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EFEITOS DA EXPOSIÇÃO À ATRAZINA NA QUALIDADE ESPERMÁTICA DE MACHOS DA ESPÉCIE *Calomys laucha* OLFERS, 1818 (MAMMALIA: RODENTIA)

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"Desconfie do destino e acredite em você. Gaste mais horas realizando que sonhando, fazendo que planejando, vivendo que esperando porque, embora quem quase morre esteja vivo, quem quase vive já morreu".

Sarah Westphal

Sumário

Resumo

A atrazina é muito utilizada no Brasil, na forma de um herbicida usado para o controle de ervas daninhas em culturas de milho e cana de açúcar. Apesar da natureza seletiva deste herbicida, diversos estudos reportam efeitos danosos da atrazina na capacidade reprodutiva de diversas espécies de animais. O *Calomys laucha* é um roedor encontrado em pastagens, margens de estrada e ao redor de campos de cultivo de cereais. Assim, por residir próxima a áreas agrícolas, esta espécie se torna suscetível à contaminação pelo herbicida atrazina. Dessa forma este trabalho avaliou os efeitos de baixas doses de atrazina na qualidade espermática de machos da espécie *Calomys laucha*. Foram realizados três experimentos, avaliando os efeitos da exposição direta de machos adultos à atrazina e outros dois experimentos avaliando a exposição indireta de filhotes via placenta durante o período da gestação (pré-natal) e de filhotes expostos ao contaminante através do leite materno durante o período de amamentação. As doses de atrazina utilizadas foram: controles (água e veículo), 0,1; 1 e 10 mg/kg do animal. Os machos adultos receberam por gavage a atrazina diariamente por um período de 21 dias, enquanto que no experimento de gestação e amamentação, as fêmeas receberam as doses da atrazina, a partir do quinto dia de gestação até o momento do parto e durante todo o período de amamentação, respectivamente. Todas as fêmeas expostas a atrazina na maior dosagem (10mg/kg), durante o período gestacional, apresentaram abortos ou tiveram filhotes natimortos. Os demais resultados obtidos demonstraram que as motilidades total e progressiva foram significativamente reduzidas nas células espermáticas dos machos expostos a todas as dosagens de atrazina em todos os períodos de exposição analisados. A integridade da membrana diminuiu em todas as dosagens de atrazina nas células espermáticas de machos adultos e nos machos expostos no período da amamentação enquanto que a fluidez da membrana aumentou em todas as dosagens testadas nessas células. A funcionalidade mitocondrial dos espermatozoides reduziu em todas as dosagens de atrazina em todos os períodos de exposição. Uma diminuição na quantidade de espécies reativas de oxigênio foi verificada na dose de 1 mg/kg nas células espermáticas de machos expostos no período de pré-natal enquanto essa redução ocorreu em todas as dosagens nos espermatozóides de machos expostos durante a amamentação. O índice de fragmentação de DNA espermático foi maior na maior dose de atrazina (1 mg/kg) em machos expostos no período de pré-natal (1 mg/kg) e na menor dose (0,1 mg/kg) nos animais expostos no período da amamentação. A integridade do acrossoma foi reduzida nas células espermáticas de machos adultos expostos a dose de 10 mg/Kg. A concentração espermática reduziu na maior dose de atrazina (1 mg/kg)

nos machos expostos durante o período do pré-natal. Esses resultados sugerem que baixas doses de atrazina administradas diretamente em machos adultos causam danos diretos nas células espermáticas, enquanto que, quando administradas indiretamente durante períodos críticos de desenvolvimento, como nos períodos gestacional e lactacional, causam alterações permanentes no processo de espermatogênese dos filhotes, reduzindo a qualidade espermática desses animais.

Palavras-chave: Atrazina; parâmetros espermáticos; *Calomys laucha*; pré-natal; amamentação.

Introdução

Sistema genital masculino

O sistema genital masculino de mamíferos é responsável pela produção contínua, nutrição e armazenamento temporário dos espermatozóides, bem como o seu transporte até o aparelho genital feminino. Além disso, também são responsabilidades do sistema genital masculino a síntese e a secreção de hormônios sexuais masculinos, os andrógenos. É constituído pelos testículos, cada qual com seu sistema de ductos (ductos eferentes, epidídimo, canal deferente e ducto ejaculador), pelas glândulas acessórias (vesículas seminais, próstata e glândulas bulbouretral) e pelo pênis (Setchell e Breed, 2006).

Os testículos apresentam função endócrina, ao produzir a testosterona, além de quantidades consideráveis de estrógenos (Carreau *et al.,* 2003). Também apresentam a função exócrina, pela liberação dos espermatozóides no plasma seminal. Os testículos são órgãos pares dispostos anatomicamente em uma região chamada de escroto na maioria das espécies mamíferas. São envoltos por uma espessa cápsula de tecido conjuntivo denso, chamada de túnica albugínea, que penetra no testículo dividindo o órgão em dois compartimentos: o intertubular e o tubular (Kerr *et al.,* 2006).

O compartimento intertubular ou interstício testicular ocupa 5% do volume testicular total e é composto por tecido conjuntivo, vasos sanguíneos e linfáticos, nervos, macrófagos e células de Leydig, as quais são responsáveis pela síntese de andrógenos (Holstein *et al.,* 2003). Em ratos, constata-se uma redução na quantidade de células de Leydig, sendo estas localizadas frequentemente próximas aos espaços linfáticos e agrupadas ao redor dos vasos sanguíneos (Foley, 2001).

O epitélio dos túbulos seminíferos é formado por camadas concêntricas de células germinativas associadas a um único tipo de célula somática, a célula de Sertoli (Hess e

França, 2008). As células germinativas e as células de Sertoli apresentam uma estreita relação entre si, e essa interação tem um importante papel na regulação da espermatogênese (Skinner e Anway, 2005).

As células de Sertoli desempenham diversas funções importantes no processo de espermatogênese, a maioria das quais relacionadas à manutenção, proliferação e diferenciação das células germinativas. Dentre essas funções destaca-se o suporte estrutural e nutricional às células germinativas, formação da barreira hematotesticular, secreção do fluido testicular e de proteínas, como a proteína de ligação a andrógenos (ABP) e a inibina. Além disso, as células de Sertoli também realizam a fagocitose das células germinativas em degeneração e de corpos residuais, resultantes da espermiogênese (Hess e França, 2008). A proliferação das células de Sertoli e das células germinativas ocorre em diferentes períodos do desenvolvimento testicular. O número de células de Sertoli, estabelecido durante o período pré-púbere, determina o tamanho final do testículo e a produção espermática diária em animais sexualmente maduros (Johnston *et al.,* 2004). Além disso, variações no número, estrutura e/ou função nas células de Sertoli podem acarretar em danos ao epitélio germinativo e prejudicar a espermatogênese (Boekelheid *et al.,* 2005).

Espermatogênese

O processo de espermatogênese divide-se em três fases distintas: mitótica ou proliferativa, meiótica e espermiogênica (Hermo *et al.,* 2010). Na fase mitótica ocorre a proliferação de espermatogônias-tronco com a finalidade de aumentar a população espermatogonial. Após, grupos de espermatogônias indiferenciadas originam os espermatócitos primários (Holstein *et al.,* 2003). Na fase meiótica há o desenvolvimento dos espermatócitos primários (diplóides) via meiose, originando na primeira divisão os

espermatócitos secundários (haplóides) e na segunda divisão, as espermátides (haplóides). Durante a fase de espermiogênese, ocorre um processo de citodiferenciação das espermátides, no qual ocorre a condensação do material genético, formação do acrossoma, reposicionamento das mitocôndrias, perda de citoplasma e formação do flagelo, originando, finalmente, os espermatozoides (Holstein *et al.,* 2003). As espermátides haplóides, advindas do processo meiótico pelo qual os espermatócitos se submeteram, passam por 19 estádios no rato, 16 no camundongo e 8 no ser humano até se transformarem no espermatozóide (Hess e França, 2008). Em ratos, a produção de espermatozóides dura 56 dias, tempo que corresponde à duração do ciclo de epitélio seminífero (Russell *et al.,* 1990).

