do

número de células imunorreativas para a proteína de ligação ao cálcio, calbindina (proteína utilizada para delimitar as bordas da SDN), nesta região do cérebro foi observado, demostrando uma hipermasculinização (Patisaul et al., 2007). Neste mesmo estudo, a exposição à genisteína levou a um aumento no volume do núcleo periventricular anteroventral (AVPV), o qual é uma área do cérebro responsável pelo controle neuroendócrino da ovulação em fêmeas e potencialmente envolvido no controle neuroendócrino da liberação do Hormônio liberador de gonadotrofina (GnRH), em ambos os sexos (Simerly, 2002). Yamamoto et al. (2005) verificaram um aumento do SDN-POA em fêmeas cujas mães foram expostas a 1,5 µg/kg/dia de dietilestilbestrol (composto não esteroide sintético com forte atividade estrogênica). Efeitos adversos no comportamento sexual também foram observados em vários estudos após exposição a DEs no período de diferenciação sexual do cérebro, como é o caso da exposição aos PCBs (Chung et al., 2001; Steinberg et al., 2007) ou fitoestrógeno (Patisaul et al., 2004). Tais substâncias, quando administradas a ratos em desenvolvimento, causaram alterações adversas no comportamento reprodutivo em fêmeas adultas.

O processo de diferenciação do cérebro pode ser afetado também por substâncias que não são consideradas DEs, como é o caso da indometacina. Este fármaco é um anti-inflamatório não esteroidal (AINE), que atua inibindo a enzima cicloxigenase (COX), interferindo desta forma na síntese de prostaglandinas (PG) (Summ & Evers, 2013). O estradiol induz a um aumento de duas vezes em COX-1 e COX-2 e cerca de sete vezes em prostaglandina E_2 (PGE_2), na área pré-óptica durante a masculinização do cérebro. Já foi constatado que ratas recém-nascidas tratadas com PGE_2 exibiram comportamento sexual masculino quando foram expostas a testosterona na fase adulta (Amateau & McCarthy, 2004). Por outro lado, machos tratados durante o período neonatal com indometacina, não apresentaram comportamento sexual masculino na vida adulta, indicando que eles foram

completamente defeminizados, mas não masculinizados (Todd et al., 2005). Assim, as mudanças induzidas pelo estradiol nos neurônios na POA e morfologia dos astrócitos via PGE_2 são necessárias para a completa masculinização do cérebro durante o período crítico (Schwarz & McCarthy, 2008).

Além de compostos químicos, situações estressantes, como imobilização, resfriamento e aquecimento durante o último terço de gestação, podem induzir a mudanças no desenvolvimento e no comportamento sexual em ratos machos adultos (Ward, 1972; Herrenkohl, 1986; Rhees et al., 1999). Dessa forma, aumento no volume de AVPV e diminuição do SDN-POA em machos foram verificados por Rhees et al. (1999), enquanto Gerecke et al. (2012) observaram alteração na morfologia dos neurônios na área pré-optica medial (MPOA), que persistiram até a vida adulta, tanto em ratos machos quanto em fêmeas após estresse pré-natal. O estresse neste período crítico também foi capaz de afetar o comportamento sexual masculino em ratos, diminuindo o número de ejaculações e atraso nas montas e intromissões (Gerardin et al., 2005), diminuição do comportamento copulatório (Wang et al., 2006) e alteração no padrão de preferência sexual em camundongos (Meek et al., 2006).

Sendo assim, vários fatores podem interferir no processo de diferenciação sexual do cérebro, e a exposição a determinados contaminantes, como inseticidas, herbicidas, fungicidas, plastificantes, entre outros, devem receber destaque, já que muitos deles foram identificados com atividades antiandrogênica e/ou estrogênica, atuando assim como desreguladores endócrinos (Fudvoye et al., 2014). Neste trabalho, a substância escolhida para investigação foi o fipronil, um inseticida amplamente utilizado, e que recentemente tem sido listado como um provável desregulador endócrino.

4. Fipronil

O uso indiscriminado de praguicidas provoca uma série de danos ambientais. A contaminação de áreas próximas a plantações agrícolas pode causar o desequilíbrio dos ecossistemas locais, trazendo uma série de problemas aos habitantes dessas regiões (Peres & Moreira, 2005). A exposição a estas substâncias pode causar não só intoxicações, mas efeitos teratogênicos, carcinogênicos, distúrbios hormonais, alterações na fertilidade e efeitos genotóxicos (Dahlgren et al., 2004; Pelaez et al., 2004; Menegaux et al., 2006).

A cultura da cana-de-açúcar é a segunda que mais utiliza agrotóxicos no país. Dentre os praguicidas utilizados neste cultivo, destaca-se o inseticida fipronil (5-amino-[(2,6-dicloro)-4-trifluormetil-fenil]-4-trifluormetil-sulfinil- 1 H-pirazol-3-carbonitrila) (Figura 4), comercialmente encontrado no Brasil como Blitz®, Klap® e Regent® para uso agrícola e Frontline®, Fiprolex®, Topline® e Termidor® para uso veterinário. Fipronil é um inseticida de segunda geração da classe fenilpirazol com classificação toxicológica de classe II (altamente tóxico), que foi introduzido no mercado em 1993, com ampla utilização na agricultura para o controle das pragas do solo e folha (Narahashi et al., 2010), sendo utilizado nas culturas de algodão, arroz, batata, cana-de-açúcar, cevada, feijão, milho e soja (ANVISA, 2005), na medicina veterinária, contra pulgas e carrapatos e na saúde pública, para combater vetores de doenças (Le Faouder et al., 2007).

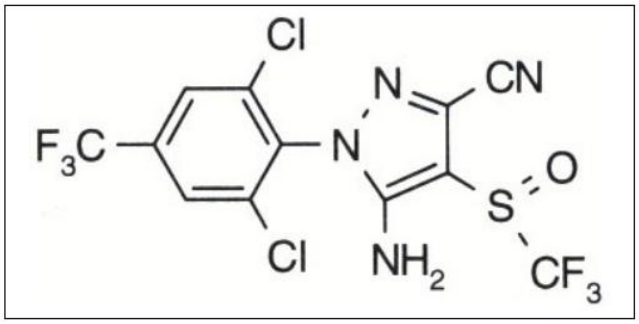

Figura 4- Fórmula estrutural do fipronil (ANVISA, 2005).

O fipronil apresenta baixa solubilidade em água e sua degradação neste meio é lenta. É um composto fotodegradável, altamente tóxico para peixes e invertebrados aquáticos e moderadamente tóxico para ratos e camundongos (Hainzl & Casida, 1996;

U.S. EPA, 2001; Tang et al., 2004; Zhao et al., 2005). Segundo a Agência Nacional de Vigilância Sanitária (ANVISA, 2005), a ingestão diária aceitável (IDA) para humanos de fipronil é de 0,0002 mg/kg de peso corpóreo, DL_{50} (Dose Letal 50) oral para ratos de 41 mg/kg (Narahashi et al., 2010) e NOAEL (no observed adverse effects level) via oral aguda para ratos é de 2,5 mg/kg (Jackson et al., 2009).

Além disso, o fipronil é rapidamente metabolizado em fipronil sulfona em várias espécies de mamíferos, tais como rato, homem, gado e cão (Mohamed et al., 2004). Esta principal via de metabolização é mediada por enzimas hepáticas do citocromo P450, e, este inseticida, como muitos outros xenobióticos, pode levar a um aumento na atividade e na expressão dessa enzima (Das et al., 2006; Leghait et al., 2009).

O fipronil quando presente no solo ou na água é degradado através de redução, hidrólise, fotólise e oxidação, formando produtos como fipronil sulfeto, fipronil amida, fipronil dessulfinilo e fipronil sulfona (Ying & Kookana, 2002; Caboni et al., 2003), sendo o fipronil sulfona o principal produto de degradação. Lao et al. (2010), detectaram fipronil sulfona em 100% das 18 amostras de sedimento, enquanto que o fipronil estava presente em apenas quatro amostras. Em outro estudo, foi constatado que o fipronil sulfona foi o produto de transformação mais abundante em amostras de peixes (Baird et al., 2013), além de ser encontrado no leite de vaca (Le Faouder et al., 2007), mostrando que este praguicida está presente na dieta humana. É importante ressaltar, que os produtos de degradação derivados do fipronil podem exercer efeitos mais tóxicos que o próprio fipronil, como é o caso do fipronil sulfona, que é extremamente estável no ambiente (Hamon et al., 1996; USEPA, 1996) e apresenta elevada toxicidade para insetos, peixes, aves e mamíferos (USEPA, 1996).

Após absorção, o fipronil é amplamente distribuído no organismo, sendo encontrado em maior quantidade no tecido adiposo e nas adrenais, e em menor quantidade

no intestino, fígado, rim, tireoide, pulmão, coração, músculo, testículo e sangue após 72 horas de administração. Resíduos detectáveis de fipronil também foram encontrados no cérebro, fato já esperado, em decorrência do seu mecanismo de ação no SNC (Cravedi et al., 2013). O tempo de meia vida do fipronil sulfona também é maior em relação ao fipronil. Mohamed et al. (2004) observaram que após administração de uma única dose de 4 mg/kg de fipronil a ratos, por via oral, foi encontrada uma meia vida de eliminação de 8,5 horas, enquanto que a do fipronil sulfona foi de 208 horas. Esta lenta eliminação do fipronil e seus metabólitos foram atribuídos à distribuição no tecido adiposo e pelo grau elevado de recirculação hepática.

Quanto ao mecanismo de ação, em insetos, esse praguicida atua especificamente no sistema nervoso central, como um bloqueador não competitivo dos canais de cloreto associados ao ácido gama-aminobutírico (GABA) (Zhao et al., 2005; Janssen et al., 2007; Narahashi et al., 2010), impedindo o influxo de íons cloreto, desregulando o potencial de membrana, o que induz a morte do inseto devido a hiperexcitação (Caboni et al., 2003; Ikeda et al., 2004; Narahashi et al., 2007). O fipronil liga-se especificamente ao receptor $GABA_A$ (Ratra et al., 2001), sendo demonstrado experimentalmente que esta ligação reduz o tempo médio de abertura do canal de cloreto (o canal abre mais rápido) e aumenta o tempo médio de fechamento do canal (fazendo permanecer mais tempo aberto), o que, consequentemente, diminui a frequência de abertura do canal (Ikeda et al., 2004).

Como o fipronil apresenta elevada afinidade pelos receptores GABA dos insetos, baixa toxicidade é esperada para mamíferos (Mohamed et al., 2004; Zhao et al., 2004). Desta forma, a Agência de Proteção Ambiental dos Estados Unidos (EPA) designou o fipronil como uma das alternativas de substituição dos organofosforados no controle de cupins e formigas. No entanto, preocupações sobre os efeitos adversos deste composto

sobre o homem foram levantadas devido à sua ampla utilização comercial e doméstica (Jennings et al., 2002; Tingle et al., 2003).

4.1 Toxicidade do fipronil

Vários efeitos adversos do fipronil já foram relatados. De Oliveira et al. (2012) observaram que esse inseticida pode ser genotóxico e mutagênico em doses elevadas (50 mg/kg) em camundongos, e recentemente um estudo aponta tais efeitos em linfócitos de sangue humano periférico, nas doses de 0,7 e 0,3 μg/mL (Çelik et al., 2014). Terçariol & Godinho (2011) verificaram que, após exposição cutânea ao fipronil (70, 140 e 280 mg/kg), os animais apresentaram efeitos centrais de comportamento, relacionados principalmente à emotividade, medo e à atividade exploratória. No fígado, animais expostos ao fipronil apresentam alterações ultraestruturais significativas em células hepáticas, com desorganização celular, esteatose hepática, morte celular e aumento das células de Kupffer, sugerindo um aumento da atividade fagocítica do fígado nos animais expostos (Ferreira et al., 2012).

Além desses efeitos, o fipronil tem sido listado como um possível desregulador endócrino. Assim, tem sido demonstrada sua atuação como um desregulador de hormônios da tireoide em ratos (Abend et al., 1991; Williams, 1995; WHO/FAO, 1997; Hurley, 1998; Hood et al., 1999). Hurley et al. (1998) verificaram que o fipronil é capaz de induzir câncer de tireoide em ratos, devido provavelmente a sua capacidade de aumentar o metabolismo dos hormônios tireoidianos e a excreção hepática. Essa alteração parece ser mediada pelo aumento na taxa de eliminação de T_4 (tiroxina), devido à maior atividade de enzimas hepáticas (Leghait et al., 2008).

Além de efeitos na tireoide, foi demonstrado que o fipronil, administrado topicamente em dose única a ratas, levou a uma alteração no funcionamento do sistema

endócrino, já que provocou distúrbios nos níveis de estrógeno e progesterona, o que acarretou efeitos adversos na reprodução das fêmeas expostas (Ohi et al., 2004). Um dos possíveis mecanismos de ação para estes efeitos do fipronil é a interferência com a síntese de esteroides, mais precisamente com a enzima citocromo P450 aromatase, que catalisa a conversão de andrógenos em estrógenos (Simpson & Davis, 2001). Alterações no comportamento materno e desenvolvimento de alguns reflexos foram observados por Udo et al. (2014) após exposição pré-natal ao fipronil a baixas doses (0,1, 1,0, ou 10,0 mg/kg/dia). Os autores sugerem que esta alteração esta relacionada a uma desregulação endócrina ou interferência do sistema GABAérgico. Recentemente, um estudo *in vitro* (ensaio de gene repórter para ERα utilizando células CHO-K1) mostrou que o principal metabólito do fipronil, o fipronil sulfona, apresenta atividade antiestrogênica (Lu et al., 2014), além de possuir atividade antiandrogênica (Aït-Aïssa et al., 2010). Essas informações demonstram a necessidade de mais estudos sobre este inseticida.

Justificativa

A exposição a substâncias presentes no ambiente suspeitas de desregulação endócrina pode comprometer várias funções reprodutivas importantes, especialmente se a exposição ocorrer durante o período gestacional e/ou lactacional. Tais alterações podem interferir com o padrão de desenvolvimento dos filhotes, induzindo mudanças permanentes no controle neuroendócrino da reprodução, que muitas vezes, não são detectadas após o nascimento, mas somente mais tarde, na vida adulta reprodutiva. A maioria dos estudos realizados com o inseticida fipronil avaliou sua toxicidade aguda, e poucos se referem aos seus possíveis efeitos reprodutivos, especialmente se a exposição ocorrer durante a gestação. Dessa forma, considerando os efeitos adversos já relatados do fipronil, tais como sua ação como um desregular endócrino, torna-se de extrema importância avaliar seus efeitos após exposição durante períodos críticos da diferenciação sexual hipotalâmica.

Objetivos

Objetivo Geral

Avaliar os possíveis efeitos resultantes da exposição perinatal ao fipronil e suas repercussões tardias sobre parâmetros reprodutivos em ratos machos e fêmeas.

Objetivos Específicos

- Avaliar sinais de toxicidade materna durante a exposição ao fipronil;
- Avaliar o desenvolvimento sexual dos descendentes machos, através da determinação do peso corporal, distância anogenital e idades de separação prepucial e de descida testicular;
- Avaliar o desenvolvimento sexual da prole feminina, através da determinação do peso corporal, distância anogenital, abertura vaginal e primeiro estro;
- Investigar se a exposição ao fipronil pode alterar, na vida adulta, parâmetros reprodutivos nos machos como: pesos de órgãos reprodutores, contagem espermática, morfologia e motilidade espermática, dosagem hormonal, análise histopatológica do testículo, epidídimo, contagem de células de Sertoli, comportamento e preferência sexual, fertilidade, e expressão de receptores de andrógenos no testículo;
- Investigar se a exposição ao fipronil pode alterar, na vida adulta, parâmetros reprodutivos em fêmeas como; ciclo estral, pesos de órgãos reprodutores, fertilidade, níveis hormonais, comportamento sexual e análise histopatológica de útero e ovário.

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Capítulo 1

O presente trabalho deu origem ao manuscrito intitulado ***"Perinatal exposure to insecticide fipronil affects sperm motility in male rats."*** que será submetido à revista Toxicology, ISSN: 0300-483X. Fator de Impacto: 3,621.

Perinatal exposure to insecticide fipronil affects sperm motility in male rats.

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Abstract

Fipronil is an insecticide widely used in agriculture, veterinary medicine and public health, and it recently has been listed as a potential endocrine disrupter. We evaluated the effects of perinatal exposure to fipronil during the period of sexual brain differentiation and its later repercussions on reproductive parameters in adult male rats. Pregnant rats were exposed (gavage) to fipronil (0.03; 0.3; 3 mg/kg) from gestational day 15 until postnatal day 7. Fipronil exposure did not alter either the organ weights or the sperm production and morphology of males in adulthood. Furthermore, there were no adverse effects on the number of Sertoli cells per seminiferous tubule, the testicular and epididymal histo-morphometry or the histopathology, nor in the expression patterns of androgen receptor in the testis. Similarly, no alterations were observed in the other reproductive parameters analyzed. However, the animals exposed to fipronil presented an alteration in sperm motility, with a decrease in motile sperm and an increase in immobile sperm. These findings demonstrate that perinatal exposure to fipronil has long-term effects on sperm parameters, and the epididymis can be a target organ for these effects. Further studies should be conducted to identify the toxic mechanisms of fipronil on sperm motility.

Keywords: endocrine disruptors, fipronil, male reproduction, sperm motility, rats.

1. Introduction

Fipronil is a phenylpyrazole insecticide used in agriculture (Narahashi et al., 2010), in veterinary medicine against fleas and ticks, and in public health to combat disease vectors (Le Faouder et al., 2007). The mechanism of action of this insecticide is to block chloride channels associated with the gamma-aminobutyric acid (GABA) in a non-competitive manner (Zhao et al., 2005; Janssen et al., 2007; Narahashi et al., 2010). The US Environmental Protection Agency has designated fipronil as an alternative for organophosphates in insect control. However, concerns about the adverse effects of this compound on non-target organisms, including humans, have been raised due to its wide commercial and domestic use (Jennings et al., 2002; Tingle et al., 2003).