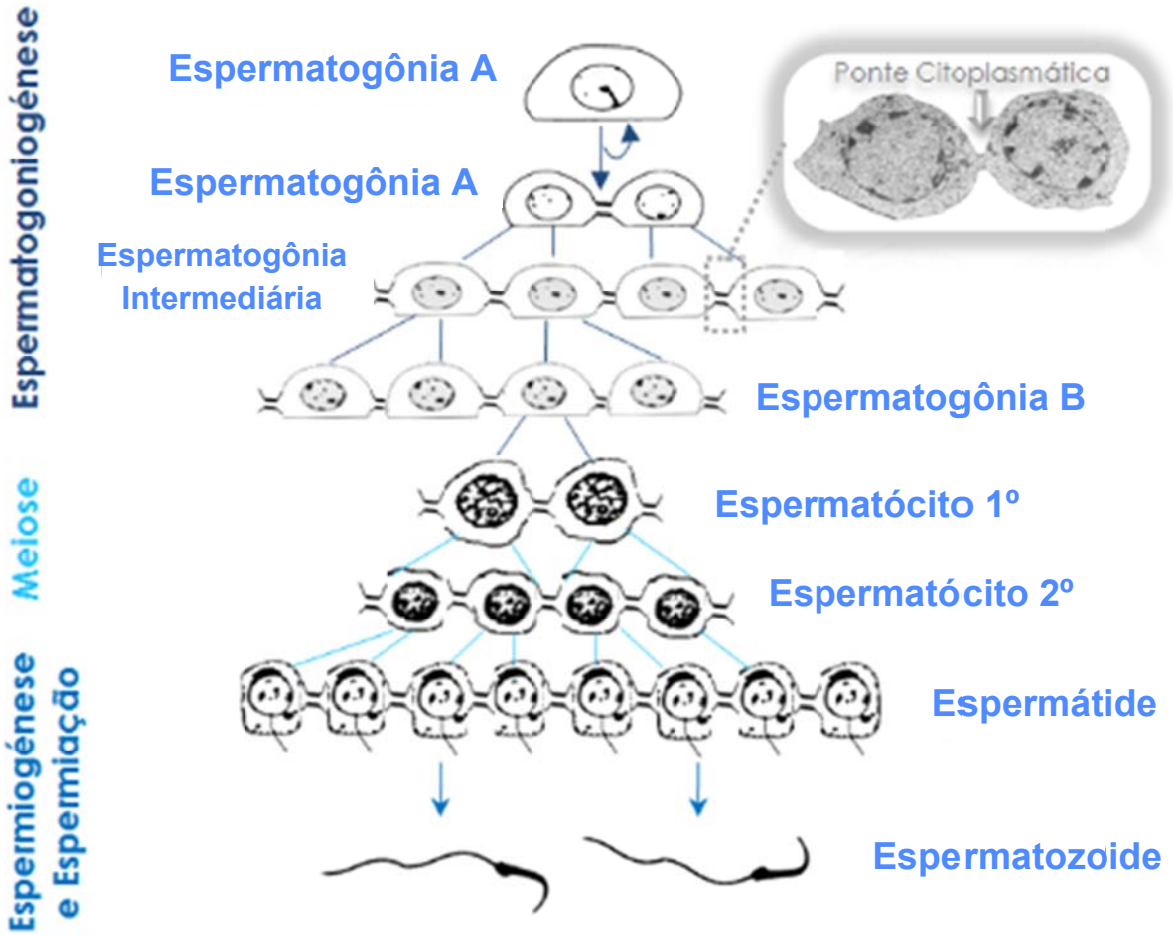

Figura 1. Esquema da espermatogênese (Adaptado de França *et al.,* 1998)

Após a liberação das espermátides para a luz dos túbulos seminíferos, elas são deslocadas para o epidídimo. Os espermatozóides adquirem a habilidade de fertilização no início do trânsito espermático, mas somente ao final do trajeto pelo epidídimo é que eles conseguem tornarem-se viáveis (Shum *et al.,* 2009). O tempo de passagem dos espermatozóides pelo epidídimo é espécie-específico, variando de 3 a 15 dias (Cosentino e Cockett, 1986). Em ratos, esse tempo é de 8 dias, sendo que uma aceleração nesse tempo pode prejudicar o processo de maturação espermática, modificando também a quantidade de espermatozóides disponíveis para a ejaculação (Fernandez *et al.,* 2006).

O espermatozoide é uma célula alongada, morfologicamente dividida em três regiões, tendo como função única à liberação do material genético para o oócito, gerando o zigoto. Na cabeça do espermatozoide está contido o núcleo haplóide com a cromatina altamente compactada e o acrossoma, que dispõe de várias enzimas hidrolíticas capazes de dissolver a zona pelúcida permitindo a penetração no oócito. No rato e no hamster a cabeça do espermatozóide é alongada em formato de gancho (Robaire *et al.,* 2006). Posteriormente, encontramos a peça intermediária, contendo as mitocôndrias, relacionadas com a produção de energia e motilidade espermática; e finalmente, a cauda formada por um longo flagelo, também responsável pelo movimento espermático (Garner e Hafez, 2004).

A membrana plasmática é organizada em uma bicamada lipídica associada a proteínas e apresenta um papel fundamental nos fenômenos celulares. A membrana espermática modifica-se ao longo da espermatogênese, trânsito e armazenamento no epidídimo, ejaculação, depósito no trato genital feminino, capacitação e na penetração no oócito, se adaptando a cada ambiente e situação (Lenzi *et al.,* 1996). Além disso, as células espermáticas apresentam grandes quantidades de ácidos graxos poliinsaturados em sua membrana plasmática, o que os tornam especialmente susceptíveis à peroxidação lipídica por espécies reativas de oxigênio (EROs) (Vernet *et al.,* 2004). A peroxidação lipídica da membrana

espermática tem sido relacionada a defeitos na peça intermediária, diminuição na motilidade espermática devido a defeitos no axonema, bem como diminuição na habilidade fertilizante (Vernet *et al.,* 2004).

Desse modo, a estrutura da membrana precisa estar íntegra, garantindo viabilidade e capacidade fertilizante à célula espermática. Igualmente, as mitocôndrias necessitam estar funcionais para garantir a demanda de Adenosina Trifosfato (ATP) para os batimentos flagelares, hiperativação e penetração, sendo também indispensável à manutenção das enzimas acrossomais para que ocorra a fertilização (Chemes e Rawe, 2003).

Controle endócrino da espermatogênese

Para o início e a manutenção do processo de espermatogênese é necessária uma complexa interação entre o hipotálamo, hipófise e testículos (Figura 2). O hipotálamo é o responsável pelo controle central deste complexo, ele secreta o hormônio liberador de gonadotrofinas (GnRH), o qual é liberado de modo pulsátil através dos terminais hipotalâmicos neurais (Ojeda *et al.,* 2006). O GnRH, liberado na eminência média, através do sistema vascular porta, alcança a hipófise estimulando a produção e a secreção dos hormônios LH (hormônio luteinizante) e FSH (hormônio folículo estimulante).

O LH atua nas células de Leydig regulando a secreção de testosterona, que por sua vez inibe a liberação de GnRH no hipotálamo com conseqüente inibição da liberação de LH pela hipófise, provocando assim, uma retroalimentação negativa da sua produção. O FSH atua nos túbulos seminíferos no estímulo a espermatogênese, atuando nas células de Sertoli, nos testículos. Assim, estimuladas pelo FSH, as células de Sertoli secretam glicoproteínas, nos túbulos seminíferos, que são essenciais para o êxito da espermatogênese, tal como a proteína de ligação a andrógenos (androgen binding protein - ABP), responsável pelo transporte e

proteção de andrógenos contra a degradação, além de garantir uma concentração adequada de testosterona para a maturação das células germinativas que resultam nos espermatozóides (Sofikitis *et al.,* 2008). Produzem ainda a inibina e a ativina, sendo que a ativina estimula a produção de FSH, enquanto a inibina exerce efeito de feedback negativo na liberação de FSH (Ojeda *et al.,* 2006). A testosterona apenas consegue inibir a liberação de FSH em altas concentrações (O'Donnel, *et al.* 2006).

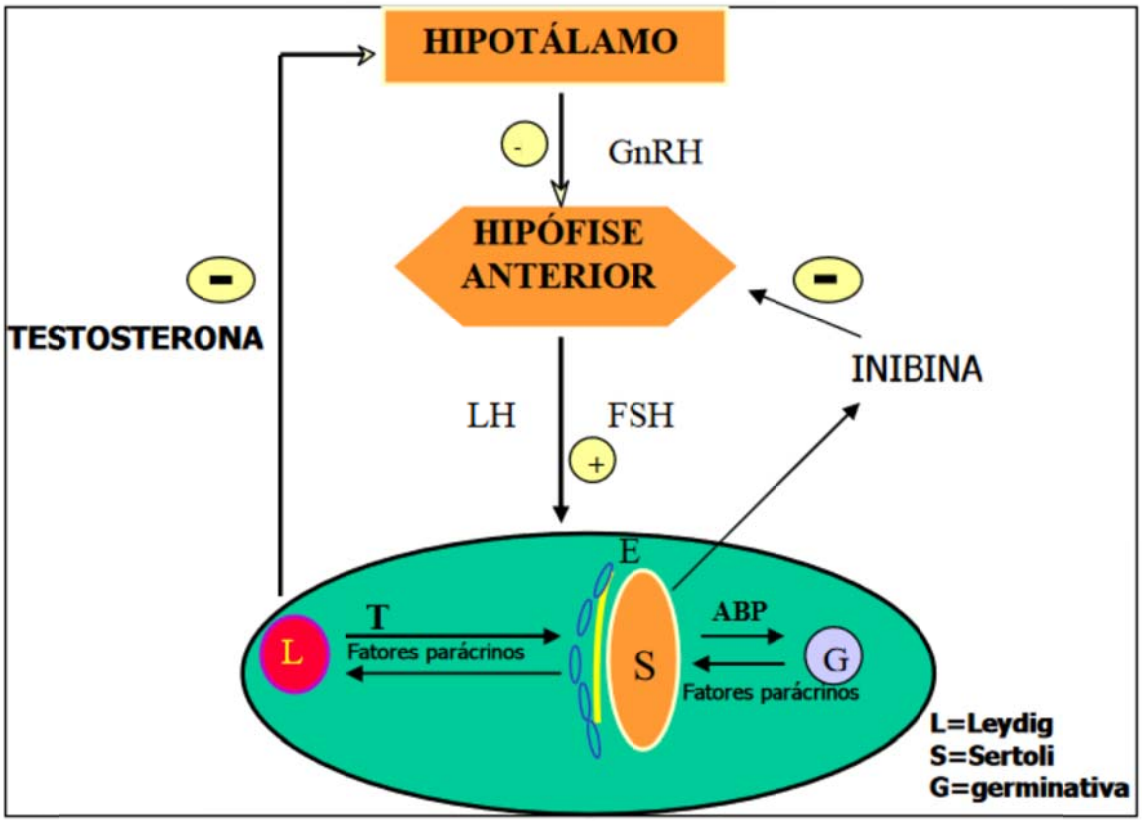

Figura 2. Regulação hormonal da espermatogênese (Adaptado de Oliveira, 2007).

O processo da espermatogênese é totalmente dependente de andrógenos. Os receptores para andrógenos estão localizados nas células de Sertoli, nas células mióides peritubulares e nas células de Leydig. Nas células germinativas não existem receptores para andrógenos, o que indica que a testosterona atua via células de Sertoli (Holdcraft e Braun, 2004). Em ratos adultos que tiveram uma importante diminuição nos níveis de andrógenos através de

hipofisectomia, observou-se uma regressão no epitélio seminífero. Além disso, na ausência de testosterona, ocorreu a perda de espermátides arredondadas e também de espermátides alongadas maduras, indicando a forte ação do andrógeno na espermiogênese (França *et al.,* 1998).

A testosterona produzida pelas células de Leydig é fundamental durante o desenvolvimento fetal, um pulso de testosterona é responsável pela diferenciação sexual masculina, e no período de pós-natal, o aumento na produção de testosterona promove o desenvolvimento das características sexuais secundárias, o comportamento sexual masculino e o início e a manutenção da espermatogênese (Contreras *et al.,* 2007).

A enzima aromatase converte a testosterona em estradiol. Em roedores, esta enzima é encontrada principalmente nas células germinativas maduras e nos espermatozóides (Akingbemi *et al.,* 2004). A presença de aromatase e/ou estrógenos nos testículos é de suma importância, pois animais deficientes para a aromatase apresentaram vários distúrbios reprodutivos, como a redução no número de espermátides arredondadas e alongadas (Shetty *et al.,* 1998), espermatogênese incomum com a suspensão da maturação das células germinativas na fase de espermátides (Fischer *et al.,* 1998), além de atrofia do testículo e infertilidade (Oliveira *et al.,* 2001). Além disso, a aromatase exerce um importante papel na motilidade e capacitação espermática, visto que a ausência de aromatase acarreta na imobilidade dos espermatozóides (Lazaros *et al.,* 2011). Assim, a importância dos estrógenos no processo de espermatogênese está associada com a proliferação das células germinativas, diferenciação e maturação final das espermátides, além da sobrevivência e inibição da apoptose das células germinativas. A maturação espermática é particularmente sensível e dependente de estrógenos (Carreau e Hess, 2010).

Desreguladores Endócrinos

Existe uma crescente preocupação sobre os possíveis efeitos danosos aos animais, inclusive a seres humanos, de contaminantes ambientais com potencial ação sobre o sistema endócrino (Bila e Dezotti, 2007; Mnif *et al.,* 2011). Os desreguladores endócrinos são substâncias naturais ou sintéticas, encontradas em enorme variedade no ambiente, que interferem na síntese, secreção, transporte, metabolismo, ação ou eliminação de hormônios naturais, por se ligarem a seus receptores e mimetizarem ou inibirem a ação dos mesmos, causando alterações no funcionamento do sistema endócrino e, consequentemente, provocando efeitos adversos sobre a saúde dos indivíduos expostos ou da sua descendência (Bila e Dezotti, 2007; Topari, 2008; Mnif *et al.,* 2011).

Desreguladores endócrinos de origem natural, como os fitoestrógenos, normalmente se decompõem mais facilmente e são excretados em pouco tempo do organismo, não se acumulando em tecidos e, portanto, normalmente não ocasionando problemas graves aos animais. No entanto, os desreguladores endócrinos de origem sintética, tais como agrotóxicos (como a atrazina), bifenilas policloradas (PCB), alquilfenóis, bisfenol, metais pesados, dentre outros, podem causar respostas antagônicas ou agônicas induzindo ou atenuando a resposta de hormônios endógenos, como os hormônios reprodutivos, iniciando uma cascata de efeitos específicos nas células, tecidos ou sistemas que apresentam os receptores específicos (Frye *et al.,* 2011). As substâncias que apresentam o efeito antagonista possuem a habilidade de acoplar-se aos receptores hormonais e bloquear a ação dos seus ligantes naturais, dessa forma, conseguem inibir ou atenuar a sua resposta. Enquanto que as substâncias agonistas conseguem acoplar-se a um sítio ativo, como os receptores de hormônios esteróides, e causar uma resposta, diminuindo ou aumentando as atividades de células ligadas a estes receptores. Além disso, os desreguladores endócrinos também podem interferir nas etapas de síntese, transporte e metabolismo de hormônios (Bila e Dezotti, 2007).