Several adverse effects of fipronil have been reported. De Oliveira et al. (2010) observed that this insecticide can be genotoxic and mutagenic in high doses (50 mg/kg) in mice. Such genotoxic effects were also observed in human peripheral blood lymphocytes in doses of 0.7 and 0.3 mg/mL (Çelik et al., 2014). In another study, the animals dermally exposed to fipronil (70, 140 and 280 mg/kg) presented central behavioral effects, primarily related to emotion, fear and exploratory behavior (Terçariol & Godinho, 2011). In addition, fipronil has been listed as a possible endocrine disruptor, acting on thyroid hormones in rats (WHO/FAO, 1997; Hood et al., 1999). In this sense, Hurley et al. (1998) found that this compound is capable of inducing thyroid cancer in rats, probably due to its ability to increase thyroid hormone metabolism and hepatic excretion.

There are few studies on fipronil exposure and reproductive effects after perinatal exposure. However, it has been demonstrated that this insecticide, administered topically as a single dose to rats (70, 140 and 280 mg/kg) can lead to a change in the functioning of the endocrine system, which may lead a disturbance in estrogen and progesterone levels,

causing adverse effects on reproduction in exposed adult females (Ohi et al., 2004). Changes in maternal behavior and in the development of some reflexes were observed by Udo et al. (2014) after prenatal exposure to fipronil at low doses (0.1, 1.0 and 10.0 mg/kg/day). The authors suggest that this change is related to endocrine disruption or interference of the GABAergic system.

As sex steroid and thyroid hormones are critical for development of the fetus and neonate, any changes in the hormonal environment can compromising their development (Fudvoye et al., 2014). Thus, more attention should be given in relation to the exposure to endocrine disruptors in this critical period of development, since most of the reported effects are observed later in life (Schwarz & McCarthy, 2008; Fudvoye et al., 2014).

Based on this information as well as the facts that fipronil and its metabolites have lipophilic characteristics and its major metabolite, fipronil sulfone is excreted in cow milk (Le Faouder et al., 2007), exposure to this insecticide can occur during the fetus developing, a phase highly sensitive to the action of substances with potential endocrine disrupting qualities. Thus, the present study aims to evaluate the effects of perinatal exposure to fipronil and its possible later effects on reproductive parameters in male rats. The treatment period we use coincides with the critical period for the occurrence of hypothalamic sexual differentiation, which depends on steroid hormones.

2. Materials and Methods

2.1 Animals

Adult male (n = 40) (90 days of age, weighing approximately 400g) and female (n = 43) (60 days of age, weighing approximately 200g) Wistar rats were supplied by Central Biotherium of São Paulo State University (UNESP). The animals were maintained under

controlled temperature (23˚C), with a constant 12 h light-dark cycle and free access to food and water. The experimental procedures were in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and was approved by the Ethics Committee for Animal Experimentation at the Institute of Biosciences of Botucatu/UNESP (Protocol number: 499/2013).

Two nulliparous female rats were mated with one male, during the dark period of the cycle, and Gestational day (GD) 0 was determined by the presence of sperm in vaginal smears of females in estrus (sexually receptive). Pregnant and lactating rats were maintained in individual cages.

2.2 Experimental groups and treatment

The study was conducted according to the experimental design depicted in Figure 1. Pregnant rats were distributed into four experimental groups. One group was treated with vehicle (1 mL/kg corn oil) and was used as control. Three groups of dams received fipronil (5-amino - [(2,6-dichloro)-4-trifluoromethyl-phenyl]-4-trifluoromethyl sulfinyl-1H-pyrazol-3-carbonitrile; Sigma-Aldrich Co. USA, 97.5% purity) at doses of 0.03; 0.3 or 3 mg/kg of body weight. The choice of doses was based on the study of Leghait et al. (2009) that used a dose of 3 mg/kg and observed changes in thyroid hormones levels. The other two doses were 10 and 100X lower than the highest dose. Fipronil was dissolved in corn oil. Experiments were conducted in the last week of pregnancy (GD 15-21) and in the first week of lactation (postnatal day (PND) 1-7) by gavage. The period of the treatment of this study coincided with the critical window of hypothalamic sexual differentiation (McLusky & Naftolin, 1981). In this experimental design, the pups were exposed to fipronil via placenta (during intrauterine period) and through milk (after birth). After birth, the number of pups per litter was reduced to eight, 4 males and 4 females. The male

offspring was weaned on PND21 and maintained into adulthood for evaluation of reproductive parameters.

2.3 Organ collection

At PND 90, male rats from each group (9-12/group; 1 male/litter) were killed by CO_2 inhalation. Organs such as testis, epididymis, seminal vesicle, vas deferens, prostate, pituitary, thyroid, adrenal, brain, liver and kidney were removed and weighed (absolute and relative to body weight) on analytical balance. The right testis and epididymis were stored for sperm analysis and the left organs for histological and immunohistochemical analyzes.

2.4 Serum testosterone levels

Blood was collected by cardiac puncture to determine the serum concentrations of testosterone. The serum was obtained by centrifugation (1200 rpm for 20 min at 4 ° C), and the testosterone hormone levels were determined by the technique of double antibody radioimmunoassay using specified kits of ImmuChem™ Double Antibody supplied by MP Biomedicals, LLC Diagnostic Division Orangeburg, NY, carried out in the Neuroendocrinology Laboratory, Dental School of Ribeirão Preto, University of São Paulo. All the samples were dosed in the same assay, to avoid interassay errors (with a 4% intra-assay error).

2.5 Sperm motility and morphology

Sperm motility was analyzed according to Perobelli et al. (2010) on spermatozoa collected from the right vas deferens. Under a light microscope, 100 spermatozoa were analyzed and classified as type A: mobile with progressive movement, type B: mobile

without progressive movement and type C: non-mobile. For evaluation of sperm morphology, the interior of the left vas deferens of mature rats was washed, with the aid of a syringe and needle, with 1.5 mL of saline solution, after which histological slides were prepared. Two hundred spermatozoa (heads only or intact sperm) per animal were evaluated for head and/or flagellar defects by phase-contrast microscopy (X 200, total magnification) in wet preparations (Filler, 1993).

2.6 Daily sperm production per testis, sperm number, and transit time in the epididymis.

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) and sperm in the caput/corpus and cauda epididymis were enumerated as described previously by Robb et al. (1978), with adaptations adopted by Fernandes et al. (2007). Briefly, each right testis, decapsulated and weighed soon after collection, was homogenized in 5 ml of NaCl 0.9% containing Triton X 100 0.5%, followed by sonication for 30s. After a 10-fold dilution a sample was transferred to Neubauer chambers (4 fields per animal), preceding a count of mature spermatids. To calculate daily sperm production (DSP) the number of spermatids at stage 19 was divided by 6.1, which is the number of days of the seminiferous cycle in which these spermatids are present in the seminiferous epithelium. In the same manner, caput/corpus and cauda epididymis portions were cut into small fragments with scissors and homogenized, and sperm counted as described for the testis. The sperm transit time through the epididymis was determined by dividing the number of sperm in each portion by the DSP.

2.7 Histological evaluation and Sertoli cells number

The left testis and epididymis were fixed in Bouin's solution, embedded in Paraplast® and sectionated in 4 μm (transversal sections of testis and longitudinal

sections of epididymis) and stained with hematoxylin and eosin (H&E) for histological analysis. The numbers of Sertoli cell nuclei were counted in histological section from the testis of rats at adulthood, in 20 seminiferous tubules per rat (Leblond & Clermont, 1952; Nassr et al., 2010).

2.8 Morphometric and stereological analysis

Images were obtained from sections in H&E (Nikon Eclipse E200MV, Infinity 1 Camera) of the testis and epididymis (caput and cauda, region 6A) and morphometric measurements were performed in Image Analysis System (Image J 1.49f software) to evaluate seminiferous epithelial height and tubular diameter. For this analysis 10 sections (n = 6) were measured per animal (stage IX of the spermatogenic cycle) at 200 X magnification. Random H&E images of 90 histological fields per experimental group were captured and analyzed by the stereological method so that histological fragments of all animals were evaluated equally (15 per animal). Stereological analyses were obtained by Weibel's multipurpose graticulate, with 120 points and 60 test lines (Weibel, 1963) to compare the epididymal components (epithelium, stroma and lumen) in the experimental groups.

2.9 Immunohistochemistry for androgen receptors (AR) on testis

The Histological sections in silanized slides, dewaxed using toluene, hydrated using decreasing concentrations of ethanol and washed in phosphate buffer (PBS), followed by antigen recovery with citrate buffer (0.01 M, pH 6.0) in a pressure cooker submitted to high temperatures for 40 min. After these steps, the slides were incubated in hydrogen peroxide in 3% of methanol during 15 min and subsequently the sections were incubated overnight with primary antibody anti-androgen receptor

(Clone SC-816 – AR N-20 from Santa Cruz Biotechnology, CA, USA). After incubating, the slides were washed with PBS buffer and during 1 h, at room temperature, the sections were incubated with secondary antibody (Biotinylated Sheap Anti-Rabbit Immunoglobulins – DAKO CYT. INC.®). In the next step, the slides were washed with PBS buffer and submitted to avidin–biotin–peroxidase solution (StreptABComplex DAKO CYT. INC.®) for 45 min. After sequential washes, the reaction was finally detected with diaminobenzidine tetrachloride-DAB (Sigma Chemical Company, St. Louis, MO, USA) as a chromogen and sections were counter-stained with Harris' hematoxylin for 5 min, then, the slides were washed in tap water and counter-stained with Harris' hematoxylin. Negative and positive controls were performed.

2.10 Fertility Test - Natural Mating

For the evaluation of fertility, rats on PND 75 were paired with females, placed in their cages (one female per male), late in the afternoon. On the following morning, vaginal smears were collected and the day sperm were found in the smear was determined to be GD0. On the GD20 the females were killed by CO_2 inhalation followed by decapitation. After collection of the uterus and ovaries, the numbers of corpora lutea, implants, reabsorptions, live fetuses, and dead fetuses were determined. From these results the following parameters were calculated: gestation rate: number of pregnant females/number of inseminated females ×100; fertility potential (efficiency of implantation): implantation sites/corpora lutea ×100; rate of pre-implantation loss: [number of corpora lutea – number of implantations/number of corpora lutea] ×100; rate of post-implantation loss: [number of implantations – number of live fetuses]/number of implantations ×100; and sex ratio: number of male fetuses/number of female fetuses ×100.

2.11 Sexual behavior

After the test of fertility the adult rats, now sexually experienced were anesthetized with sodium Ketamine and Xylazine (25 and 10 mg/kg, respectively) and bilaterally castrated. Then, all of these males received testosterone cipionate (Sigma Pharma) at 1 mg/day, sc, 3 times a week, for 2 weeks (Ribeiro & Pereira, 2005). The testosterone replacement schedule was set up so that the first injection was given on the day after orchidectomy, and the last one was always applied on the day immediately before the male sexual behavior test. The evaluation of sexual behavior was performed under red-light illumination during the dark phase of the diurnal cycle. This procedure was done in order to obtain the same hormonal condition in male rats of both experimental groups during the male sexual behavior evaluation. The female sexual behavior was assessed in the same experimental animals 15 days after the male sexual behavior test.

2.11.1 Male sexual behavior

Male rats (1 per litter) were placed individually in cages of polycarbonate crystal, measuring 44 ×31 ×16 cm, 5 min before introduction of 1 adult female in natural estrus (sexually receptive) determined by vaginal smear. The animals were observed in the dark period of the cycle with the aid of red lamps. The following parameters were observed for 40 min: latency to the first mount, intromission, and ejaculation; number of intromissions until the first ejaculation; latency of the first post-ejaculatory intromission; number of post-ejaculatory intromissions; and number of ejaculations (Ågmo, 1997). The males that did not mount in the initial 10 min were considered sexually inactive (Gerardin et al., 2005; Pereira et al., 2006; Oliva et al., 2006).

2. 11.2 Female sexual behavior

Twenty-four hours prior to the test, experimental males were treated with estradiol benzoate (Sigma Co., USA) at 20 µg/kg, ip (Ribeiro & Pereira, 2005) since the female behavior is dependent on estrogen receptors in the brain and their stimulation by the female hormone. A sexually experienced intact male rat was first placed into an acrylic cage for 10 min for adaptation and then cohabited with each experimental male. The animals were observed for 10 min to assess female sexual behavior (lordosis) and mount acceptance.

2.12 Sexual partner preference

The assessment of sexual preference was performed on a semicircular arena (100×50 cm) with 2 cages (25×15 cm) positioned on opposite sides, outside the arena, in which the stimulus animals, a sexually active male and a receptive female in estrus, were placed. The partition between the stimulus animals and the experimental sexually in experienced adult male rats (gonadally intact) consisted of a wire mesh allowing both animals to see, smell, and hear each other. The floor in front of the stimulus animals was demarcated in zones (30×20 cm) and the test lasted 20 min under red-light illumination during the first half of the dark phase of their cycle. The following measures were recorded: number of visits to each of the stimulus animal zones; total time spent with in each of the stimulus animal zones; and the duration of each visit to each stimulus animal's zone (adapted from Vega-Matuszczyk & Larsson, 1995). Following each test, a partner preference score was calculated by subtracting the time spent in the zone containing the sexually active male from the time spent in the zone containing the estrus female. Thus, a positive score indicates a preference for the estrus female, a negative score a preference for the sexually active male.

2.13 Statistical Analysis

Values are expressed in mean ± SEM and medians (Q1–Q3). For comparison of results among the experimental groups, statistical tests for analysis of variance were utilized – ANOVA, with the "a posteriori" Tukey-Kramer test or Kruskal-Wallis, with the "a posteriori" Dunn test. Differences were considered significant when $p<0.05$.

3. Results

At PND 90, there were no significant differences among experimental groups in body weight or absolute and relative organs weights (Table 1). Similarly, perinatal exposure to fipronil did not alter the DSP per testis, number of sperm in the caput/corpus and cauda epididymis, or sperm transit time (Table 2).

Perinatal fipronil led to a significant decrease in the number of motile sperm with a mobile progressive path (Type A) for all doses tested and an increase in the number of non-mobile sperm (Type C) for the highest dose (3 mg/kg) (Figure 2). However, morphological analysis of sperm extracted from the vas deferens showed that the percentages of normal (Control: 97%; 0.03 mg/kg: 95%; 0.3 mg/kg: 96% and 3 mg/kg: 96%) and abnormal forms were similar among the groups.

Table 3 and Figure 3 show the results of sexual behavior and Table 4, sexual preference. None of the parameters investigated were markedly different among groups. Again, in relation to fertility, there were no significant differences among groups for most of the parameters evaluated, except for an increase of the mother's body weight in the group exposed to the highest dose of fipronil (Table 5). Furthermore, there were no significant differences in testosterone levels (Control: 3.78 ± 0.77; 0.03 mg/kg: 2.00 ± 0.55; 0.3 mg/kg: 3.79 ± 0.80 and 3 mg/kg: 3.08 ± 0.64; ng/ml).

Neither the histopathology of the testis and epididymis (data not shown) nor morphometric testicular and epididymal stereological analysis (Table 6) revealed

treatment-related morphological alterations. The number of Sertoli cells per seminiferous tubule (Control: 18.40 ± 0.23; 0.03 mg/kg: 18.32 ± 0.20; 0.3 mg/kg: 18.63 ± 0.27 and 3 mg/kg: 18.55 ± 0.30) and the expression patterns of androgen receptors in the testis (data not shown) showed similarity among groups.

4. Discussion

Exposure to endocrine disruptors, particularly in fetal life and infancy, may interfere with the sexual development of the brain, resulting in disturbances in the masculinization and feminization processes in females and males (Negri-Cesi et al., 2008; Patisaul & Polston, 2008; Gore, 2010). The present study demonstrates that fipronil exposure during the sexual differentiation of the brain can have long-term effects on sperm parameters in male rats.

In order to evaluate the later effects of perinatal exposure to a xenobiotic, the assessment of body and organ weights provides important information about the general health of an individual (OECD, 1996). Changes in the wet weight of reproductive organs (androgen dependent), such as the testis, epididymis, seminal vesicle and prostate, are used as parameters to indicate changes in the levels of sex hormones (Zenick et al., 1994). In the present study, significant differences in the body and organ weights of adult animals were not found, suggesting that the hormone levels are normal in this phase. This was confirmed after testosterone analyzes, which was similar among groups.

The effects of endocrine disruptors can be manifested as changes in reproductive development and may alter the fertility in adulthood (Gore, 2010). For this reason, we evaluated sperm motility, an important parameter used to assess the quality of sperm obtained from semen *in vitro* (Mahadevan & Trounson, 1984) and *in vivo* (Bostofte et al., 1990; Barratt et al., 1993). The reduction in the percentage of mobile spermatozoa (Type

A) and the increase in the non-mobile spermatozoa (Type C) observed here demonstrate that perinatal exposure to fipronil can compromise the sperm quality of animals in adult life. Changes in sperm motility can make it difficult for male gametes to penetrate the cervical mucus and the zona pellucida and may thus inhibit fertilization (Aitken et al., 1985; Mortimer et al., 1986).

Note that the fertility of sperm, which includes the acquisition of motility and the ability to recognize and fuse with the plasma membrane of the oocyte, is acquired only during their transit through the epididymis (Gatti et al., 2004). The environment inside the epididymis is regulated to ensure sperm maturation and the acquisition of functional characteristics important to sperm cells (Robaire et al., 2006). Exposure to chemicals can disrupt the biochemical balance of the epididymis and thus adversely affect the maturation of sperm (Clegg et al., 2001; Foster et al., 2010), and there is a need for further studies on the impact of toxic substances on the function of the epididymis (Bonde et al., 2008).

Despite the changes in sperm motility, fipronil exposure did not compromise the fertility of the rats after natural mating. It is important to emphasize that the reduction in number and quality of sperm is not a direct measure of fertility unless a drastic effect has been induced (Neubert, 1997). It is known that in some subspecies of rats and mice, sperm production can be reduced by 90% without compromising fertility. However, less severe reductions may have dramatic consequences for human males who function nearer the threshold for the number of sperm needed to ensure reproductive competence (Zenick et al., 1994).