Assim, uma vez que o sistema endócrino desempenha papel crítico sobre o desenvolvimento e função do sistema genital masculino, este pode ser considerado um alvo particularmente vulnerável a potenciais perturbações endócrinas. Sabe-se que os efeitos da exposição à desreguladores endócrinos na vida adulta podem ser revertidos ou compensados por mecanismos hormonais, no entanto, quando a exposição ocorre durante a gestação ou nas primeiras semanas de vida, pode provocar danos irreversíveis (Cremonese, 2014).

Nesse contexto, os agrotóxicos são considerados como o maior grupo de substâncias classificadas como desreguladores endócrinos (Bila e Dezotti, 2007). Um estudo de Mckinlay *et al*. (2008) verificou 127 agrotóxicos com propriedades de desreguladores endócrinos.

Os agrotóxicos são substâncias químicas utilizadas na produção agrícola e na pastagem, com a finalidade de alterar a composição destes e, assim, preservá-los frente à ação danosa de pragas e doenças, que podem reduzir os ganhos econômicos, diminuindo a produtividade agrícola (Rodrigues, 2008). Sua eficácia ocorre devido a sua alta toxicidade frente a organismos vivos e também na dificuldade de sua degradação. No entanto, um dos maiores problemas relacionados com o consumo de agrotóxicos é que a maior parte aplicada acaba não atingindo os organismos-alvo, sendo carreado pelas águas das chuvas, percolando ou volatizando, representando uma ameaça ao ambiente, principalmente os aquáticos. Dessa forma, a sua acumulação em animais e vegetais, bem como a contaminação do solo, alimentos, águas subterrâneas e/ou superficiais é frequente (Spadotto, 2006).

Colaborando com isso, o uso de agrotóxicos tem se intensificado na agricultura, principalmente nos últimos 30 anos. O Brasil vem destacando-se entre os maiores consumidores de agrotóxicos no mundo, ficando atrás somente do Japão e dos Estados Unidos (Carneiro, 2012). Assim, os impactos desse alto consumo e principalmente da utilização inadequada desses produtos, são amplos, colocando em risco a saúde dos ecossistemas e do homem. O uso indiscriminado causa a contaminação dos solos, das águas superficiais e

subterrâneas, dos alimentos, apresentando, consequentemente, efeitos negativos em organismos terrestres e aquáticos e intoxicação humana pelo consumo de água e alimentos contaminados (Spadotto, 2006). Segundo Carneiro *et al.* (2015), estima-se que um terço dos alimentos consumidos cotidianamente pelos brasileiros está contaminado por agrotóxicos. Assim, os agrotóxicos além de cumprirem o papel de proteger as culturas agrícolas das pragas, doenças e plantas daninhas, podem oferecer riscos à saúde humana e ao ambiente.

É conhecido que várias classes de agrotóxicos apresentam atividade estrogênica e/ou antiestrogênica, como os agrotóxicos organoclorados e piretróides, e androgênica e/ou antiandrogênica, como os organoclorados, organofosforados e atrazina (Mnif *et al.,* 2011). Exposições a agrotóxicos com características de desreguladores endócrinos nos períodos fetal, juvenil ou adulto podem causar danos em todo o ciclo de vida do indivíduo, iniciando no processo de embriogênese e diferenciação sexual e atingindo até a fase reprodutiva (Jeng *et al.,* 2014). Estudos da exposição de animais a esses contaminantes na fase embrionária e fetal apontam abortos espontâneos e alterações na diferenciação sexual (Fernandez *et al.,* 2007; Rosemberg *et al.,* 2008). Quando a exposição ocorre no período juvenil, acarreta em alterações nas características sexuais secundárias, puberdade precoce ou retardo na maturação sexual e mudanças na espermatogênese (Meeker *et al.,* 2010). E finalmente, quando as exposições ocorrem na fase adulta, resultam em mudanças nos níveis de hormônios reprodutivos, alterações na morfologia de órgãos reprodutivos, diminuição da qualidade espermática (Fernandez *et al.,* 2012; Jeng *et al.,* 2014).

Como citado anteriormente, os agrotóxicos estão entre o grupo com maior número de substâncias classificadas como desreguladores endócrinos. Entre esses compostos está incluído o herbicida atrazina, mundialmente utilizado (Stanko *et al*., 2010).

Atrazina

A atrazina (2-cloro-4-etilamino-6-isopropilamino-1,3,5-triazina) é um herbicida muito utilizado na agricultura em uma variedade de culturas, especialmente no cultivo do algodão, milho e cana-de-açúcar, e no preparo de áreas para plantio; sendo utilizado para o controle seletivo de gramíneas e ervas daninhas (Garcia *et al.,* 2012). Seu mecanismo de ação é a inibição do fotossistema II, bloqueando a síntese de clorofila, e com isso, impedindo a fotossíntese (Sawicki *et al.,* 1998). A atrazina apresenta uma alta estabilidade em sua molécula química, que é explicada pela presença do anel heterocíclico, semelhante ao benzeno (Figura 3). Esse anel também confere grande resistência à degradação biológica (Pereira, 2011). Assim, a atrazina é considerada um potente contaminante devido as suas propriedades físico-químicas, que incluem baixa hidrólise, alta persistência no solo, moderada a baixa solubilidade em água, e maior solubilidade em matéria orgânica e tecido adiposo (Ross *et al.,* 2009; Akdogan *et al.,* 2013). A atrazina pode alcançar o ambiente de diversas maneiras, e é frequentemente introduzida em águas superficiais e subterrâneas por escoamento e pela filtração do solo, ou então pode permanecer adsorvida às partículas do solo após a sua aplicação. Dessa forma, as concentrações de atrazina encontradas no ambiente são bastante variadas. A atrazina pode ser encontrada até um ano após a aplicação e, por essa razão, torna-se um contaminante potencial, sendo encontrada comumente em solos (Brodeur *et al.,* 2009), águas superficiais, subterrâneas e potáveis (Jablonowski *et al.,* 2011), além de alimentos (Garcia *et al.,* 2012).

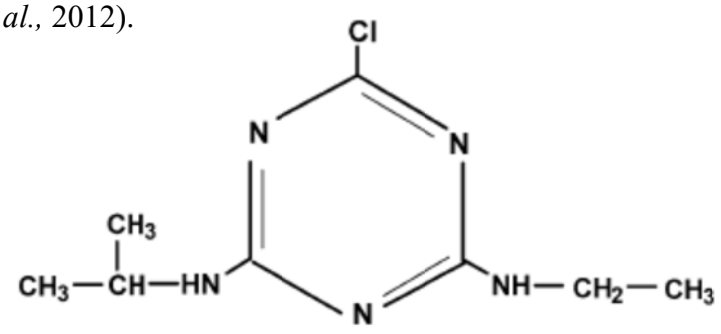

Figura 3. Estrutura química da atrazina (Adaptado de Cox, 2001).