In the present study, the treatment period also coincided with the proliferation of Sertoli cells that begins on DG 16 in rats, with a peak in proliferation on DG 20 (Orth, 1982). In this period, the blood-testis barrier is already being formed in order to achieve the ability to sustain the spermatogenesis (França et al., 2012). Substances capable of

interfering with the proliferation of this cell population, whose importance is crucial for maintenance of spermatogenesis, can affect DSP and, consequently, fertility. However, in the present study, the number of spermatids in the testis and DSP were not affected after perinatal exposure, as well as the Sertoli cells count. The absence of effects on the testis agrees with the histological findings discussed below.

Histopathological evaluation is one of the most sensitive parameters for detecting the adverse effects of chemicals on male reproductive function (Creasy, 2003). In this study, the absence of histological changes in the testis and epididymis as well as seminiferous tubular diameter and the stereology of the epididymis, suggest that perinatal exposure to fipronil did not interfere with spermatogenesis or testicular function. In the same way, changes in sperm morphology were also not observed among the experimental groups.

The levels of androgen receptor (AR) are essential for the normal functioning of the male reproductive system. AR can be detected in Sertoli cells, peritubular myoid cells, Leydig cells and perivascular smooth muscle cells in the testis (Vornberger et al., 1994). Changes in the expression of this receptor may impair the function of Sertoli cells (O'Shaughnessy et al., 2010), steroidogenesis and sperm production (Wang et al., 2009). In this study, the results of AR expression in the testis were similar among groups, indicating that fipronil exposure during hypothalamic sexual differentiation did not interfere with the expression pattern of AR in adult life.

Although the prenatal testosterone surge is important to sexual differentiation of brain and behavior, the cellular and molecular consequences that follow steroid receptor activation during the sexual differentiation of the brain are largely unknown. Previous research has focused on quantitative differences in the levels of neurotransmitters or other cellular proteins. The activation of $GABA_A$ receptors in the hypothalamus of neonatal

males is an important step in this process (Auger et al., 2001; McCarthy et al., 2002). Thus, substances able to block this receptor, such as the insecticide fipronil, may impair hypothalamus masculinization, compromising sexual behavior.

It is known that $GABA_A$ receptor antagonists, such as picrotoxin and pentylenetetrazol, activate the hypothalamic-pituitary-adrenal axis and release of corticosteroids (Lal & Emmett-Oglesby, 1983; Silva et al., 1995), as occurs in a stress scenario. Perinatal and prenatal stresses can lead to the demasculinization of the fetal brain (Gerecke et al., 2012). Silva et al. (1998) and Yasuhara et al. (2005) showed that perinatal exposure to picrotoxin alter sexual dimorphism manifest as altered reproductive performance and sexual behavior of male rats. However, in this study, we did not observe alterations in behavior parameters, nor were there apparent changes in sexual partner preferences, which develop neonatally, presumably by estradiol derived from endogenous testosterone (Brand et al., 1991).

Recently, Lu et al. (2014) (*in vitro* reporter gene assays using CHO-K1 cells) showed that for estrogenic and anti-estrogenic activities, fipronil and its metabolite fipronil sulfone, present no agonistic characteristics, but exhibited the similarly antagonistic activities via estrogen receptor α. Furthermore, the antagonistic activity of fipronil sulfone *via* thyroid hormone receptor β was revealed, demonstrating that fipronil sulfone may play an important role in the disruption of thyroid function in animals, and even in humans. Thus, for any of the mechanisms demonstrated, fipronil and its metabolites could interfere with the determining of sex differences in the brain and compromise the reproductive capacity of animals.

These findings demonstrate that perinatal exposure to fipronil has long-term effects on sperm parameters, and the epididymis can be a target organ for these adverse effects. The importance of understanding epididymal function and sperm maturation is

underscored by the fact that up to 40% of infertile men have idiopathic infertility, which may reflect disturbances in sperm maturation (Cornwall, 2009). Therefore, further studies are needed to determine the molecular mechanisms underlying the role of fipronil in reduced sperm motility, especially regarding the quality of the male gametes.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Figures and tables

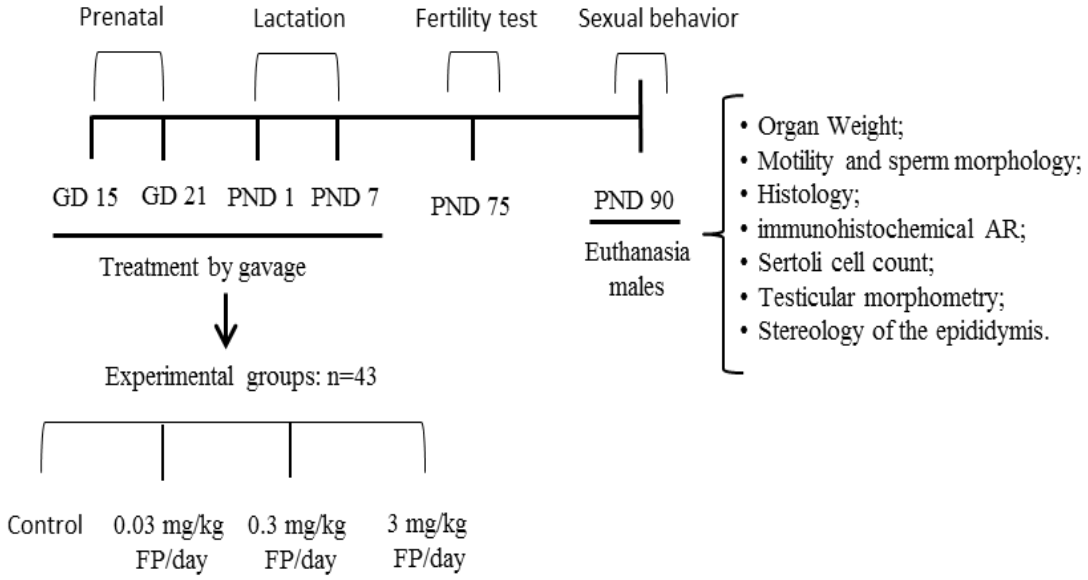

Figure 1 - Experimental design

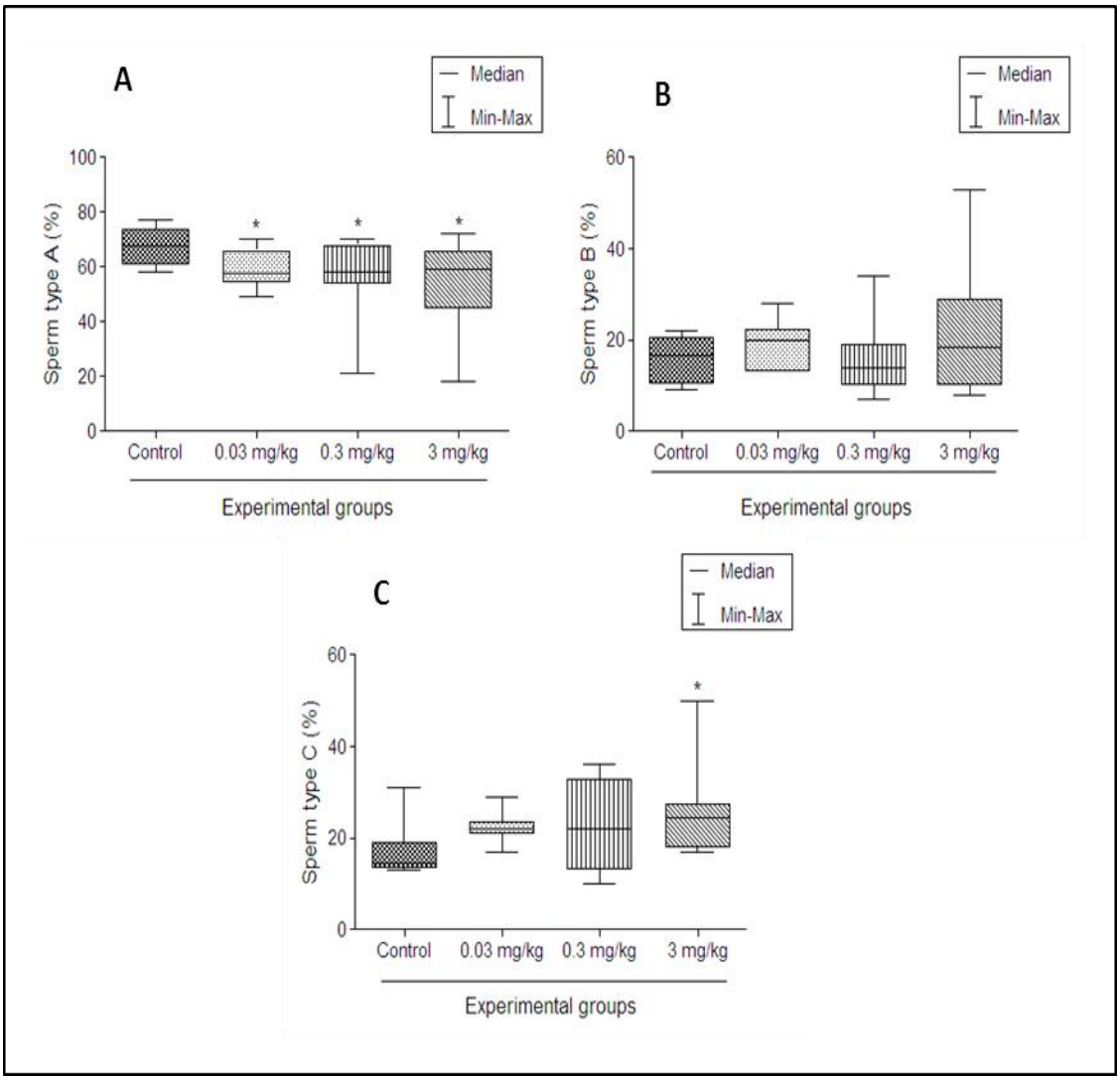

Figure 2 - Sperm motility of adult male rats exposed to 0.03. 0.3 and 3 mg/kg of fipronil. The sperm type A (mobile progressive path). Type B (mobile without progressive path). Type C (non-mobile sperm). Values are expressed as median and interquartile range. 9-12 rats/group. * $p < 0.05$. Kruskal-Wallis followed by Dunn test.

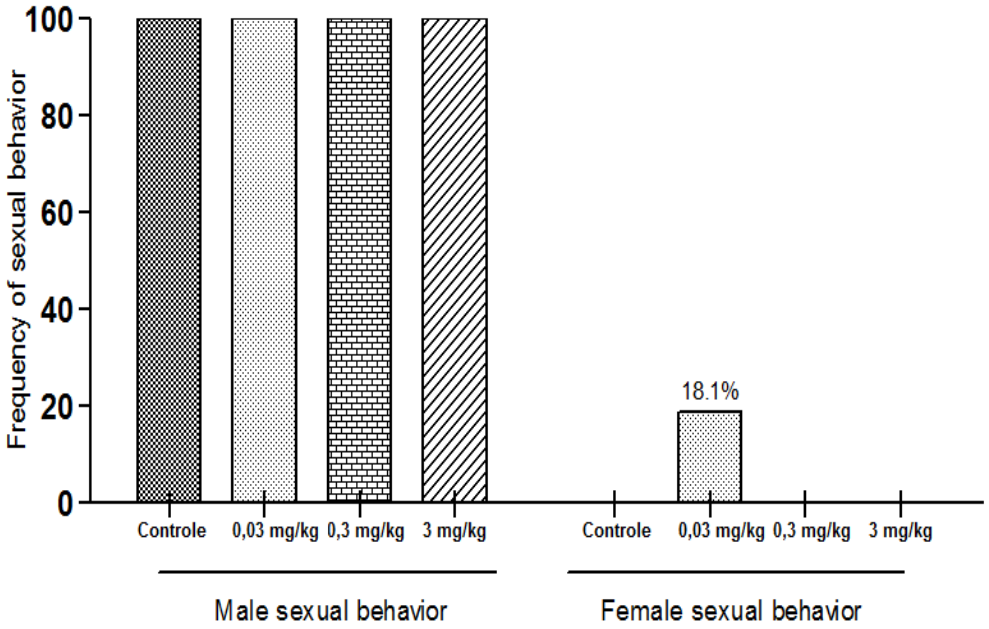

Figure 3 - Frequency of rats that showed male sexual behavior after replacement with testosterone and female sexual behavior after administration of estrogen, 10-12 rats/group.

Table 1 - Final body weight and absolute and relative organ weights of rats exposed to 0.03, 0.3 and 3 mg/kg of fipronil.

Parameters	Experimental Groups			
	Control	**0.03 mg/kg**	**0.3 mg/kg**	**3 mg/kg**
Body Weight (g)	439.64 ± 9.89	423.21 ± 13.34	440.96 ± 12.40	454.28 ± 10.31
Brain (g)	1.84 ± 0.04	1.80 ± 0.04	1.69 ± 0.09	1.75 ± 0.05
Brain (g/100g)	0.42 ± 0.01	0.43 ± 0.01	0.38 ± 0.02	0.38 ± 0.01
Pituitary (mg)	11.05 ± 0.30	9.17 ± 0.41	9.71 ± 0.41	11.58 ± 1.76
Pituitary (mg/100g)	2.53 ± 0.09	2.17 ± 0.22	2.20 ± 0.07	2.51 ± 0.33
Thyroid (mg)	22.58 ± 0.85	22.05 ± 1.36	21.01 ± 1.28	21.27 ± 1.26
Thyroid (mg/100g)	5.13 ± 0.14	5.19 ± 0.24	4.79 ± 0.34	4.70 ± 0.30
Liver (g)	17.04 ± 0.66	16.40 ± 0.68	16.91 ± 0.59	18.20 ± 0.83
Liver (g/100g)	3.87 ± 0.11	3.87 ± 0.10	3.83 ± 0.07	3.99 ± 0.12
Right kidney (g)	1.54 ± 0.04	1.52 ± 0.03	1.51 ± 0.05	1.61 ± 0.04
Right kidney (g/100g)	0.35 ± 0.00	0.36 ± 0.00	0.34 ± 0.01	0.35 ± 0.00
Left kidney (g)	1.51 ± 0.04	1.45 ± 0.04	1.48 ± 0.05	1.57 ± 0.05
Left kidney (g/100g)	0.31 ± 0.02	0.34 ± 0.00	0.33 ± 0.01	0.34 ± 0.00
Right adrenal (mg)	39.58 ± 2.13	32.66 ± 2.25	35.44 ± 2.21	37.70 ± 1.33
Right adrenal (mg/100g)	9.14 ± 0.53	7.76 ± 0.60	8.00 ± 0.37	8.30 ± 0.26
Left adrenal (mg)	42.58 ± 2.24	36.09 ± 2.57	37.53 ± 2.10	41.21 ± 1.31
Left adrenal (mg/100g)	9.70 ± 0.49	8.56 ± 0.67	8.50 ± 0.41	9.07 ± 0.22
Testis (g)	1.71± 0.05	1.67 ± 0.03	1.75 ± 0.04	1.64 ± 0.06
Testis (g/100g)	0.38 ± 0.00	0.39 ± 0.01	0.40 ± 0.01	0.36 ± 0.01
Epididymis (g)	0.63 ± 0.01	0.63 ± 0.02	0.63 ± 0.01	0.59 ± 0.02
Epididymis (g/100g)	0.14 ± 0.00	0.14 ± 0.00	0.14 ± 0.00	0.13 ± 0.00
Ventral prostate (mg)	379.11 ± 34.51	405.71 ± 56.17	405.70 ± 27.17	419.35 ± 49.57
Ventral prostate (mg/100g)	86.53 ± 7.73	93.82 ± 12.03	96.15 ± 6.68	92.73 ± 11.19
Full seminal gland (g)	1.14 ± 0.06	1.10 ± 0.04	1.18 ± 0.05	1.17 ± 0.06
Full seminal gland (g/100g)	0.24 ± 0.02	0.26 ± 0.00	0.26 ± 0.01	0.25 ± 0.01

Values are expressed as mean ± SEM, 9-12 rats/group. $p > 0.05$ by ANOVA.

Table 2 - Sperm counts in the testis and epididymis and sperm transit time of rats exposed to 0.03, 0.3 and 3 mg/kg of fipronil.

Parameters	Experimental Groups			
	Control	**0.03mg/kg**	**0.3mg/kg**	**3mg/kg**
Sperm counts in the testis				
Daily sperm production (x 10^6 / testis / day)	28.83 ± 1.20	25.77 ± 1.36	24.85 ± 1.31	27.81± 1.51
Daily sperm production (x10^6 / g / testis / day)	18.47 ± 0.88	17.42 ± 1.20	15.48 ± 0.74	18.70 ± 0.89
Number of spermatids in the testis (x 10^6)	175.90 ± 7.32	157.22 ± 8.31	151.68 ± 7.97	169.68 ± 9.24
Number of spermatids in the testis (x 10^6/g/testis)	109.71 ± 4.99	105.45 ± 7.62	94.60 ± 4.52	114.20 ± 5.45
Sperm counts in the epididymis Caput/corpus				
Number of sperm (x 10^6)	88.01 ± 4.20	95.37 ± 5.27	100.61 ± 4.79	80.64 ± 6.81
Number of sperm (x 10^6/g/organ)	290.72 ± 6.48	318.57 ± 14.73	314.62 ± 16.63	277.05 ± 17.01
Sperm transit time (days)	3.10 ± 0.19	3.75 ± 0.22	3.90 ± 0.25	3.04 ± 0.27
Sperm counts in the epididymis Cauda Region				
Number of sperm (x 10^6)	190.95 ± 9.30	187.09 ± 11.44	190.32 ± 7.99	193.26 ± 13.54
Number of sperm (x 10^6/g/organ)	870.37± 30.93	821.77 ± 43.76	820.99 ± 31.54	903.73 ± 46.83
Sperm transit time (days)	6.70 ± 0.35	7.36 ± 0.60	7.41 ± 0.40	7.12 ± 0.61

Values are expressed as mean ± SEM, 9-12 rats/group. $p > 0.05$ by ANOVA.