Devido a sua toxicidade, o uso da atrazina está proibido na União Européia desde 2001, porém este herbicida ainda é amplamente utilizado em muitos países, incluindo os Estados Unidos, China, México, Argentina e Brasil (LeBaron *et al.* 2008). No Brasil, a ANVISA (Agência Nacional de Vigilância Sanitária) estabelece a quantidade máxima de resíduos de atrazina oficialmente aceita em alimentos, sendo os limites máximos de resíduos (LMRs) permitidos em abacaxi e sorgo de 0,02 mg/kg, e para o milho e a cana-de-açúcar de 0,25 mg/kg (ANVISA, 2003). Na água, o CONAMA (Conselho Nacional do Meio Ambiente) estabelece a concentração máxima aceitável de 2μg/L para a água potável (CONAMA, 2005). No entanto, concentrações acima das permitidas são frequentemente encontradas em águas superficiais, subterrâneas e precipitações (Nwani, 2010; Moreira *et al.,* 2012).

Recentemente, diversos estudos têm reportado os efeitos da atrazina como desregulador endócrino em mamíferos, anfíbios e peixes (Jin *et al.,* 2014; Papoulias *et al.,* 2013; Papoulias *et al.,* 2014). O mecanismo pelo qual a atrazina desregula o sistema endócrino foi revelado recentemente, ela se liga a enzima fosfodiesterase (PDE) causando a sua inibição na célula. A inibição da fosfodiesterase aumenta os níveis de adenosina monofosfato cíclico (AMPc), o que resulta em uma elevação na transcrição do gene Cyp19 que codifica para a enzima aromatase. Esse efeito foi verificado em uma linhagem celular humana de carcinoma adrenocortical, na qual a exposição à atrazina elevou a atividade da aromatase em paralelo com um aumento nos níveis de AMPc (Sanderson *et al.,* 2000). A função da aromatase é converter a testosterona em estrógeno, assim o efeito final causado pela atrazina é uma elevação dos níveis de estrógenos nos organismos (Fan *et al.,* 2007).

Efeitos estrogênicos provocados pela atrazina já foram demonstrados em diversos animais, tais como, mamíferos (Victor-Costa *et al.,* 2010) e peixes (Spanò *et al.,* 2004); assim como a redução na concentração de testosterona em machos adultos de sapos (Hecker *et al.,*

2005), ratos (Zirkin *et al.,* 2001) e peixes (Spanò *et al.,* 2004). Dessa forma, pesquisas sugerem que esse desequilíbrio hormonal causado pela atrazina seja responsável pela feminilização parcial verificada em gônadas de machos de peixes (Tillitt *et al.,* 2010) e anfíbios (Hayes *et al.,* 2010).

Pesquisas recentes têm focado nos potentes efeitos reprodutivos causados pela atrazina em mamíferos. Em ratos machos, foram relatadas alterações morfológicas em diversos órgãos após exposições a atrazina, como redução no peso dos testículos, aumento da glândula adrenal, dilatação e desorganização dos túbulos seminíferos (Victor-Costa *et al.,* 2010). Nas células de Leydig, foram verificadas irregularidades no núcleo com aumento de heterocromatina (Victor-Costa *et al.,* 2010), enquanto nas células de Sertoli foram observadas menores conexões intercelulares, reduzindo as suas junções com as células germinativas, causando reduções nos níveis de testosterona (Song *et al,* 2014). Além disso, estudos apontam que a exposição à atrazina causa atrofia testicular, diminuições no número de espermatozóides nos testículos e epidídimo, reduções na motilidade e na viabilidade, mudanças na morfologia e um decréscimo na produção diária de esperma (Abarikwu *et al.,* 2010). Ademais, outros efeitos relatados foram a diminuição da taxa de migração espermática, mudanças histopatológicas nos testículos e redução da concentração de proteínas testiculares (Rosenberg *et al.,* 2008).

A produção de ATP nas células espermáticas também é altamente afetada pela exposição à atrazina como mostrou um estudo realizado por Hase *et al.,* (2008), no qual foi verificado que a atrazina é capaz de prejudicar a função da mitocôndria espermática. A atrazina se liga com a ATP sintase, inibindo a sua atividade e, consequentemente diminuindo a produção de ATP, o que pode resultar na diminuição da viabilidade e, principalmente, da motilidade espermática, como foi verificado por Betancourt *et al.* (2006), Abarikwu *et al.* (2010 e 2015) e Dehkhargani *et al.* (2011).

A exposição à atrazina nos períodos gestacional e lactacional também provocam diversos danos reprodutivos nos filhotes. Estudos reportam que a exposição à atrazina durante período gestacional e no início do desenvolvimento dos filhotes de ratos causou diminuição do peso corporal, dos testículos e epidídimos (Victor-Costa *et al.*, 2010; DeSesso *et al.,* 2014), atrasos na puberdade (Rayner *et al.,* 2007), redução da distâcia anogenital (Rosenberg *et al.,* 2008), diminuição da expressão de genes estereidogênicos e de andrógenos (Pogmic *et al.,* 2009), além de aumento no percentual de espermatozóides anormais (DeSesso *et al.,* 2014) .

Esses efeitos demonstram que a atrazina e seus metabólitos são transferidos para os filhotes através do leite materno e pela placenta. Isso foi verificado por Stoker e Cooper (2007) que identificaram a presença de ^{14}C-atrazina e de seus resíduos ^{14}C-CITRI, no estômago e cérebro dos filhotes que foram expostos à atrazina no período da amamentação. Além disso, Fraites *et al.* (2011) também verificaram a presença de atrazina e de seus metabólitos nos tecidos das mães, fetos e neonatos após exposições realizadas nas fêmeas durante os períodos gestacional e lactacional. A atrazina é um composto altamente lipofílico e com baixo peso molecular, o que facilita a sua difusão da mãe para os filhotes tanto pelo leite materno quanto através da placenta (Thalacker, 1996; Breitzka *et al.,* 1997).

Além de efeitos reprodutivos, a exposição à atrazina pode causar também, alterações na expressão de enzimas relacionadas ao metabolismo energético, detoxificação celular e sistema de defesa antioxidante (Jin *et al*., 2010). Estudos analisando os efeitos da atrazina nos testículos e epidídimos de ratos, verificaram aumento nos níveis de peroxidação lipídica com consequente diminuição das atividades das enzimas antioxidantes glutationa-S-transferase (GST) e superóxido dismutase (SOD) em testículos e epidídimos e da catalase (CAT) nos epidídimos (Abarikwu *et al.,* 2010), indicando que a presença desse composto causa uma situação de estresse oxidativo no animal.

Calomys laucha

O gênero *Calomys,* pertencente à família Cricetidae, é um dos gêneros que apresenta maior distribuição entre os roedores neotropicais (Prioto e Polop, 2003). As espécies do gênero *Calomys* têm hábito terrestre e são principalmente granívoras. Constroem ninhos esféricos, com material vegetal, em depressões camufladas no solo ou entre folhagens ou tronco de árvores mortas. Reproduzem-se durante todo o ano, com menor intensidade no inverno dependendo da disponibilidade de alimento (Colares, 1997).

A espécie *Calomys laucha* é encontrada normalmente em pastagens, áreas agrícolas, margens de estrada, ao redor dos campos de cultivo de cereais (Mills *et al.,* 1992) e de dunas costeiras (Colares, 1997), onde se alimentam e constroem ninhos para a reprodução. Esses animais são encontrados com frequência em culturas de milho e trigo. Essa espécie se distribui nas áreas do sul da Bolívia, sul do Brasil, Uruguai e centro da Argentina (Reis *et al.,* 2006) onde é responsável por prejuízos na agricultura e também é o transmissor do vírus Junin, causador da febre hemorrágica Argentina (Alche e Coto, 1993).

Relevância do estudo

O conhecimento de aspectos reprodutivos de animais silvestres, como a espécie do presente estudo, *Calomys laucha*, é imprescindível para o manejo desses espécimes, tanto em seu habitat natural como em condições de laboratório. Esse conhecimento é importante para estudar as respostas fisiológicas desses animais frente a substâncias químicas, presentes no ambiente, que atuam como desreguladores endócrinos e interferem em sua reprodução.

Sabe-se que para o sucesso reprodutivo masculino, deve ocorrer a produção de espermatozóides funcionais aptos à fertilização. No entanto, é conhecido que contaminantes ambientais, como a atrazina, afetam o sistema reprodutivo por vários mecanismos e vias. Os

efeitos na reprodução masculina atingem o processo de espermatogênese, que é um processo contínuo de divisão celular, diferenciação e maturação, causando uma baixa qualidade do sêmen, como alterações morfológicas nos espermatozoides e uma baixa concentração espermática, podendo resultar em infertilidade ou em uma fertilidade diminuída.

Assim, a prevalência do uso do herbicida atrazina e a sua persistência no ambiente revela a importância de estudos que busquem o entendimento dos impactos deste composto em aspectos reprodutivos de organismos expostos a esse contaminante. A atrazina é utilizada largamente na agricultura sendo uma fonte de contaminação dos ambientes terrestres e aquáticos, devido a sua alta persistência no ambiente. Dessa forma, levando em conta os diversos estudos que mostram a toxicidade e o potencial de desregulação endócrina da atrazina, além dos aspectos ambientais como a suscetibilidade de exposição do *Calomys laucha* a este contaminante por residir próximo a pastagens em áreas agrícolas, salienta-se a importância da realização deste estudo para verificar os possíveis efeitos desse contaminante na qualidade espermática desse roedor silvestre.

Objetivos

Objetivo Geral

Verificar os efeitos da atrazina sobre a qualidade espermática de machos adultos da espécie *Calomys laucha*.

Ojetivos Específicos

- Verificar a motilidade nas células espermáticas de machos adultos e filhotes expostos durante os períodos do pré-natal ou amamentação a diferentes doses da atrazina;

- Analisar os parâmetros espermáticos (integridade, rompimento e fluidez de membrana, integridade do acrossomo, funcionalidade mitocondrial, dano de DNA, concentração espermática) em machos adultos e filhotes expostos durante os períodos do pré-natal ou amamentação a diferentes doses da atrazina;

- Verificar a quantidade de ERO e a peroxidação lipídica nas células espermáticas de machos adultos e filhotes expostos durante os períodos do pré-natal ou amamentação a diferentes doses da atrazina.

Manuscrito 1:

Low atrazine dosages reduce sperm quality of *Calomys laucha* mice

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Low atrazine dosages reduce sperm quality of *Calomys laucha* mice

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Abstract

Reproductive effects caused by the exposure to environmentally relevant dosages of atrazine on wild animals are poorly understood. This study evaluated the effects of three dosages of atrazine on sperm parameters of adult *Calomys laucha* males. Adult mice were orally exposed to dosages of 0 (water and vehicle control), 0.1, 1 and 10 mg/kg of animal weight for a 21-day period. Following exposure, analyses were performed to determine sperm motility parameters, plasma membrane integrity and fluidity, mitochondrial functionality, acrosome integrity, DNA damage, lipid peroxidation, and production of reactive oxygen species (ROS) in the sperm samples. Total and progressive motility were reduced in all dosages in comparison to control groups. Membrane integrity and mitochondrial functionality of sperm were reduced in all dosages, and the sperm membrane fluidity increased in the higher dosages of atrazine (1 and 10 mg/kg), in comparison with the vehicle control. A decreased in the acrosome integrity was noted at 10 mg/kg of atrazine, compared to the control groups. The integrity of DNA, ROS generation, and lipid peroxidation of sperm showed no significant differences when compared with the control groups. These results suggest that exposure to low dosages of atrazine can affect sperm parameters of *Calomys laucha* and therefore reduce the reproductive capacity of wild rodent species.

Keywords: Atrazine; sperm parameters; *Calomys laucha*; sperm motility; herbicide; wild rodent.

Introduction

Pesticides are used to improve agricultural production. However, if improperly used, these products and their metabolites pose a threat to environmental and human health. Among the most popular pesticides used worldwide are the herbicides from the group of triazines, which represent approximately 30% of total pesticides in use (Cabral *et al.*, 2003). Notable within this class is atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine), a herbicide widely used for weed control in corn, sorghum and sugarcane (Singh *et al.,* 2008). This herbicide is used extensively, particularly in the United States (Barr *et al.*, 2007), Brazil (Ludovice *et al.*, 2003), and in Europe, within which its use has been banned by several countries (Sass and Colangelo, 2006).

Certain characteristics of atrazine may raise concern, as it is promptly bioaccumulated, it is resistant to biodegradation, and it is easily found in both surface and groundwater (Solomon *et al.*, 1996). Furthermore, atrazine has been detected in dairy foods, such as milk and yogurt (Garcia *et al.*, 2012; Li *et al.*, 2013).

Despite being prohibited in the European Union, and restricted in other countries, atrazine is still found in concentrations above recommended limits (US EPA: 3 μg/L, European Union: 0.1 μg/L). Recent reports indicate that, with respect to US EPA (Environmental Protection Agency) tolerances and drinking water, atrazine acute dietary exposures in humans range from 0.234 to 0.857 μg/kg/day, and chronic dietary exposures range from 0.046 to 0.286 μg/kg/day (Gammon *et al.*, 2005). ANVISA, the Brazilian National Health Protection Agency, has established maximum residue limits (MRLs) of atrazine in foods such as maize (0.25 mg/kg), sugarcane (0.25 mg/kg), and pineapple (0.02 mg/kg) (ANVISA, 2003). Further, the Brasilian National Environmental Council – CONAMA – has established maximum acceptable concentrations of atrazine in drinking water at 2 μg/L (CONAMA, 2005).

Numerous studies have been carried out in order to verify the effects of atrazine and its residues in humans and other animals. Many of these studies have shown atrazine as a potent endocrine disrupter, which may affect reproduction in different groups of vertebrates, such as mammals (Breckenridge *et al.*, 2015), birds (Hussain *et al.*, 2010), amphibians (Hayes *et al.*, 2010), reptiles (Parsley *et al.*, 2015) and fish (Wirbisky *et al.*, 2016). The mechanism by which atrazine deregulates the endocrine system consists of a connection and consequent inhibition of the phosphodiesterase (PDE) enzyme in the cell. From the inhibition of PDE, the cyclic adenosine monophosphate levels (AMPc) are increased, resulting in an increase in the

transcription of the Cyp19 gene, which codifies for the aromatase enzyme (Fan *et al.,* 2007). This effect was noted in a human adrenocortical carcinoma cell line, in which exposure to atrazine increased aromatase activity and induced an increase in AMPc levels (Sanderson *et al.*, 2000). As the function of aromatase is to convert testosterone into estrogen; the overall effect of atrazine is an elevation of estrogen levels in organisms.

The increase in estrogen hormones caused by exposure to atrazine has been observed in several animals, including mammals (Victor-Costa *et al.*, 2010) and fish (Spanò *et al.*, 2004). In addition, male testosterone levels are reduced following exposure to atrazine, as shown by studies of adult frogs (Hecker *et al.*, 2005), rats (Trentacoste *et al.*, 2001) and fish (Spanò *et al.*, 2004). This hormonal imbalance caused by exposure to atrazine is related to the partial feminization observed in male fish, amphibians and reptiles (Tillitt *et al.*, 2010; Hayes *et al.*, 2010; DeSolla *et al.*, 2006).

Studies on atrazine reproductive damages in mammals have observed similar effects. Abarikwu *et al.* (2010 and 2015) and Feyzi-Dehkhargani *et al.* (2012) exposed adult male rats to 100 and 300 mg/kg of atrazine observing decreases in the number of sperm in the testis and epididymis, reductions in motility and viability, morphology changes, and a reduction in daily sperm production. Song *et al.* (2014) also reported an increase in sperm abnormalities in adult male rats exposed to 77 and 154 mg/kg of atrazine, along with decreases in testosterone levels and in the expression of inhibin B.