Table 3 - Sexual behavior of sexually experienced adult rats exposed to 0.03, 0.3 and 3 mg/kg of fipronil.

Parameters	Experimental Groups			
	Control	**0.03 mg/kg**	**0.3 mg/kg**	**3 mg/kg**
Latency of the first mount	15.66 ± 3.05	26.33 ± 5.11	25.30 ± 8.19	21.33 ± 6.73
Latency of the first intromission	22.58 ± 5.50	29.87 ± 5.78	27.44 ± 7.16	17.25 ± 2.89
Number of intromissions	25.00 ± 3.77	21.90 ± 1.94	28.40 ± 5.88	27.22 ± 6.67
Latency of the first ejaculation	675.08 ± 94.06	589.90 ± 92.95	559.33 ± 134.18	506.22 ± 101.17
Latency of the first intromission post-ejaculation	309.67 ± 20.60	338.27 ± 29.32	314.13 ± 12.29	302.88 ± 13.76
Number of intromissions post-ejaculation	16.00 ± 2.25	15.72 ± 1.45	13.62 ± 3.22	17.62 ± 2.52
Number of ejaculations	2.91 ± 0.46	2.63 ± 0.15	2.77 ± 0.32	3.00 ± 0.26

Values are expressed as mean ± SEM, 9-12 rats/group. $p > 0.05$ by ANOVA. The latencies are expressed in seconds.

Table 4 - Sexual preference in sexually experienced of rats exposed to 0.03, 0.3 and 3 mg/kg of fipronil.

Parameters	Experimental Groups			
	Control	**0.03 mg/kg**	**0.3 mg/kg**	**3 mg/kg**
Number of visits to the female zone	19.90 ± 1.37	19.54 ± 1.36	18.00 ± 1.51	20.60 ± 1.32
Number of visits to the male zone	18.72 ± 1.99	18.54 ± 1.83	16.40 ± 1.39	15.80 ± 1.55
Total time in the female zone (s)	681.55 ± 58.26	714.45 ± 48.05	706.60 ± 47.14	735.90 ± 30.87
Total time in the male zone (s)	342.55 ± 44.68	310.18 ± 36.81	308.50 ± 46.68	228.50 ± 23.83
Time of each visit to female (s)	37.18 ± 5.46	39.59 ± 5.06	40.96 ± 3.29	37.91 ± 4.35
Time of each visit to male (s)	18.84 ± 2.37	17.40 ± 1.93	21.09 ± 4.73	14.75 ± 0.88
Preference score	+339.00±99.28	+404.27±80.67	+398.10±89.63	+507.40±47.94

Values expressed as mean ± SEM, 11 -10 rats/group. $p > 0.05$ by ANOVA.

Table 5 - Fertility after natural mating of rats exposed to 0.03, 0.3 and 3 mg/kg for fipronil.

Parameters	Experimental Groups			
	Control	**0.03 mg/kg**	**0.3 mg/kg**	**3 mg/kg**
[1]Body Weight of mothers (g)	365.04 ± 7.90	367.65 ± 8.28	372.40 ± 7.75	399.02 ± 8.49 *
[1]Uterine weight fetuses	73.35 ± 3.19	69.17 ± 4.61	72.83 ± 3.14	73.35 ± 5.83
[1]Number of corpora lutea	12.50 ± 0.54	12.72 ± 0.40	13.00 ± 0.66	13.20 ± 0.46
[1]Number of implantations	12.41 ± 0.51	11.90 ± 0.65	12.80 ± 0.57	12.70 ± 0.70
[1]Number of resorptions	0.00 ± 0.00	0.72 ± 0.63	0.50 ± 0.30	0.50 ± 0.30
[1]Number of live fetuses	12.41 ± 0.51	11.18 ± 0.79	12.30 ± 0.59	12.20 ± 0.94
[1]Weight of fetus (g)	3.92 ± 0.08	3.96 ± 0.08	3.94 ± 0.04	3.88 ± 0.06
[2]Pregnancy rate (%)	100	100	90.9	100
[2]Sex ratio (%)	85.71 (67.85 - 117.85)	71.42 (57.14 - 71.42)	85.71 (71.42 - 85.71)	85.71 (71.42 - 85.71)
[2]Fertility potential (%)	100 (100-100)	100 (92.30-100)	100 (100-100)	100 (100-100)
[2]Pre-implantation loss(%)	0.00 (0.00-0.00)	0.00 (0.00-1.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)
[2]Post-implantation loss (%)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)

[1]Values are expressed as mean ± SEM, 10-12 rats/group. * $p < 0.05$. ANOVA followed by Tukey test.

[2]Values expressed as median and interquartile range.

Table 6 – Stereological and morphometric (μm) analysis of rats exposed to 0.03, 0.3 and 3 mg/kg of fipronil.

Parameters	Experimental Groups			
	Control	**0.03 mg/kg**	**0.3 mg/kg**	**3 mg/kg**
Testicular morphometry				
Tubular diameter	297.07 ± 5.20	297.32 ± 3.49	298.59 ± 6.73	290.93 ± 7.92
Epithelial height	80.96 ± 1.28	81.36 ± 0.82	82.49 ± 0.62	78.63 ± 0.90
[1]Epididymal stereology				
Caput				
Epithelium	18.19 (17.43 - 19.02)	18.31 (16.89 - 19.73)	21.52 (19.24 - 22.35)	19.52 (18.40 - 22.63)
Stroma	9.02 (8.16 - 9.56)	9.30 (8.91 - 9.63)	10.29 (9.90 - 11.20)	9.44 (7.70 - 11.15)
Lumen	73.96 (73.11 - 74.55)	72.50 (70.90 - 73.91)	69.84 (67.87 - 71.39)	71.25 (63.22 - 73.68)
Cauda				
Epithelium	7.08 (6.19 - 7.67)	5.37 (4.72 - 5.90)	6.78 (6.33 - 6.98)	5.63 (5.29 - 6.03)
Stroma	9.46 (8.77 - 10.99)	9.24 (8.85 - 10.04)	10.19 (9.01 - 10.92)	9.35(8.74 - 11.23)
Lumen	83.35 (82.79 - 83.87)	85.02 (84.75 - 86.57)	83.03 (82.69 - 83.52)	84.96 (83.24 - 85.75)

Values are expressed as mean ± SEM, 6 rats/group.
[1]Values expressed as median and interquartile range. $p > 0.05$ by ANOVA.

Capítulo 2

O presente trabalho deu origem ao manuscrito intitulado **"Onset of puberty and estrous cycle of rats perinatally exposed to the insecticide fipronil"** que será submetido à revista Toxicology Letters, ISSN: 0378-4274. Fator de Impacto: 3.262.

Onset of puberty and estrous cycle of rats perinatally exposed to the insecticide fipronil.

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Abstract

Fipronil, a phenylpyrazole insecticide, is used in agriculture, veterinary medicine, and public health. Because it is considered a potential endocrine disruptor, this study evaluated the effects of perinatal exposure to fipronil on the female reproductive system. Pregnant rats were exposed (via gavage) to fipronil (0.03, 0.3, and 3 mg/kg) from gestational day 15 to day 7 after birth, and its long-term effects on the reproductive functions were evaluated. Perinatal exposure to the highest dose of fipronil (3 mg/kg) delayed the age of vaginal opening and the first estrus, without affecting the anogenital distance. Moreover, exposure to 0.3 mg/kg of fipronil led to a significantly shorter estrous cycle and reduced the number of cycles during the period of evaluation. However, the other reproductive parameters analyzed (fertility, hormone levels, sexual behavior, histology of ovaries and uterus) showed no alterations. In this experimental model, fipronil interfered with the development of the female reproductive system, without long-term effects on fertility. Further studies are needed to identify its action mechanisms on the female reproductive system.

Keywords: fipronil, estrous cycle, fertility, female offspring, rats.

1. Introduction

Female reproductive disorders, including polycystic ovary syndrome, endometriosis, accelerated puberty, masculinized genitalia, breast cancer, and disrupted hypothalamic-pituitary-gonadal function, can be considered late-onset symptoms of developmental abnormalities of the reproductive system, which may be the result of genetic and/or environmental factors (Balabanic et al., 2011). Several synthetic or natural agents have been identified as interacting with the endocrine system and triggering these alterations. These exogenous substances, which are referred to as endocrine disruptors (EDs) (Kavlock et al., 1996; US EPA, 1997), can give rise to adverse health effects, especially if the exposure occurs in critical periods of development (Dickerson & Gore, 2007).

Fipronil is an insecticide that belongs to the phenylpyrazole chemical group. This substance is widely used in the control of many agricultural and domestic pests (Le Faouder et al., 2007; Narahashi et al., 2010). In insects, the mechanism of action of this compound is to competitively block chloride channels associated with gamma-aminobutyric acid (GABA), causing death by neuronal hyperexcitation and paralysis (Zhao et al., 2004; Janssen et al., 2007; Narahashi et al., 2010). Although fipronil is considered an insecticide with selective toxicity, its major metabolite, fipronil sulfone, is at least 20 times more potent in blocking mammalian $GABA_A$ receptors (Zhao et al., 2005). Moreover, fipronil and its metabolites have been identified as possible anti-androgen chemicals, interfering with the AR signaling pathway. This information underscores the need to consider fipronil as an environmental hazard, given its endocrine disruption potential (Aït-Aïssa et al., 2010).

Fipronil has been designated as an alternative to replace organophosphates in insect control by US Environmental Protection Agency, but some studies have

demonstrated its adverse effects on non-target organisms (Tingle et al., 2003). This pesticide, which has been found to have genotoxic and mutagenic effects on mice and humans (De Oliveira et al., 2010; Çelik et al., 2014), is able to induce thyroid cancer in rats (Hurley et al., 1998) and is considered a possible endocrine disruptor (WHO/FAO, 1997; Hood et al., 1999). In another study, dermal exposure to fipronil (Frontline®, Top Spot®) in rats caused behavioral effects on the central nervous system, primarily involving emotions, fear and exploratory behavior (Terçariol & Godinho, 2011), indicating that this insecticide may be neurotoxic.

Studies have shown that fipronil can be toxic to the female reproductive system (Ohi et al., 2004; Udo et al., 2014). Ohi et al. (2004) observed that fipronil can alter the endocrine system function and cause adverse reproductive effects in female rats, after the application of a single dose (70, 140 and 280 mg/kg). In another study, Udo et al. (2014) observed that prenatal exposure to low doses of fipronil can impair maternal behavior, and suggested that these alterations may be due to the GABAergic system or to endocrine disruption, since fipronil also acts as an endocrine disruptor.

Based on these findings, and considering that GABA inhibitors have been described as substances that can alter the endocrine system (McCarthy, 1995; Davis et al., 1996, 2000), this study aimed to evaluate the effects of perinatal exposure to fipronil and its possible later effects on reproductive functions in female rats.

2 Materials and Methods

2.1 Animals and Treatments

Adult male (n = 40) (90 days of age) and females (n = 43) (60 days of age) Wistar rats, from the Central Biotherium of State University of São Paulo (UNESP) were used. The animals were maintained under controlled temperature (23˚C), with a constant 12 h light-dark cycle and free access to food and water. Two non-pregnant female rats were mated with one male, during the dark portion of the lighting cycle, and the day of sperm detection in the vaginal smear was considered day 0 of gestation (GD 0). The pregnant females were randomly assigned between the experimental groups and housed individually in cages. At birth, the offspring were weighed and reduced to eight pups per litter, 4 males and 4 females. The female offspring was weaned on PND21 and maintained into adulthood for evaluation of reproductive parameters. The experimental procedures were in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and was approved by the Committee for Ethics in Animal Experimentation at the Institute of Biosciences/UNESP (Protocol number: 499/2013).

Pregnant rats were divided into four groups. One group was treated with vehicle (1 mL/kg corn oil) and served as control. For the evaluation of the effects of fipronil (purity 97.5%) of 5-amino - [(2,6-dichloro)-4-trifluoromethyl-phenyl]-4-trifluoromethyl sulfinyl-1H-pyrazol-3-carbonitrile (Sigma-Aldrich Co. Ltd.) three groups were treated with doses of 0.03; 0.3 and 3 mg/kg body weight. The dose regimens were based on the study of the Leghait et al. (2009) that used a dose of 3 mg/kg and observed changes in thyroid hormones. The other two doses were 10 and 100X lower than the highest dose. Fipronil was dissolved in corn oil. Experiments were conducted in the last week of

pregnancy (GD 15 -21) and in the first week of lactation (postnatal day (PND) 1-7) by gavage (orally) (Figure 1). Based on this experimental design, the pups were exposed to fipronil via placenta (during intrauterine period) and through milk (after birth). The treatment period coincides with the critical period for the occurrence of hypothalamic sexual differentiation, which depends on steroid hormones (McLusky & Naftolin, 1981).

2.2 Anogenital Distance and Number of Nipples/areola

At birth and at 22 days of age, the anogenital distance (AGD, the distance from the anus to the genital tubercle) was measured in female pups, using a vernier calipers. AGD was normalised against the cube root of body weight. Male rodents have AGD values that are approximately twice the length of those of females (Gallavan et al., 1999). On PND 13, the number of areolas was recorded. Observations were scored based on the presence or absence of a nipple bud or a discoloration of the skin surrounding the nipple (Mylchreest et al., 2000). These data are expressed as litter mean.

2.3 External Signs of Puberty Onset

On PND 30, all females were evaluated daily for vaginal opening (VO). The day of full opening of the vaginal orifice was recorded. On the day of VO, the female rats were weighed and daily vaginal fluid was collected as described by Marcondes et al. (2002), to detect the day of first estrus (predominance of cornified epithelial cells). Ten microliters of 0.9% saline was instilled into the vagina and subsequently aspirated. Vaginal fluids were placed in a slide and analyzed under a light microscope (Leica MicroStar IV) at 200×magnification. These data are expressed as litter mean.

2.4 Estrous Cycle

The estrous cycle was assessed on the basis of vaginal smears collected every morning over a period of 15 days (PND 65 to PND 80). The material was observed under a light microscope and the estrous cycle phases were classified as diestrus, proestrus, estrous and metaestrus.

A proestrus phase consists of a predominance of nucleated epithelial cells and the estrous consists of anucleated cornified cells. A metestrus phase consists of the same proportion among leukocytes, cornified, and nucleated epithelial cells and the diestrus phase primarily consists of a predominance of leukocytes (Marcondes et al., 2002). The estrous cycle duration was calculated as the number of days between one estrous phase to the next.

2.5 Collection and Analysis of Organs

On PND 80, one female rat from each litter was killed by CO_2 inhalation followed by decapitation in estrus phase, for collection and determination of the weight of organs including brain, pituitary, thyroid, liver, kidney, adrenal, uteri (with fluid) and ovaries on precision balance. For histological evaluation of reproductive organs the uterus and ovaries were fixed in Bouin's solution, dehydrated in ethanol, embedded in Paraplast® and sectionated in 5 μm (three sections per animal), and stained with hematoxylin and eosin (H&E).

In each ovary, ovarian follicles and corpora lutea were counted in 3 sections per animal. Follicles were classified according to Guerra et al. (2010). Primordial and primary follicles were enumerated together; oocytes surrounded by a single layer of either squamous or cuboidal epithelial cells were included. Follicles were classified as pre-antral when containing 2-4 layers of granulosa cells with no antral space. Antral

follicles were classified when containing three or more layers of granulosa cells and a clearly defined antral space. Characteristics of atretic follicles included pyknotic granulosa cells, disorganized granulosa cells, degenerating oocyte and detachment from the basement membrane.

In the uterus, the endometrial height was measured, in 3 sections per animal using a light microscope. In each section, five different regions were analyzed, resulting in a total of 15 measurements per animal.

2.6 Serum estradiol and progesterone levels

Blood was collected by cardiac puncture to determine the serum concentrations of estradiol and progesterone. The serum was obtained by centrifugation (1200 rpm for 20 min at 4 °C), and the progesterone hormone levels were determined by the technique of double antibody radioimmunoassay using specified kits of ImmuChem™ Double Antibody supplied by MP Biomedicals, LLC Diagnostic Division Orangeburg , NY. The estradiol dosage was performed by ELISA using specific kits of DRG® Estradiol ELISA (EIA-2693) supplied by DRG International, Inc., USA, carried out in the Neroendocrinology Laboratory, Dental School of Ribeirão Preto, University of São Paulo-USP. All the samples were dosed in the same assay, to avoid interassay errors (with a 4% intra-assay error).

2.7 Sexual behavior

During the first estrus after PND 80, female rats from different experimental groups (1 or 2 per litte) were used for the fertility test. Rats were maintained under controlled temperature conditions on an inverted 12-h light–dark cycle, for at least seven days, with food and water *ad libitum*. For the evaluation of female sexual

behavior, sexually experienced males were allowed ten mounts on the female and the presence of lordosis was measured. Results were expressed as the lordosis quotient (LQ, number of lordosis/ten mounts×100) (Beach, 1976). All females were used only once.

2.8 Fertility Tests - Natural Mating

This analysis was performed by natural mating. Female offspring (1 per litter) were placed with sexually experienced males for additional 4 h after the end of the sexual behavior evaluation. At the end of afternoon, rats were separated and vaginal smears collected and sperm detection were determined to show GD 0. On GD 20 females were killed by decapitation. After collection of the uterus and ovaries the numbers of corpora lutea, implants, reabsorptions, live fetuses, and dead fetuses were determined. From these results the following parameters were calculated: gestation rate: number of pregnant females/number of inseminated females ×100; fertility potential (efficiency of implantation): implantation sites/corpora lutea ×100; rate of pre-implantation loss: [number of corpora lutea – number of implantations/number of corpora lutea] ×100; rate of post-implantation loss: [number of implantations – number of live fetuses]/number of implantations ×100; and sex ratio: number of male fetuses/number of female fetuses ×100.

2.9 Statistical analyses

Values are expressed in mean ± SEM and medians (Q1–Q3). For comparison of results among the experimental groups, statistical tests for analysis of variance were utilized – ANOVA, with the "a posteriori" Tukey-Kramer test or Kruskal-Wallis, with the "a posteriori" Dunn test. Differences were considered significant when $p<0.05$.

3. Results

No signs of toxicity were observed during the daily treatment with fipronil. Similarly, the perinatal treatment did not alter the body weight or the anogenital distance of female offspring at birth and at PND 22. The experimental groups also showed a similar number of nipples/areola (Table 1).

Perinatal exposure to the highest dose of fipronil (3 mg/kg) delayed the age of vaginal opening and the first estrus (Figure 2). Furthermore, at a dose of 0.3 mg/kg of fipronil, the duration of the estrous cycle was significantly increased and the number of cycles was significantly reduced during the period of evaluation (Table 2).

At PND 80, there were no significant differences among the experimental groups in the body weight and in the absolute and relative weight of most of the organs. However, animals exposed to the highest dose of fipronil showed a statistically significant decline in the absolute and relative weight of the thyroid (Table 3).

The ovaries and uterus analysis by light microscopy did not reveal any morphological changes related to the treatment (data not shown). The ovarian follicles and corpora lutea numbers and the uterine endometrial height were similar among the experimental groups (Table 4).

The lordosis quotient also showed no differences among the groups (Figure 3). Similarly, the reproductive performance was not affected after perinatal exposure to fipronil (Table 5). In terms of hormone dosage, although there was a tendency for augmented estradiol levels in animals exposed to the highest dose of fipronil (3 mg/kg), this increase was not statistically significant ($p = 0.0561$). The groups also showed similar progesterone levels (Figure 4).

4. Discussion

The developing organism is highly dependent on sex steroids and thyroid hormones for its maturation; thus, the fetus and newborn are very sensitive to any change in their hormonal environment. According Fudvoye et al. (2014), studies have identified the perinatal period as the most sensitive to these effects; however, most of the reported effects were observed later in life. In the present study, we observed that the exposure of female rats to fipronil during the perinatal period can interfere in their reproductive development, albeit without affecting their adult reproductive parameters.

Female offspring exposed to fipronil showed no signs or symptoms of toxicity during the treatment, as expressed by body mass on postnatal days 1 and 22. Similarly, the anogenital distance (AGD) and the number of nipples did not differ among experimental groups. It has long been known that, in rodents, the AGD and nipple count are important parameters for the evaluation of endocrine-disrupting substances (Wolf et al., 2004; Liu et al., 2014). The AGD, in particular, reflects fetal androgen action (Wolf et al., 1999) and can be used as a biomarker of exposure to androgen. Animal studies have shown that this parameter is a sensitive indicator of prenatal exposure to androgens, and can be used to retrospectively assess fetal exposure to androgen (Hsieh et al., 2008). In this regard, exposure to endocrine disruptors with androgenic or anti-androgenic activity can cause the AGD to decrease (in males) or increase (in females) (Liu et al., 2014). Based on the absence of change in the AGD in this study, it is suggested that fipronil did not present androgenic activity. However, other variables should also be evaluated.

Multiple genetic and environmental factors, particularly endocrine-disrupting chemicals, can influence the physiological age range of the onset of puberty (Parent et al., 2003). In rodents, the ages of vaginal opening and first estrus indicate the onset of

puberty. This event is characterized by rapid physiological changes, such as growth and maturation of the gonads and brain. The main hormone involved in the regulation of onset of puberty is gonadotropin-releasing hormone (GnRH) from the hypothalamus, which stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the anterior pituitary (Terasawa & Fernandez, 2001). These gonadotropins, in turn, act in the ovaries, inducing an increase in estradiol levels (Walker Jr. & Homberger, 1997). As the vaginal opening and first estrus are dependent on increased estradiol during puberty, a delay in this process may suggest antiestrogen or androgenic activities of a substance. However, other factors that are not specifically controlled by estrogen/androgen may also influence the timing of normal puberty (Grande et al., 2006).

In this study, we detected delayed pubertal onset (vaginal opening and first estrus) in response to the highest dose of fipronil. Some studies show that this compound exhibits antiestrogenic (Lu et al., 2014) and antiandrogen (AIT-Aïssa et al., 2010) activities. Thus, this delay may be due to dysfunction of the hypothalamus–pituitary–gonad axis caused by exposure to fipronil. Furthermore, these disorders may be related to changes in receptor expression, as well as morphological changes in hypothalamic nuclei relevant to the female reproductive system.

Estrous cycle monitoring is a basic method of evaluating the reproductive capacity of a female animal, and abnormality in reproductive function is often associated with estrus disruption (Shaikh et al., 1971). It has been demonstrated that change in estrous cyclicity and cycle length may be related to stress, light/dark cycle (Everett et al., 1989), the presence of males (Mora et al, 1985; Barkley et al, 1993) and prolactin and progesterone levels (Van der Schoot & Uilenbroek, 1983; Sanchez-Criado, 1996). In adult life, the female offspring exposed to a dose of 0.3 mg/kg fipronil

showed changes in the estrous cycle, such as an increase in estrous cycle length and a decrease in the number of cycles. Ohi et al. (2004) also demonstrated that fipronil treatment disrupted the normal cyclicity of female rats, which showed an increase in the estrous cycle length, with a persistent diestrus. The aforementioned authors also observed a decrease in estradiol and increase of progesterone levels after exposure to fipronil, and attributed these results to the stimulatory effect of the GABAergic action of fipronil at pituitary level. In our study, differences in estradiol or progesterone levels among the groups were not detected. It is possible that conflicting results may be due to differences in the experimental protocol of these two studies, such as the treatment period, dosages and administration route.

Although the female offspring showed disturbed pubertal onset and cyclicity, most of the reproductive organs showed no differences in weight. However, the thyroid of the animals exposed to the highest dose of fipronil presented a significant reduction in weight, possibly indicating alterations in the functioning of this gland, such as hormone production. This can be explained by the ability of fipronil to deregulate the thyroid hormones, as has been noted in several studies (Abend et al., 1991; Williams, 1995; WHO/FAO, 1997; Hurley, 1998; Hood et al., 1999). Although the thyroid hormones (T_3 and T_4) were not dosed in this study, the change observed in the weight of this organ may be attributed to a deficiency in hormone production. Viluksela et al. (2014) detected a decrease in thyroid weight and correlated it with a reduction in T_3 and T_4 levels observed in rats exposed to polychlorinated biphenyls (PCBs).

The classical view of female brain sexual differentiation is backed by the idea of non-participation of estradiol in brain sexual differentiation, through the protection afforded by alpha-fetoprotein (AFP) to areas of the hypothalamus. However, it has been postulated that estradiol can participate in female brain differentiation, and that

antiandrogenic and antiestrogenic substances may also compromise this event, and consequently, sexual behavior (Bakker & Baum, 2008).

Numerous mechanisms of action of estradiol act upon regions of the hypothalamus during the period of brain sexual differentiation. Estradiol-induced sex differences in the arcuate nucleus, a small nucleus that regulates feeding (Stricker-Krongrad et al., 1998), anterior pituitary function (Micevych et al., 2003), and female sexual receptivity (Dewing et al., 2007) require GABAergic transmission (Sinchak et al., 2013). Since fipronil can act as an antagonist of GABA receptors, this process of differentiation could be affected. However, in the classical view of female sexual differentiation, this event would be relevant only to the male brain, since estradiol in females would not participate in this process owing to the action of AFP. In females, changes in this nucleus would probably be detectable after exposure to GABAergic agonists or estrogenic substances, which does not seem to be the case of fipronil. This could explain the absence of changes in the sexual behavior of rats in this study, due to the mechanism of action of fipronil.

In addition to participating in the brain differentiation process, the sex steroids also influence the growth, differentiation and function of female reproductive organs (Boutin & Cunha, 1997). The main period of differentiation and maturation of the uterus occurs in rodents after birth (Brody & Cunha, 1989), and exposure to steroid hormones during this period may compromise endometrial adrenogenesis (Crain et al., 2008). EDs with androgenic activity can also induce uterine abnormalities when administered to rats during the intrauterine period (Lobl & Gorski, 1974; Pinilla et al., 1993; Wolf et al., 2002; Hotchkiss et al., 2007). Our results indicated that there was no impairment in the uterus and ovary histology, indicating that differentiation of the female reproductive tract occurred normally. These data corroborate those obtained by

estradiol and progesterone assays, which were not compromised by exposure to fipronil. Similarly, the changes observed in puberty did not affect the fertility of these females in adult life. These results are similar to those reported by Ohi et al. (2004), who suggested that fipronil does not interfere in the regular development of the fetus after implantation, since significant differences were not observed in the fetal resorption rate, average number newborns per brood, and average body mass at birth.

It can be concluded that, in these experimental conditions, fipronil was able to disrupt the development of the female reproductive system, since it altered the onset of puberty and the normal cyclicity of the animals. However, these changes did not persist into adulthood, since the adult reproductive parameters were not affected. Further studies are needed to determine how the mechanisms of fipronil toxicity affect the female reproductive functions.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Figures and tables

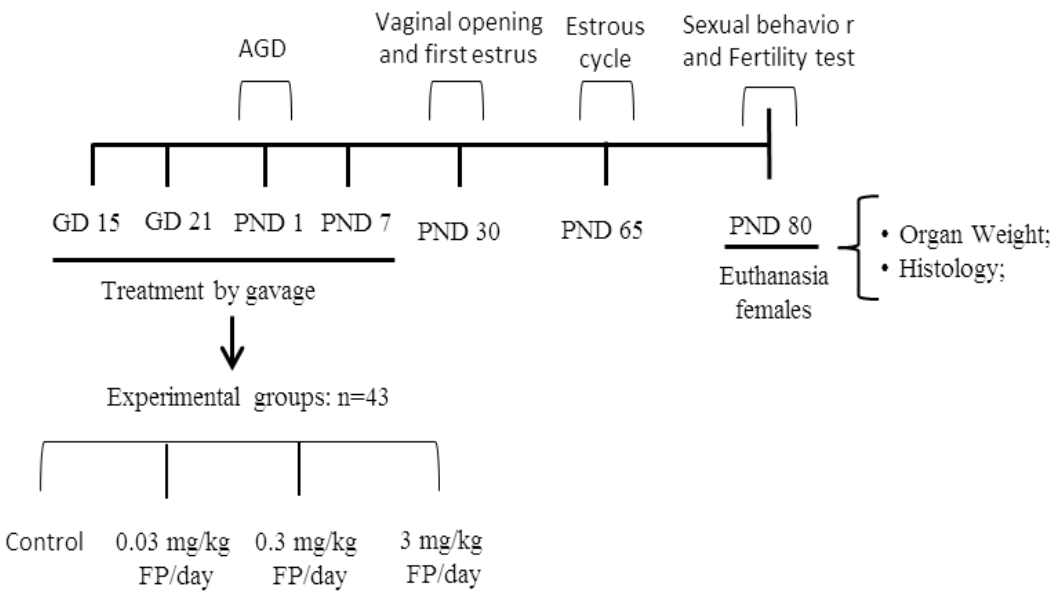

Figure 1 – Experimental design

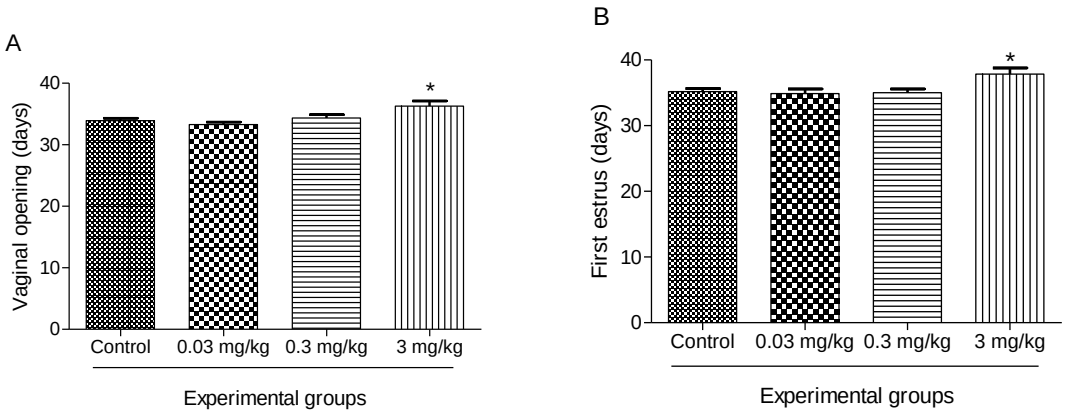

Figure 2 – Vaginal opening (A) and first estrus (B) in control and exposed 0.03, 0.3 and 3 mg/kg fipronil. Values are expressed as mean ± SEM, 9-12 rats/group. * $p < 0.05$. ANOVA followed by Tukey test.

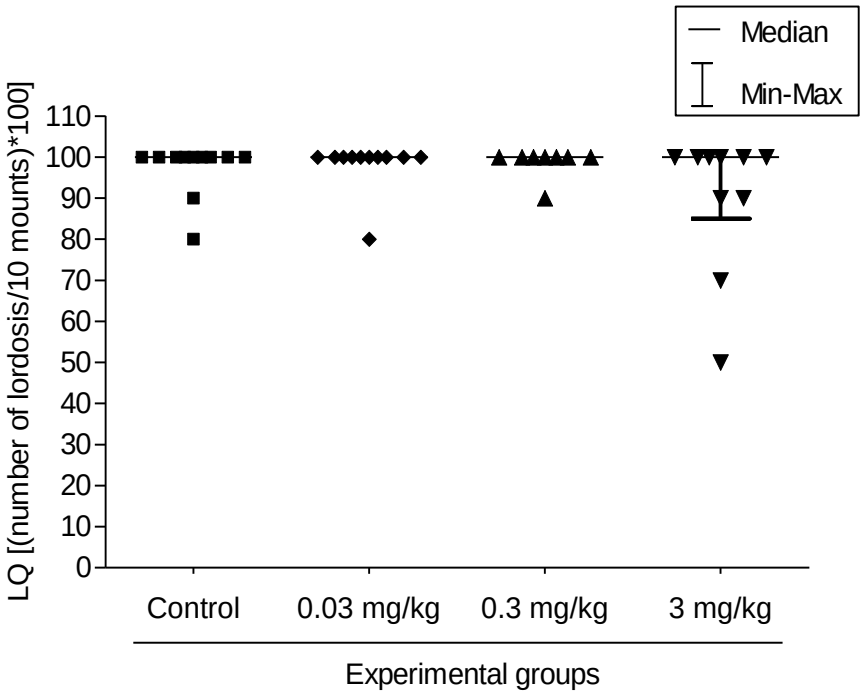

Figure 3 - Lordosis quociente (LQ), obtained by sexual behavior test, from famale at PND 80, on per litter (8 – 11 rats/group), at estrus phases. Values expressed as median and interquartile range.

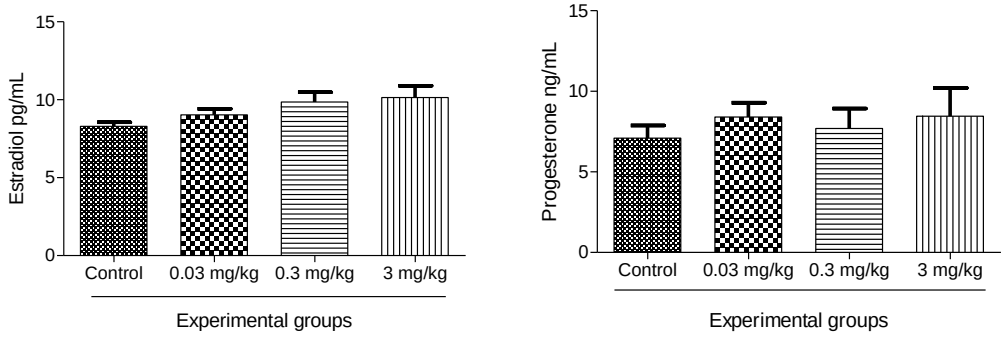

Figure 4 - Serum estradiol and progesterone levels of adult rats exposed to 0.03, 0.3 and 3 mg/kg fipronil. Values are expressed as mean ± SEM, 9-12 rats/group. $p > 0.05$ by ANOVA.

Table 1 – Body weight, relative anogenital distance (AGD) (at birth and at PND 22) and nipple counting at PND 13 of females offspring exposed to 0.03, 0.3 and 3 mg/kg of fipronil.

Groups	Body weight (g)		Relative AGD (mm/g$^{1/3}$)		Number of nipples[A]
	At birth	PND 22	At birth	PND 22	
Control	6.75 ± 0.14	45.36 ± 1.24	1.22 ± 0.02	2.20 ± 0.04	12.00(12-12)
0.03 mg/kg	7.32± 0.24	44.11 ± 1.52	1.20 ± 0.03	2.14 ± 0.03	12.00 (12-12)
0.3 mg/kg	6.83 ± 0.15	43.68 ± 1.66	1.36 ± 0.11	2.21 ± 0.06	12.00 (12-12.12)
3 mg/kg	6.71 ± 0.19	41.45 ± 1.88	1.19 ± 0.01	2.29 ± 0.08	12.00 (12-12.25)

Values are expressed as mean ± SEM, 9-12 rats/group. $p > 0.05$ by ANOVA.

[A]Values expressed as median and interquartile range.

Table 2 - Estrous cyclicity of female rats exposed to 0.03, 0.3 and 3 mg/kg fipronil.

	Experimental Groups			
	Control	**0.03 mg/kg**	**0.3 mg/kg**	**3 mg/kg**
Proestrus	3.70 ± 0.19	3.08 ± 0.14	3.25 ± 0.23	3.36 ± 0.12
Estrus	4.53 ± 0.15	4.60 ± 0.16	4.64 ± 0.18	4.58 ± 0.27
Metaestrus	1.62 ± 0.14	1.96 ± 0.07	2.13 ± 0.14	1.83 ± 0.18
Diestrus	4.52 ± 0.19	4.93 ± 0.16	4.36 ± 0.14	5.03 ± 0.17
Number of estrous cycles	2.96 ± 0.05	2.81 ± 0.09	2.54 ± 0.11*	2.92 ± 0.07
Estous cycle length (days)	4.19 ± 0.06	4.35 ± 0.14	4.81 ± 0.26*	4.18 ± 0.10

Values are expressed as mean ± SEM, 9-12 rats/group. * $p < 0.05$. ANOVA followed by Tukey test.