Calomys laucha is a wild rodent that is broadly distributed throughout South America (Reis *et al.*, 2006) in terrestrial habits, and is considered primarily a granivore. This species is mostly distributed among grasslands, agricultural areas, roadsides, around cereal crop fields, and it is often found in crops of maize and wheat (Mills *et al.*, 1992).

There is no information in the literature about the effects of low dosages of atrazine on *Calomys laucha* sperm parameters. When this rodent inhabits sites near pastures and agricultural areas, it becomes susceptible to contamination. Thereby, the present study evaluated the effects of exposure to low dosages of atrazine in sperm parameters of *Calomys laucha*.

Methods

Animal handling

Specimens were obtained from the non-conventional animal's vivarium of the Federal University of Rio Grande (FURG), Rio Grande do Sul State, Brazil. The capture and maintenance of the captive animals was properly approved by the Brazilian Institute of Environmental Protection (IBAMA). The animals selected for this study were from the F5 generation, kept in vivarium.

For this study, 40 adult male *Calomys laucha* rodents were used. The mice were placed individually in plastic boxes (35 x 20 x 13 cm) and kept in an environment with controlled temperature (20 ± 2 °C), photoperiod of 12h light / 12h dark, and received food and water *ad libitun*. The experimental procedures of this study were approved by the Ethics Committee of FURG (CEUA – report number P031/2013).

Experimental design

The mice were exposed daily to atrazine by gavage in the dosages of 0.1 mg/kg, 1 mg/kg and 10 mg/kg of animal weight. Atrazine was diluted in 1% carboxymethylcellulose and administered daily in the amount of 100 µL for each animal. Control animals received water (control) or 1% carboxymethylcellulose (vehicle control). The mice were exposed to the atrazine dosages or controls for 21 days.

Following the last exposure day, specimens were sacrificed by cervical dislocation. Gonads were collected through the opening of the abdominal cavity. Then the cauda of both epididymes and part of the vas deferens were removed, ruptured with a needle and placed in a Petri dish containing Beltsville Thawing Solution (BTS) for sperm dilution (Corcini *et al.,* 2012). Sperm quality evaluations were performed at the Laboratory of Animal Reproduction at the Faculty of Veterinary Medicine, Federal University of Pelotas.

Sperm analysis:

Motility

In this work, total and progressive motility of mice sperm cells were evaluated using an automatic sperm analysis program: Computer Assisted Sperm Analysis (CASA) -

Automatic system of sperm analysis by computer, Sperm Vision©. The software was adjusted to pre-existing movement patterns of rodent sperm. Samples with 3μL of sperm were placed in a standard count analysis chamber, and then incubated at 37ºC. Ten automatized fields were considered for the analysis, with a minimum total of 1000 cells.

Flow cytometry

To evaluate membrane integrity and fluidity, DNA and acrosome integrity, mitochondrial functionality, reactive oxygen species and lipid peroxidation, an Attune Acoustic Focusing™ apparatus from Life Technologies was used. Analyses were performed using Attune Cytometric Software, version 2.1. A violet laser (405 nm wavelength) was used for detection in all evaluations. Sperm cell populations, were stained with Hoechst 33342 at a concentration of 16.2 mM, except for in DNA fragmentation. Non-spermatic events were discarded by FSC x SSC dispersion graphics and negative Hoechst 33342. To read all parameters, the cells stained with fluorophores were added to calcium-free PBS (80g of NaCl, 11.5g of KCl, 24g of Na_2HPO_4, 2g of KH_2PO_4 and 1L of Milli-Q water) using a total of 10000 sperm events per analysis with a flow of 200 cells/s. Specific fluorescent dyes were used for each evaluation, in which they were exposed in the dark at 37ºC for 5 minutes. Then, 500 μL of calcium-free PBS was added. All samples were then homogenized and analyzed via flow cytometry.

The evaluation of plasma membrane integrity was performed combining fluorescent dyes: 27 μM carboxyfluorescein diacetate (CFD) (green fluorescence) and 7.3 μM propidium iodide (PI) (red fluorescence). CFD penetrates the spermatozoa where it is then converted by esterases (enzymes that catalyze the hydrolysis of an ester bond) in viable cells; into a permeable fluorescent compound that is not retained in the cytoplasm. Conversely, PI only enters the nucleus of cells with damaged membranes. Therefore, only the damaged cells were marked with PI, and the intact membrane cells were marked with CFD. The sperm cells were classified as not injured (CFD+ and PI-), and injured (CFD+ and PI+, CFD- and PI- or CFD- and PI+) and expressed the percentage of live cells with membrane integrity (Fernández-Gago *et al.,* 2013).

Plasma membrane fluidity was analyzed using the florescent dye merocyanine 540 (2.7 μM), and YO-PRO, which fluoresces green at a final concentration of 0.1 μM (Invitrogen, Eugene, OR, USA). Only live sperm (YO-PRO negative) were selected and

classified into high fluidity cells (high M540 concentration) or low fluidity cells (low M540 concentration). Results were expressed as percentage of live cells with high fluidity (Fernández-Gago *et al.,* 2013).

Evaluation of mitochondrial functionality was performed using the fluorescent dye (13 μM) rhodamine 123. This dye, emits a stronger green fluorescence in the presence of high mitochondrial activity (higher electrochemical potential) and PI (7.3 μM). Only intact sperm (PI-) were selected and classified into cells with high functionality (high fluorescence, high accumulation of Rhodamine 123) and low functionality (low fluorescence, low accumulation of Rhodamine 123). Results were expressed as a percentage of cells with high mitochondrial functionality (Silva *et al.,* 2016).

The fluorescent dye lectin, extracted from peanut, *Arachis hypogaea* (PNA) and conjugated with fluorescein isothiocyanate (1 μg/mL: FITC) and propidium iodide (7.3 μM: PI) dye were used to evaluate acrosome integrity. Sperm were classified as unreacted with full acrosomes (FITC/PNA- and PI-), and reacted and/or harmed acrosomes (FITC/PNA+ and PI-; FITC/PNA- and PI+; FITC/ PNA+ and PI+). Results were expressed as a percentage of cells with intact acrossome (Martinez-Pastor *et al.,* 2006).

Orange acridine dye was used to evaluate DNA integrity. Orange acridine dye fluoresces green when combined with double-stranded helical DNA (intact DNA): however fluoresces orange-red when combined with methylated spermatic DNA (damaged DNA). In this evaluation, 10 μL of semen was mixed with 5 μL of TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA, pH 1.2). After a 30 second incubation period, 10 μL of Triton (0.1% Triton X-100) was added to this mixture. Then, after another 30 second period, 5 μL of orange acridine (0.037 M citric acid, 0.126 M Na_2HPO_4, 0.0011 M EDTA dissodium, 0.15 M NaCl, pH 6.0) was added to the solution. The samples were analyzed less than 5 minutes after staining, and the results were expressed as percentage of sperm cells with intact DNA (unfragmented).

The production of intracellular reactive oxygen species (ROS) was analyzed using fluorescent dye (1 μM) 2'7' dichlorofluorescein diacetate (H_2DCF-DA). This dye fluoresces green when oxidized by intracellular ROS and with 7.3 μM of PI (final concentration). Median green fluorescence intensity of the living sperm (PI-) was used to represent these measurements (Domínguez-Rebolledo *et al.,* 2011).

Lipid peroxidation (LPO) was measured using the BODIPY 581/591 C11 compound, at a final concentration of 0.002 mM. BODIPY 581/591 C11 is a fatty acid which carries a LPO sensitive fluorophore that changes its fluorescence from red (absence of lipoperoxidation) to green when lipid peroxidation occurs. The samples were homogenized and incubated in the dark for 5 (background) and 60 minutes at 37°C, and then washed by centrifugation at 600 g for 5 minutes to remove unbound dye. Sperm cells were measured as a percentage of non-lipid peroxidation by red and green median fluorescence intensity. The non-lipidic peroxidation percentage was calculated with red fluorescence / (green + red fluorescence) * 100 (Balao da Silva *et al.,* 2013).