Table 3 - Final body weight and absolute and relative organ weights of rats exposed to 0.03, 0.3 and 3 mg/kg of fipronil.

Parameters	Experimental Groups			
	Control	0.03 mg/kg	0.3 mg/kg	3 mg/kg
Body Weight (g)	260.93± 3.82	237.34 ± 2.99*	248.51 ± 5.45	257.11 ± 5.39
Brain (g)	1.75 ± 0.04	1.78 ± 0.03	1.78 ± 0.03	1.80 ± 0.03
Brain (mg/100g)	0.67 ± 0.01	0.74 ± 0.01	0.72 ± 0.02	0.70 ± 0.01
Pituitary (mg)	11.74 ± 0.39	12.10 ± 0.73	11.54 ± 0.84	12.39 ± 0.84
Pituitary (mg/100g)	4.50 ± 0.14	5.21 ± 0.33	4.68 ± 0.37	4.81 ± 0.31
Thyroid (mg)	17.22 ± 0.79	15.90 ± 0.84	17.81 ± 1.35	13.16 ± 0.77*
Thyroid (mg/100g)	6.62 ± 0.33	6.85 ± 0.38	6.73 ± 0.59	5.11 ± 0.28*
Liver (g)	9.99 ± 0.22	9.07 ± 0.34	9.11 ± 0.28	9.65 ± 0.27
Liver (g/100g)	3.82 ± 0.06	3.89 ± 0.11	3.66 ± 0.08	3.76 ± 0.10
Right kidney (g)	0.93 ± 0.02	0.91 ± 0.02	0.88 ± 0.01	0.95 ± 0.02
Right kidney (g/100g)	0.35 ± 0.00	0.39 ± 0.00	0.35 ± 0.00	0.37 ± 0.00
Left kidney (g)	0.91 ± 0.01	0.87 ± 0.02	0.87 ± 0.01	0.90 ± 0.02
Left kidney (g/100g)	0.35 ± 0.00	0.37 ± 0.00	0.35 ± 0.00	0.35 ± 0.00
Left adrenal (mg)	44.95 ± 1.93	43.49 ± 2.40	45.44 ± 2.59	44.48 ± 2.97
Left adrenal (mg/100g)	17.21 ± 0.66	18.62 ± 0.87	18.37 ± 1.14	17.24 ± 1.03
Left adrenal (mg)	50.05 ± 2.11	47.49 ± 2.45	46.89 ± 2.58	47.49 ± 2.62
Left adrenal (mg/100g)	19.17 ± 0.73	20.36 ± 0.93	18.90 ± 1.01	18.44 ± 0.88
Úterus (g)	0.44 ± 0,02	0.49 ± 0.03	0.42 ± 0.02	0.47 ± 0.04
Úterus (g/100g)	0.17 ± 0.00	0.21 ± 0.01	0.17 ± 0.01	0.18 ± 0.01
Right ovary (mg)	62.97 ± 2.17	61.90 ± 2.59	54.33 ± 5.98	57.90 ± 2.24
Right ovary (mg/100g)	24.18 ± 0.87	26.76 ± 1.54	24.24 ± 1.44	22.52 ± 0.72
Left ovary (mg)	56.40 ± 1.71	58.41 ± 2.40	56.42 ± 2.22	57.27 ± 3.32
Left ovary (mg/100g)	21.70 ± 0.79	25.25 ± 1.38	22.70 ± 1.02	22.31 ± 1.29

Values are expressed as mean ± SEM, 9-12 rats/group. * $p < 0.05$. ANOVA followed by Tukey test.

Table 4 - Ovarian follicle and corpora lutea counting and uterine endometrium thickness (μm) of adult rats exposed to 0.03, 0.3 and 3 mg/kg of fipronil.

Structures	Experimental Groups			
	Control	**0.03 mg/kg**	**0.3 mg/kg**	**3 mg/kg**
Primordial and primary follicles	15.36 ± 1.42	14.85 ± 1.56	15.45 ± 1.84	15.70 ± 1.94
Pre-antral follicles	3.63 ± 0.38	2.70 ± 0.48	3.70 ± 0.69	3.86 ± 0.55
Antral follicles	8.93 ± 1.80	7.43 ± 0.95	9.83 ± 1.86	10.70 ± 1.98
Corpora lutea	7.50 ± 0.85	7.93 ± 0.96	6.20 ± 0.77	8.46 ± 1.50
Uterine endometrium thickness	518.72 ± 14.36	544.72 ± 21.38	483.02 ± 17.33	532.56 ± 18.10

Values are expressed as mean ± SEM, 9-12 rats/group. $p > 0.05$ by ANOVA.

Table 5 – Fertility test of female after natural mating of rats exposed to 0.03, 0.3 and 3 mg/kg for fipronil.

Parameters	Experimental Groups			
	Control	**0.03 mg/kg**	**0.3 mg/kg**	**3 mg/kg**
[1]Body Weight of mothers (g)	394.45 ± 14.98	383.04 ± 22.80	358.14 ± 16.89	392.01 ± 20.63
[1]Uterine weight fetuses	65.36 ±3.43	74.75±2.36	65.57±5.90	67.82±5.18
[1]Number of corpora lutea	14.77 ± 0.40	16.12 ± 0.29	14.37 ± 0.62	14.28 ± 0.42
[1]Number of implantations	13.80 ± 0.69	15.37 ± 0.26	13.62 ± 1.01	13.71 ± 0.68
[1]Number of resorptions	0.70 ± 0.26	0.12 ± 0.12	1.00 ± 0.73	0.42 ± 0.42
[1]Number of live fetuses	13.10 ± 0.65	15.00 ± 0.32	12.37 ± 1.23	13.14 ± 0.76
[1]Weight of fetus (g)	2.98 ± 0.05	2.94 ± 0.06	2.88 ± 0.13	3.01 ± 0.07
[2]Pregnancy rate (%)	90.9	80	80	75
[2]Sex ratio (%)	150.00 (100 - 166.66)	82.63 (60 - 116.66)	116.67 (82.29. 165)	100.00 (86.60 - 115.47)
[2]Fertility potential (%)	100 (93.08-100)	94.11 (93.64-100)	100 (96.42-100)	100 (96.87-100)
[2]Pre-implantation loss (%)	0.00 (0.00-0.00)	1.00 (0.75-1.00)	0.00 (0.00-0.50)	0.00 (0.00-0.50)
[2]Post-implantation loss (%)	1.00 (0.00-1.75)	0.00 (0.00-0.25)	0.50 (0.00-1.25)	0.00 (0.00-0.50)

[1]Values are expressed as mean ± SEM, 10-12 rats/group. $p > 0.05$ by ANOVA.

[A]Values expressed as median and interquartile range.

Conclusão

Concluiu-se que o fipronil, nestas condições experimentais, foi capaz de perturbar o desenvolvimento reprodutivo de fêmeas, sem afetar os parâmetros reprodutivos tardios. Em machos, os resultados demonstraram que a exposição perinatal ao fipronil tem efeitos a longo prazo sobre parâmetros espermáticos, e que o epidídimo pode ser um órgão-alvo. Entretanto, estas alterações não afetaram a fertilidade destes animais, devido provavelmente a grande eficiência reprodutiva que os roedores apresentam. Sobretudo, tais resultados demostram os potencias efeitos tardios deste inseticida sobre a reprodução.

Apêndice

As figuras e tabelas pertencentes a esta seção não foram incluídas nos manuscritos, entretanto as análises foram realizadas durante a execução do projeto de pesquisa que originou esta tese.

Peso corpóreo, consumo de água e ração durante o tratamento

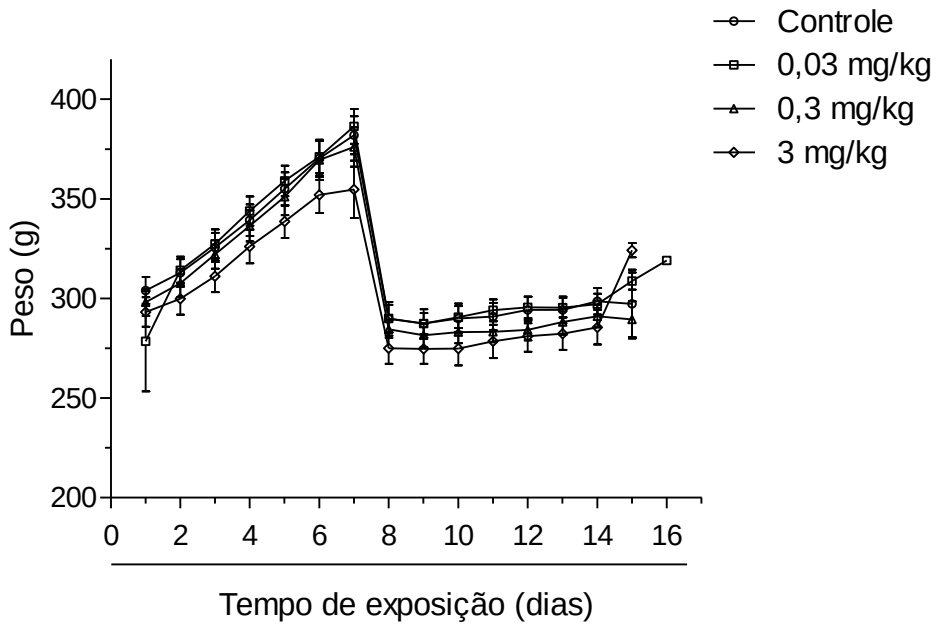

Figura 1 – Evolução de massa corporal (g) durante o tratamento de ratas dos grupos controle e expostas a 0,03, 0,3 e 3 mg/kg de fipronil. Valores expressos como média ± erro padrão da média de 9 - 12 animais/grupo. Teste análise de variância (ANOVA) com "a posteriori" de Tukey.

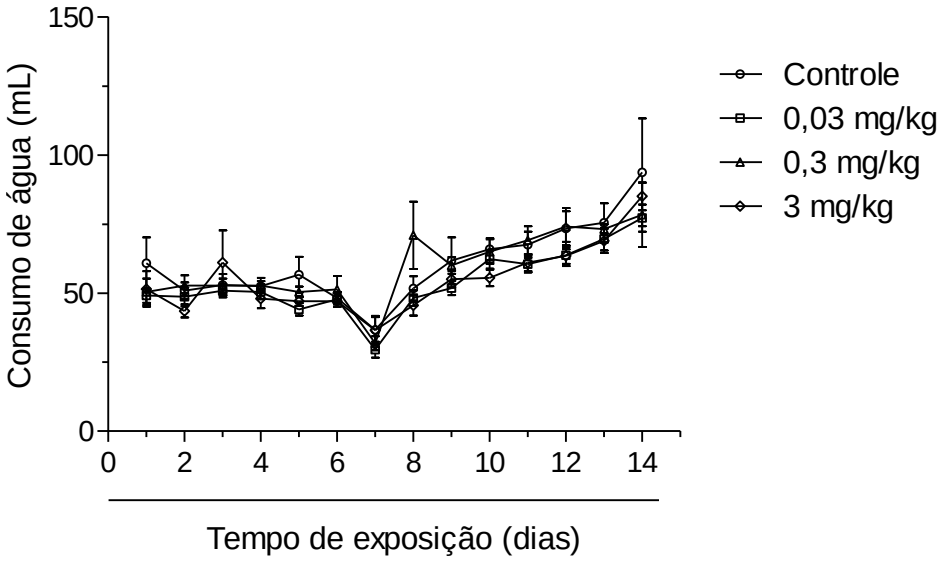

Figura 2 – Consumo de água (mL) durante o tratamento de ratas expostas a 0,03, 0,3 e 3 mg/kg de fipronil. Valores expressos como média ± EPM, 9 - 12 ratas/grupo. $p > 0.05$ por ANOVA.

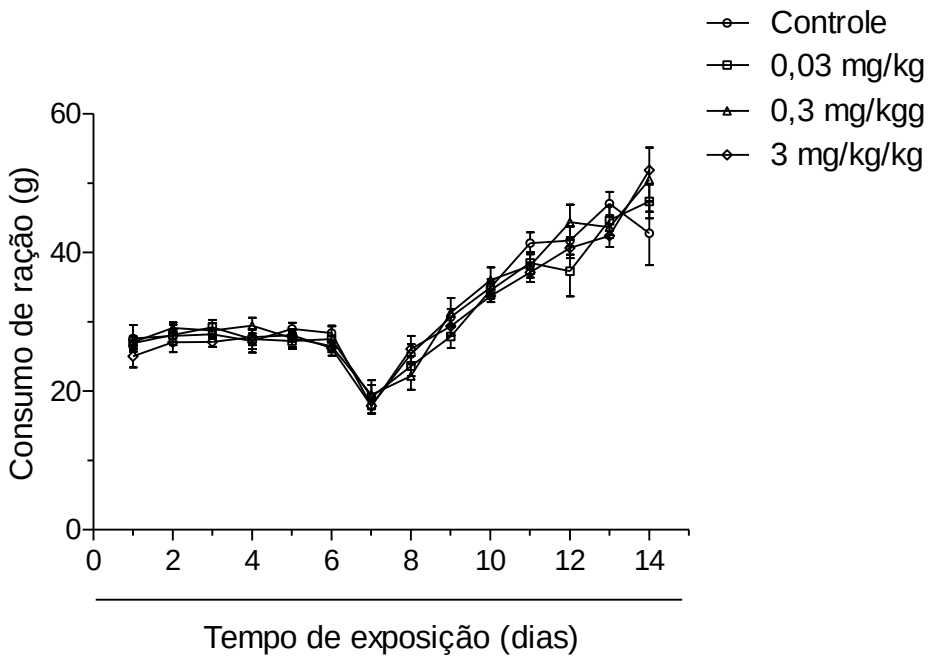

Figura 3 – Consumo de ração (g) durante o tratamento de ratas expostas a 0,03, 0,3 e 3 mg/kg de fipronil. Valores expressos como média ± EPM, 9 - 12 ratas/grupo. $p > 0.05$ por ANOVA.

Parâmetros bioquímicos e peso de órgãos das ratas após desmame expostas ao fipronil

Tabela 1 – Parâmetros bioquímicos de ratas adultas expostas a 0,03, 0,3 e 3 mg/kg de fipronil.

Parâmetros	**Grupos Experimentais**			
	Controle	**0,03 mg/kg**	**0,3 mg/kg**	**3 mg/kg**
ALT (IU/L)	92,90 ± 4,09	99,00 ± 5,35	94,80 ± 8,37	86,10 ± 5,49
AST (IU/L)	113,45 ± 2,89	116,40 ± 3,82	114,30 ± 3,31	120,89 ± 6,92
FA (IU/L)	276,18 ± 29,34	234,60 ± 29,95	209,13 ± 25,45	188,40 ± 29,62
GGT (IU/L)	2,19 ± 0,31	2,23 ± 0,27	2,13 ± 0,23	2,04 ± 0,25
Ureia (mg/dL)	65,18 ± 3,04	64,80 ± 4,22	70,20 ± 2,99	59,90 ± 2,82
Creatinina (mg/dL)	0,50 ± 0,02	0,49 ± 0,02	0,52 ± 0,03	0,49 ± 0,03
Sódio (mEq/L)	142,27 ± 0,88	142,20 ± 0,53	143,30 ± 0,78	142,10 ± 0,97
Potássio (mEq/L)	6,26 ± 0,26	6,38 ± 0,44	6,75 ± 0,35	6,17 ± 0,18
Cálcio (mg/dL)	9,44 ± 0,31	9,32 ± 0,41	9,99 ± 0,27	9,36 ± 0,31
Colesterol (mg/dL)	95,09 ± 4,94	80,50 ± 4,09	81,50 ± 4,88	77,77 ± 6,92
Glicose (mg/dL)	203,36 ± 11,71	189,90 ± 17,26	166,30 ± 11,92	180,60 ± 11,78
Proteína Total (g/dL)	6,25 ± 0,12	6,01 ± 0,14	6,21 ± 0,12	6,14 ± 0,12
Albumina (g/dL)	3,44 ± 0,04	3,38 ± 0,07	3,35 ± 0,04	3,45 ± 0,05

Valores expressos como média ± EPM, 9 – 12 ratos/grupo. $p > 0.05$ por ANOVA.

Tabela 2 - Peso corporal final e pesos absoluto e relativo de órgãos de ratas adultas (mães) expostas a 0,03, 0,3 e 3 mg/kg de fipronil.

Parâmetros	**Grupos Experimentais**			
	Controle	**0,03 mg/kg**	**0,3 mg/kg**	**3 mg/kg**
Peso Corporal (g)	259,18 ± 5,39	260,32 ± 5,74	259,72 ± 6,21	257,19 ± 7,28
Cérebro (g)	1,87 ± 0,02	1,92 ± 0,06	1,84 ± 0,02	1,81 ± 0,02
Cérebro (g/100g)	0,72 ± 0,01	0,73 ± 0,02	0,71 ± 0,01	0,71 ± 0,01
Hipófise (mg)	10.21± 0.50	7.37 ± 0.78 *	8.02 ± 0.49	9.28 ± 1.15
Hipófise (mg/100g)	3.94 ± 0.17	2.89 ± 0,03	3.09 ± 0.18	3.73 ± 0.57
Tireoide (mg)	13.73 ± 1.48	16.18 ± 1.81	13.93 ± 1.05	14.77 ± 1.67
Tireoide (mg/100g)	5,34 ± 0,60	6,24 ± 0,69	5,41 ± 0,48	5,71 ± 0,56
Coração (g)	1,17 ± 0,05	1,12 ± 0,02	1,14 ± 0,02	1,15 ± 0,04
Coração (g/100g)	0,45 ± 0,01	0,43 ± 0,00	0,43 ± 0,00	0,45 ± 0,02
Pulmão (g)	1,53 ± 0,05	1,62 ± 0,07	1,60 ± 0,03	1,61 ± 0,07
Pulmão (g/100g)	0,59 ± 0,01	0,62 ± 0,02	0,61 ± 0,01	0,63 ± 0,03
Fígado (g)	11,74 ± 0,36	11,61 ± 0,45	11,90 ± 0,42	11,32 ± 0,32
Fígado (g/100g)	4,52 ± 0,08	4,41 ± 0,10	4,57 ± 0,08	4,41 ± 0,10
Rim direito (g)	0,96 ± 0,02	0,94 ± 0,02	0,95 ± 0,02	1,03 ± 0,10
Rim direito (g/100g)	0,37 ± 0,00	0,36 ± 0,00	0,36 ± 0,00	0,40 ± 0,03
Rim esquerdo (g)	0,93 ± 0,02	0,91 ± 0,02	0,92 ± 0,02	0,92 ± 0,03
Rim esquerdo (g/100g)	0,36 ± 0,00	0,35 ± 0,00	0,35 ± 0,00	0,36 ± 0,00
Adrenal direita (mg)	42,55 ± 2,80	38,84 ± 1,87	42,22 ± 1,90	38,88 ± 1,97
Adrenal direita (mg/100g)	16,41 ± 1,01	14,90 ± 0,58	16,26 ± 0,66	15,13 ± 0,65
Adrenal esquerda (mg)	47,30 ± 2,70	45,10 ± 2,48	50,24 ± 2,63	48,69 ± 1,76
Adrenal esquerda (mg/100g)	18,25 ± 0,96	17,29 ± 0,79	19,34 ± 0,90	18,95 ± 0,49
Baço (g)	0,56 ± 0,03	0,51 ± 0,03	0,54 ± 0,03	0,54 ± 0,05
Baço (g/100g)	0,21 ± 0,01	0,19 ± 0,01	0,21 ± 0,01	0,20 ± 0,01
Útero (g)	0,28 ± 0,02	0,34 ± 0,04	0,25 ± 0,04	0,30 ± 0,02
Útero (g/100g)	0,11 ± 0,01	0,13 ± 0,01	0,09 ± 0,01	0,11 ± 0,00
Ovário direito(mg)	50,30 ± 3,18	51,60 ± 1,96	47,97 ± 3,91	45,37 ± 3,99
Ovário direito (mg/100g)	19,43 ± 1,19	19,90 ± 0,83	18,40 ± 1,31	17,51 ± 1,36
Ovário esquerdo (mg)	45,04 ± 2,00	42,14 ± 4,19	52,07 ± 2,76	48,22 ± 1,85
Ovário esquerdo (mg/100g)	17,42 ± 0,75	16,43 ± 1,39	19,89 ± 0,87	18,77 ± 0,60

Valores expressos como média ± EPM, 9 - 12 ratas/grupo. * p <0,05. ANOVA seguido pelo teste de Tukey.

Parâmetros iniciais e de instalação de puberdade dos descendentes machos

Tabela 3 - Peso corporal, distância anogenital absoluta (ao nascer e aos 22 dias de idade) e idade dos filhotes machos em relação à descida testicular dos grupos expostos a 0,03, 0,3 e 3 mg/kg de fipronil.

	Massa Corporal (g)		Distância anogenital (mm)		Descida Testicular (dias)
Grupos	**ao nascer**	**22 dias**	**ao nascer**	**22 dias**	
Controle	7,05 ± 0,16	45,17 ±1,15	4,43 ± 0,04	17,85 ± 0,11	15,89 ± 0,18
0,03 mg/kg	7,68 ± 0,22	46,55 ±0,94	4,50 ±0,05	18,32 ± 0,29	15,64 ± 0,11
0,3 mg/kg	7,29 ± 0,13	45,69 ±1,61	4,46 ± 0,08	18,50 ± 0,38	16,10 ± 0,20
3 mg/kg	7,18 ± 0,16	44,00 ±1,52	4,43 ± 0,06	17,95 ± 0,47	15,85 ± 0,15

Valores expressos como média ± EPM, 10 – 12 ratos/grupo. $p > 0.05$ por ANOVA.

Tabela 4 - Peso corporal e idade dos filhotes machos em relação à separação prepucial dos grupos expostos a 0,03, 0,3 e 3 mg/kg de fipronil.

Grupos	Massa Corporal (g)	Separação Prepucial (dias)
Controle	186,20 ± 3,25	42,16 ± 0,32
0,03 mg/kg	184,88 ± 3,28	42,13 ± 0,40
0,3 mg/kg	192,90 ± 3,20	43,24 ± 0,47
3 mg/kg	189,97 ± 2,94	42,74 ± 0,37

Valores expressos como média ± EPM, 10 – 12 ratos/grupo. $p > 0.05$ por ANOVA.

Tabela 5 - Peso corporal final e pesos absoluto e relativo de órgãos de ratos machos com 45 dias de idade expostos a 0,03, 0,3 e 3 mg/kg de fipronil.

Parâmetros	Grupos Experimentais			
	Controle	**0,03 mg/kg**	**0,3 mg/kg**	**3 mg/kg**
Peso Corporal Final (g)	217,80 ± 4,70	215,20 ± 4,47	208,86 ± 7,61	215,52 ± 5,18
Cérebro (g)	1,76 ± 0,02	1,73 ± 0,03	1,68 ± 0,03	1,74 ± 0,01
Cérebro (mg/100g)	0,81 ± 0,01	0,80 ± 0,02	0,81 ± 0,02	0,81 ± 0,02
Hipófise (mg)	7,29 ± 0,26	6,35± 0,35	6,60 ± 0,40	6,88 ± 0,83
Hipófise (mg/100g)	3,32 ± 0,11	2,75 ± 0,23	3,21 ± 0,24	3,15 ± 0,34
Tireoide (mg)	12,92 ± 0,94	13,22 ± 1,18	12,28 ± 0,48	13,17 ± 0,69
Tireoide (mg/100g)	5,94 ± 0,43	6,18 ± 0,57	5,96 ± 0,37	6,13 ± 0,34
Fígado (g)	11,20 ± 0,29	11,04 ± 0,33	10,83 ± 0,52	11,36 ± 0,34
Fígado (g/100g)	5,14 ± 0,07	5,13 ± 0,12	5,17 ± 0,09	5,27 ± 0,07
Rim direito (g)	0,94 ± 0,02	0,90 ± 0,01	0,86 ± 0,02	0,98 ± 0,02
Rim direito (g/100g)	0,43 ± 0,01	0,42 ± 0,00	0,41 ± 0,01	0,45 ± 0,00
Rim esquerdo (g)	0,93 ± 0,02	0,90 ± 0,01	0,84 ± 0,02	0,96 ± 0,02
Rim esquerdo (g/100g)	0,43 ± 0,00	0,41 ± 0,01	0,40 ± 0,01	0,44 ± 0,00
Adrenal direita (mg)	18,99 ± 0,62	17,67 ± 0,98	17,85 ± 1,06	18,29 ± 1,33
Adrenal direita (mg/100g)	8,73 ± 0,25	8,17 ± 0,35	8,56 ± 0,42	8,48 ± 0,59
Adrenal esquerda (mg)	21,17 ± 0,99	18,93 ± 1,41	18,66 ± 1,09	21,85 ± 0,91
Adrenal esquerda (mg/100g)	9,77 ± 0,55	8,77 ± 0,60	8,93 ± 0,39	10,13 ± 0,33
Testículo (g)	0,98 ± 0,02	1,00 ± 0,02	1,00 ± 0,03	0,91 ± 0,04
Testículo (g/100g)	0,45 ± 0,00	0,46 ± 0,01	0,48 ± 0,01	0,42 ± 0,02
Epidídimo (mg)	124,65 ± 6,70	137,26 ± 5,14	129,02 ± 6,89	127,41 ± 4,15
Epidídimo (mg/100g)	57,14 ± 2,65	63,79 ± 2,01	62,30 ± 2,86	59,27 ± 1,90
Próstata (mg)	109,37 ± 6,11	113,17 ± 4,44	99,45 ± 9,42	102,70 ± 9,41
Próstata (mg/100g)	50,33 ± 2,81	52,84 ± 2,50	49,37 ± 4,26	50,22 ± 4,26
Vesícula Seminal (mg)	114,45 ± 12,24	121,53 ± 10,83	110,38 ± 9,10	121,12 ± 10,47
Vesícula Seminal (mg/100g)	51,78 ± 4,73	56,77 ± 5,33	53,20 ± 4,18	56,15 ± 4,42

Valores expressos como média ± EPM, 9 – 11 ratos/grupo. $p > 0.05$ por ANOVA.

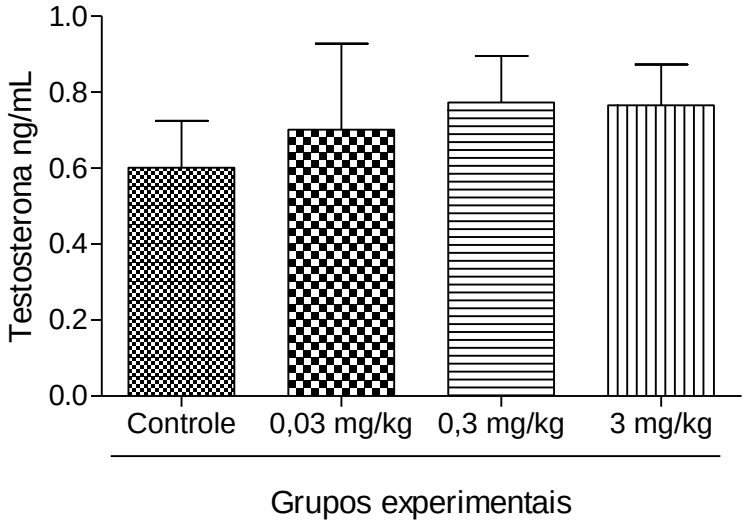

Figure 4 – Níveis séricos de testosterona de ratos com 45 dias de idade expostos a 0,03, 0,3 e 3 mg/kg de fipronil. Valores expressos como média ± EPM, 8 – 11 ratos/grupo. $p > 0.05$ por ANOVA.

Análise histológica do testículo de ratos machos adultos

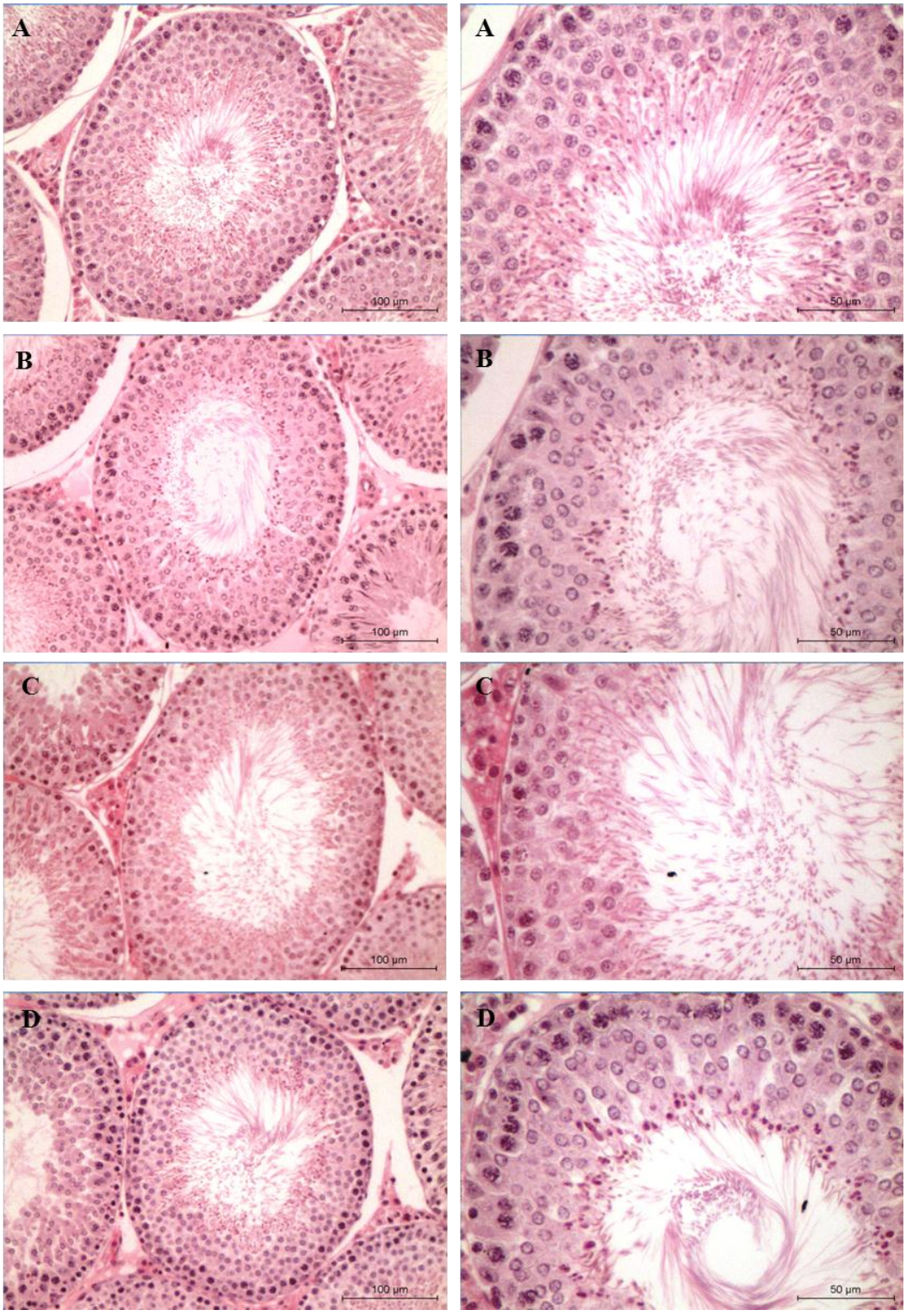

Figura 5 - Fotomicrografias de cortes transversais de testículo de ratos adultos no estágio VIII da espermatogênese. (A) Grupo Controle, (B) Grupo tratado com 0,03 mg/kg/dia de fipronil, (C) Grupo tratado com 0,3 mg/kg/dia de fipronil, (D) Grupo tratado com 3 mg/kg/dia de fipronil. Coloração em HE. 20x e 40x.

Análise histológica do epidídimo, região segmento inicial, de ratos machos adultos

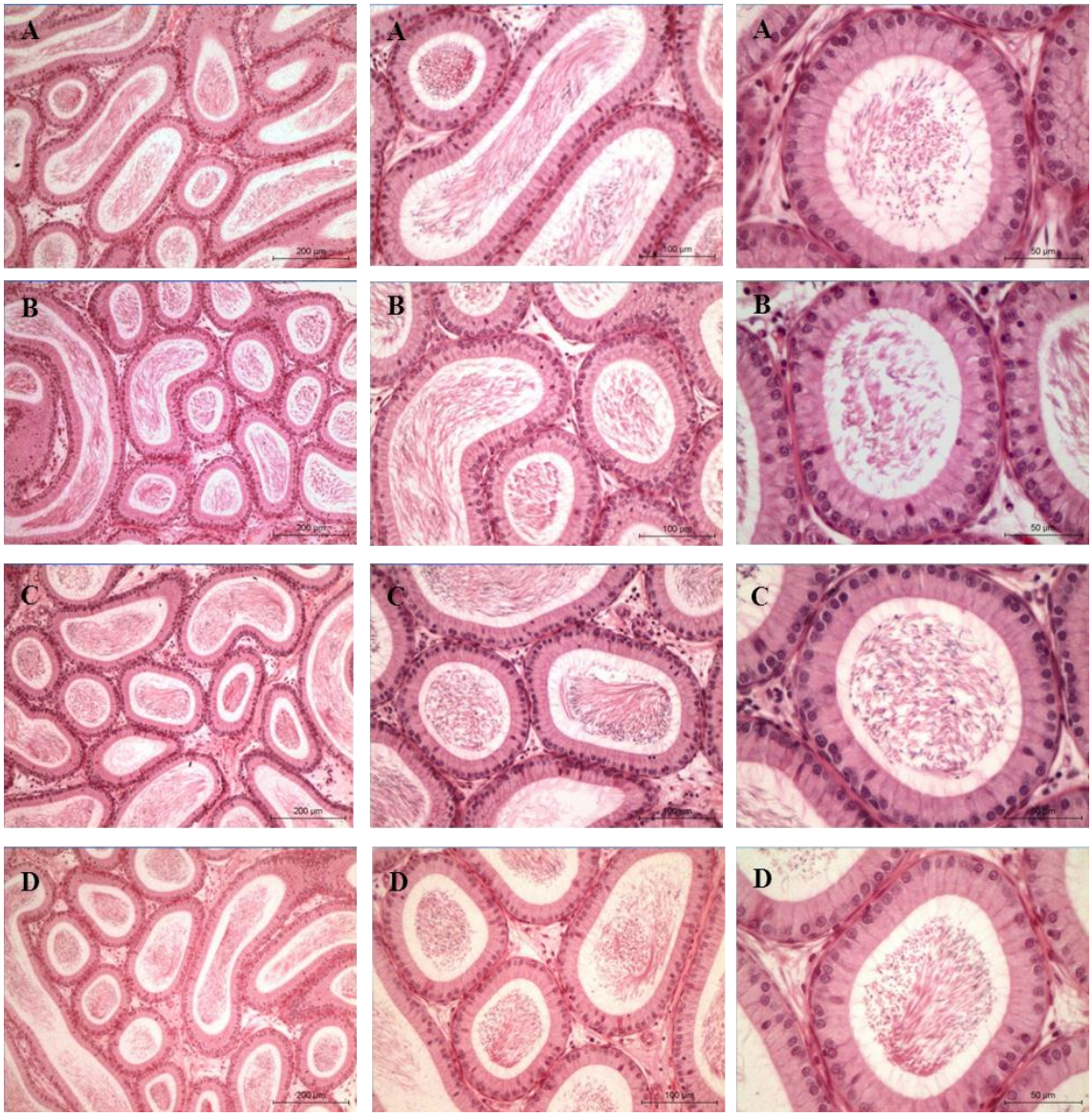

Figura 6 - Fotomicrografias de cortes longitudinais do segmento inicial do epidídimo de ratos adultos. (A) Grupo Controle, (B) Grupo tratado com 0,03 mg/kg/dia de fipronil, (C) Grupo tratado com 0,3 mg/kg/dia de fipronil, (D) Grupo tratado com 3 mg/kg/dia de fipronil. Coloração em HE. 10x, 20x e 40x.

Análise histológica do epidídimo, região da cauda de ratos machos adultos

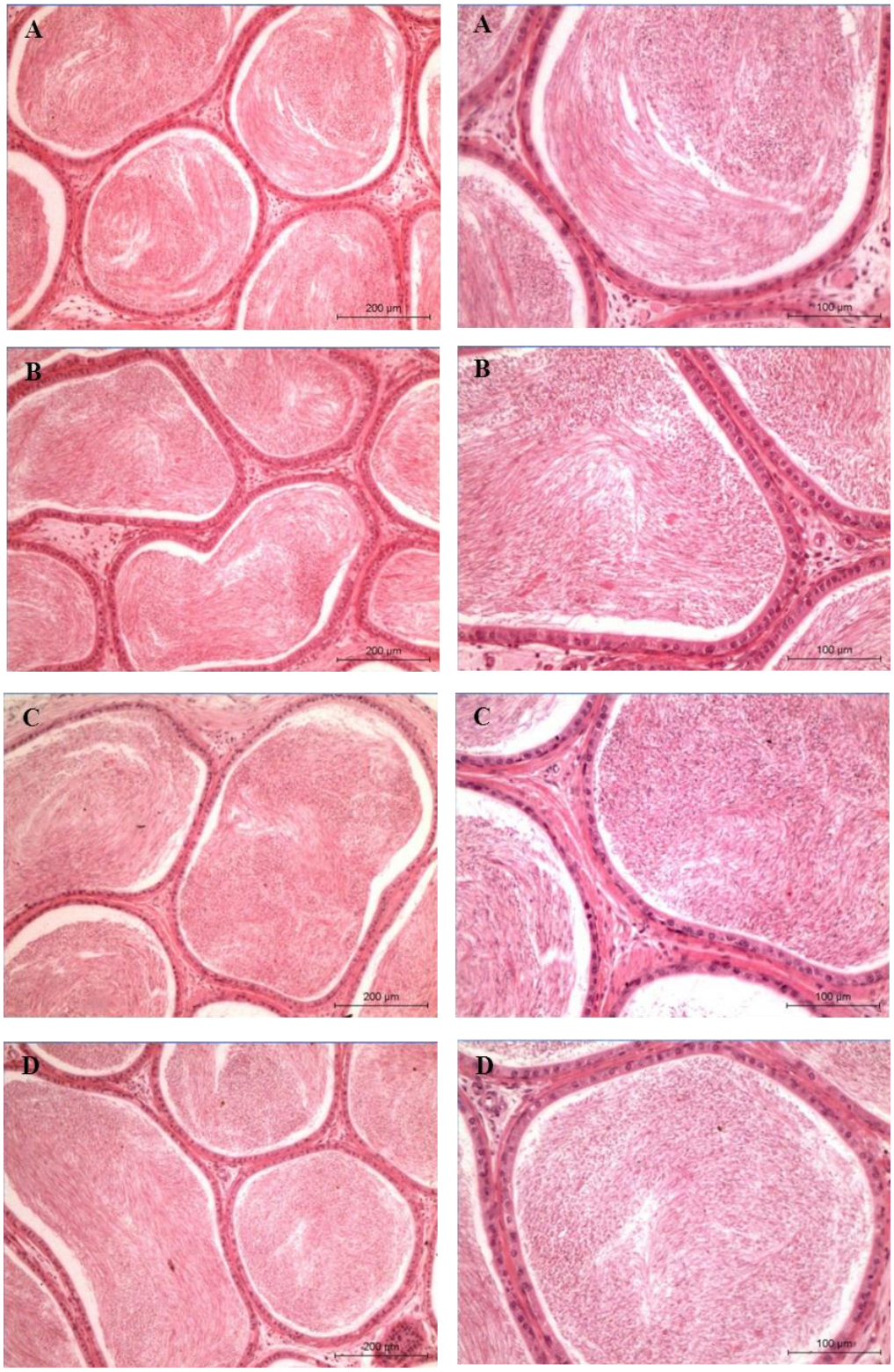

Figura 7 - Fotomicrografias de cortes longitudinais da região da cauda do epidídimo de ratos adultos. (A) Grupo Controle, (B) Grupo tratado com 0,03 mg/kg/dia de fipronil, (C) Grupo tratado com 0,3 mg/kg/dia de fipronil, (D) Grupo tratado com 3 mg/kg/dia de fipronil. Coloração em HE. 10x e 20x.

Imuno-histoquímica para receptor de andrógeno no testículo de ratos machos adultos

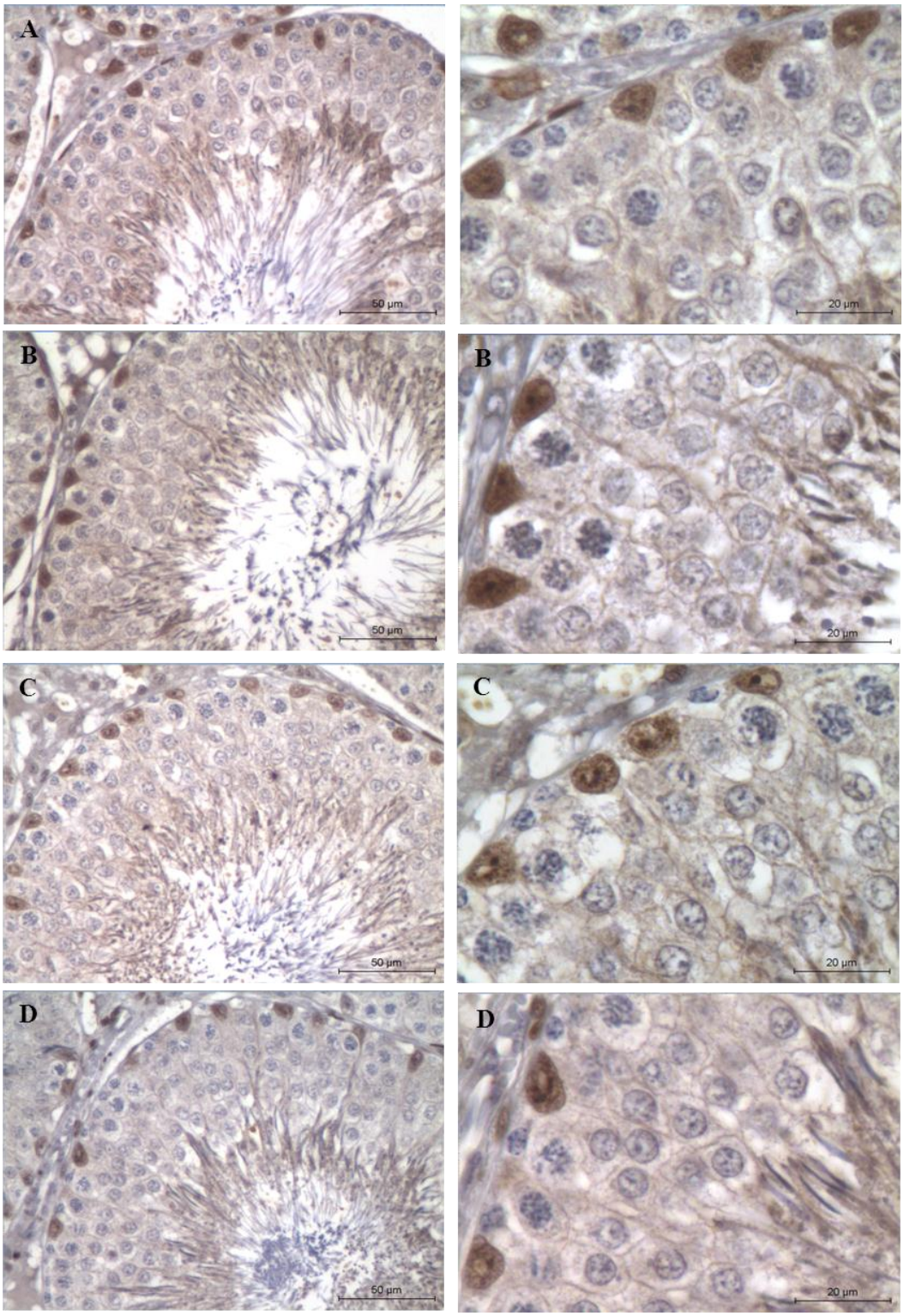

Figura 8– Imuno-histoquímica para receptor de andrógeno no testículo de ratos adultos. (A) Grupo Controle, (B) Grupo tratado com 0,03 mg/kg/dia de fipronil, (C) Grupo tratado com 0,3 mg/kg/dia de fipronil, (D) Grupo tratado com 3 mg/kg/dia de fipronil. Coloração em HE. 40x e 100x.

Histo-morfometria ovariana de ratas adultas

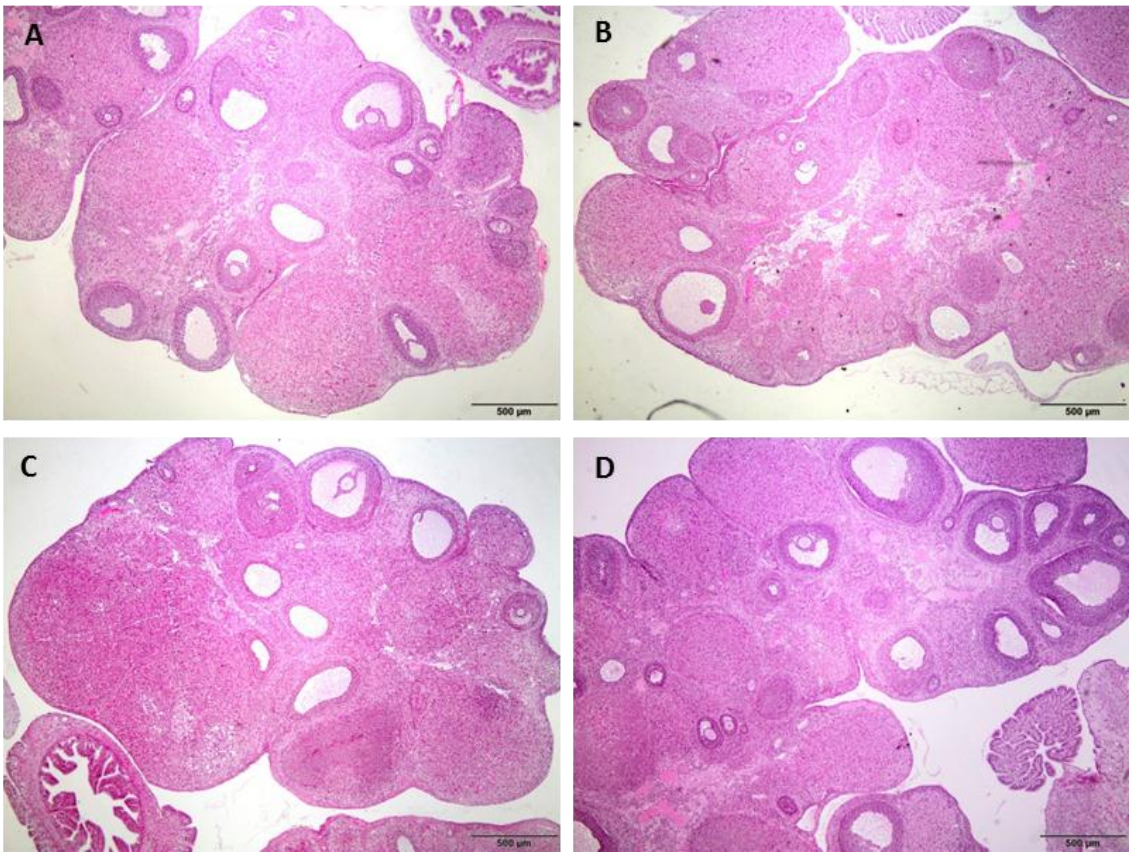

Figura 9 - Fotomicrografias de cortes de ovários de ratas em estro. (A) Grupo Controle, (B) Grupo tratado com 0,03 mg/kg/dia de fipronil, (C) Grupo tratado com 0,3 mg/kg/dia de fipronil, (D) Grupo tratado com 3 mg/kg/dia de fipronil. Coloração em HE. 5x

Histo-morfometria uterina

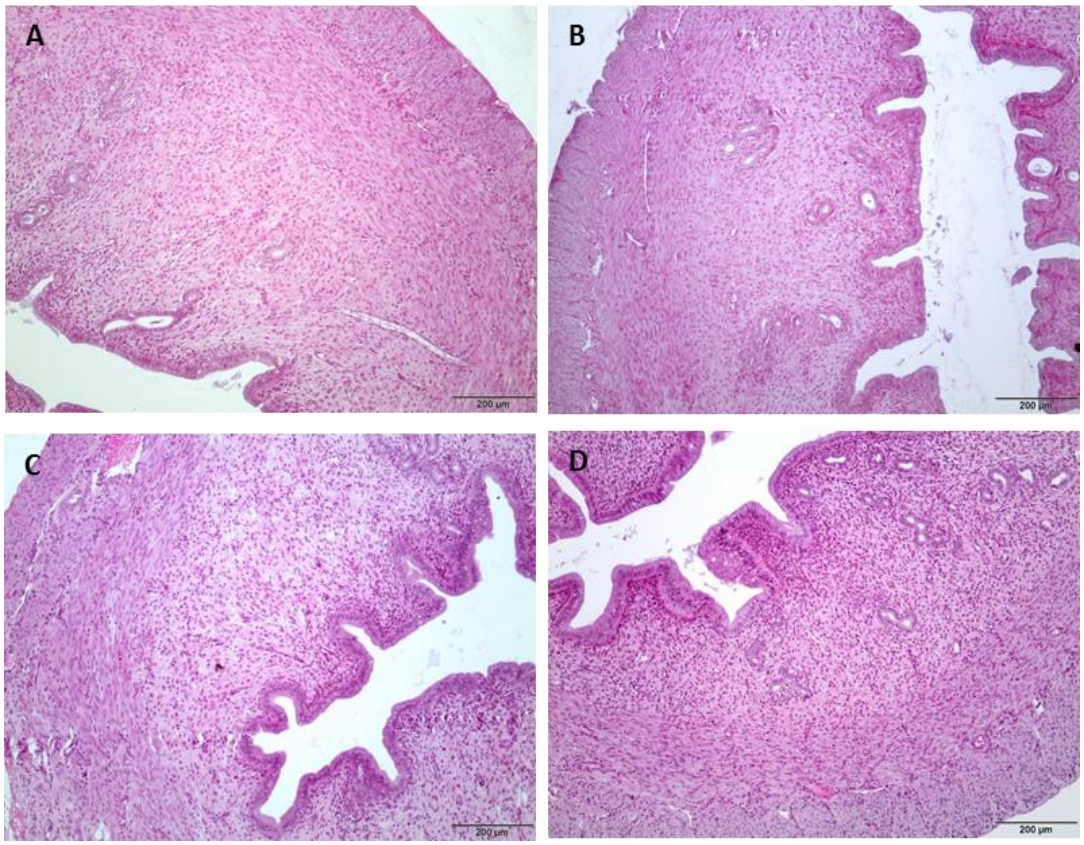

Figura 10 - Fotomicrografias de cortes de útero de ratas em estro. (A) Grupo Controle, (B) Grupo tratado com 0,03 mg/kg/dia de fipronil, (C) Grupo tratado com 0,3 mg/kg/dia de fipronil, (D) Grupo tratado com 3 mg/kg/dia de fipronil. Coloração em HE. 5x

Comissão de Ética

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

Certificado

Certificamos que o Protocolo nº **499-CEUA**, sobre "Influência da Exposição Perinatal ao Inseticida Fipronil: repercussão tardia em parâmetros reprodutivos masculinos, em ratos", sob a responsabilidade de **Wilma de Grava Kempinas e Arielle Cristina Arena**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado "*Ad referendum*" da ***COMISSÃO DE ÉTICA NO USO DE ANIMAIS*** (CEUA), nesta data.

Botucatu, 11 de novembro de 2013.

Prof. Dr. Wellerson Rodrigo Scarano
Presidente da CEUA

Instituto de Biociências – Diretoria Técnica Acadêmica
Distrito de Rubião Júnior s/n CEP 18618-970 Botucatu SP Brasil
Tel 14 3880 0851 fax 14 3815 3744 e-mail: secdta@ibb.unesp.br

Universidade Estadual Paulista Júlio de Mesquita Filho
Instituto de Biociências

unesp

Botucatu, 16 de julho de 2014.

Para:
Prof. Dr. Wellerson Rodrigo Scarano
Presidente do CEUA

Assunto: Alteração no protocolo n° 499-CEUA

Prezado Senhor,

Solicito a Comissão de Ética no Uso de Animais (CEUA) a inclusão de 40 animais (ratas Wistar fêmeas) no protocolo n° 499-CEUA já apreciado. O projeto de pesquisa já aprovado objetiva avaliar os efeitos da exposição perinatal ao fipronil sobre a reprodução apenas em machos. No entanto, como o tratamento ocorre durante a gestação e lactação, seria interessante aproveitar para avaliar os efeitos deste inseticida nas fêmeas. Os parâmetros a ser avaliados são: distância anogenital e contagem de mamilos, instalação de puberdade (abertura vaginal e primeiro estro), assim como, ciclicidade estral, comportamento sexual, teste de fertilidade e coleta de sangue e órgãos após a eutanásia. Os procedimentos de manutenção, manuseio e eutanásia serão os mesmos do protocolo n° 499-CEUA já aprovado e as análises citadas acima que não estão descritas no protocolo n° 499-CEUA, estão em anexo.

Estou disponível para qualquer esclarecimento.

Atenciosamente,

Profa. Dra. Arielle Cristina Arena
Departamento de Morfologia - IBB – UNESP

Anexar ao protocolo original 499

Aprovado em 21/07/14

Prof. Dr. Wellerson Rodrigo Scarano
Departamento de Morfologia
IBB/UNESP