Statistical analysis:

In this study, descriptive data (mean and standard error mean) were generated for the dependent variables. Normality was tested for all dependent variables using the Shapiro-Wilk test. After that, a Kruskal Wallis test for nonparametric data was performed. The software Statistix® version 2009 was used for the analyses.

Results

Both Total Motility and Progressive Motility were significantly reduced ($p < 0.05$) at all dosages of atrazine, as compared to water and vehicle controls (Figure 1).

Sperm membrane integrity was significantly damaged in all tested dosages of atrazine, in comparison to control groups (Figure 2). A reduction by more than 15% was observed at 0.1 mg/kg of atrazine, when compared to vehicle control. In the dosages of 1 mg/kg and 10 mg/kg of atrazine the observed reduction was 13% and 22%, respectively. A significantly higher sperm membrane fluidity ($p < 0.05$) was observed at 1 mg/kg and 10 mg/kg of exposure to atrazine, when compared to vehicle control (Figure 2).

Mitochondrial functionality was significantly affected by exposure to atrazine ($p<0.05$). Mitochondrial activity became reduced by 27% at 1 mg/kg, and by more than 21% at the dosages of 0.1 and 10 mg/kg, when compared to water and vehicle control groups (Figure 3).

Exposure to atrazine at the highest dosage (10 mg/kg) significantly reduced ($p < 0.05$) the acrosome integrity in the spermatozoa (44.5% ± 7.6%), in comparison to vehicle control group (72.8 % ± 5.8%) and water control group (82.1% ± 3.3%) (Figure 4).

No significant differences were observed in the DNA integrity of sperm cells of animals exposed to different dosages of atrazine, comparing with water and vehicle controls. Analyses regarding reactive oxygen species (ROS) and lipid peroxidation in sperm cells showed no significant differences from water and vehicle controls, in all atrazine dosages evaluated (Data not shown).

Discussion

Several studies have demonstrated damaging effects of atrazine on different male reproductive functions (Kniewald *et al.,* 2000; Stoker *et al.,* 2002; Abarikwu *et al.,* 2010, 2015; Victor-Costa *et al.,* 2010; Dehkhargani *et al.,* 2011). Among the above listed studies, the majority used laboratory specimens, and often with high dosages of atrazine. However, in this study, a significant disturbance to sperm capacity was observed when wild mice were exposed to much lower dosages of atrazine.

In this study, both total motility and progressive motility were reduced in all tested exposure rates of atrazine. These results are consistent with other studies in mice, where in the atrazine dosages used were 120 and 200 mg/kg (Abarikwu *et al.*, 2010); 100, 200 and 300 mg/kg (Dehkhargani *et al.*, 2011) and 12.5 mg/kg (Abarikwu *et al.*, 2015), which all also showed drastic reduction in sperm motility. It is important to highlight that atrazine dosages used in this study were lower than the above mentioned. In addition, *in vitro* studies performed by Betancourt *et al.* (2006) with pig sperm cells showed decreases in sperm motility, velocity and linearity. These results demonstrate that atrazine may act directly on the sperm cell, reducing its motility, which was also observed in the present study.

Sperm motility of rats exposed to the three dosages of atrazine (0.1 mg/kg, 1 mg/kg and 10 mg/kg) was significantly affected such that most of the observed parameters showed reductions following exposure. Reductions in sperm motility may be associated with observed mitochondrial dysfunction; mitochondria provide the required energy for flagellar motility in sperm. In all dosages of atrazine, mitochondrial functionality of spermatozoa was significantly reduced. This reduction may be related to the capacity of atrazine to establish a connection with the ATP synthase (Hase *et al.* 2008). Atrazine binding with this enzyme

would inhibit the oxidative phosphorylation process, which would culminate in a sharp decrease in ATP production. This reduction in energy production through aerobic metabolism would then directly affect the driving ability of the sperm. Furthermore, Song *et al.* (2014) demonstrated a decrease in the activity of the lactate dehydrogenase (LDH) and succinate dehydrogenase (SDH) enzymes in male rat testes when exposed to concentrations of 77 and 154 mg/kg of atrazine. Such enzymes are associated with the energy metabolism of spermatozoa, and consequently with the maturation of sperm cells. Thus, Song *et al* (2014) suggested that atrazine significantly influences the energy metabolism and maturation of sperm, which may explain the reduction in sperm motility observed in the present study.

Damages to sperm membrane integrity were found in animals treated with all dosages of atrazine, as well as an increase in spermatozoa membrane fluidity. These factors may have contributed to damages in sperm motility processes. Similar results were demonstrated by Dehkhargani *et al.* (2011) and Feyzi-Dehkhargani *et al.* (2012), where in the effects of exposure to 100, 200 and 300 mg/kg of atrazine in rat sperm were analyzed. It is worth highlighting that the present study found similar effects to the afore mentioned works using dosages of a lower magnitude order. The reduction in sperm membrane integrity may be due to a significant rise in sperm membrane flow. Importantly, membrane fluidity is inversely proportional to cholesterol levels. Therefore, it may be inferred that atrazine can reduce cholesterol levels in the sperm membrane, which would result in the increase in membrane fluidity found following exposure to higher dosages (1 mg/kg and 10 mg/kg). This observation agrees with the results of Singh *et al.* (2008), who reported a reduction in total cholesterol and phospholipids in erythrocyte membrane cells of rats exposed to 300 mg/kg of atrazine. The authors indicated that this reduction may be associated with the lipid peroxidation process. In contrast, the present study analyses of ROS and lipid peroxidation found no significant differences when compared to the control groups, which would indicate that the damage and the increased fluidity of sperm membranes were not due to oxidative stress processes. Thus, the results obtained here indicate that reductions in the integrity and increase in the fluidity of sperm membranes were not caused by oxidative stress, which may reinforce the notion that disturbances in lipid levels were responsible for the observed changes.

In the present study, integrity of spermatic DNA showed no significant differences between the control groups and any of the evaluated dosages. In contrast, Feyzi-Dehkhargani *et al.* (2012) showed an increase in DNA disintegration and nuclear immaturity in rat sperm

cells following exposure to 100, 200 and 300 mg/kg of atrazine and during different periods (12, 24 and 48 days), demonstrating a dosage/time dependent effect. The absence of DNA damage in sperm cells in the present study can be attributed to the low dosages of atrazine used, in comparison with those used by Feyzi-Dehkhargani *et al.* (2012). This observation could further be attributed to the absence of oxidative stress, which was one of the mechanisms of DNA damage for all three dosages tested.

Exposure to higher dosages of atrazine (10 mg/kg) reduced acrosome integrity in comparison to control groups. However, results observed by Maravilla-Galvan *et al.* (2009), showed a significant increase in the acrosome reaction of boar spermatozoa after an *in vitro* exposure using 8, 20 and 40 μM of atrazine. These contrasting results may be due to different dosages used and the mechanism of exposure. The decrease in acrosome integrity observed in this study may be directly related to the change in sperm membrane fluidity and to the observed damages to sperm membrane.

Studies reporting harmful effects of atrazine to sperm functionality in wild rodents such as *Calomys laucha* are rare, and the fact that their populations often live in areas adjacent to farming settlements reinforces the ecological and environmental importance of this study. It is worth emphasizing that this study used very low dosages of atrazine, in which the lowest (0.1 mg/kg) was less than the limits enforced by environmental and health agencies for residues in corn and sugarcane crops (ANVISA, 2003). Therefore, the observed impacts on the reproductive capacity in the present results suggest that even legally accepted dosages of atrazine may cause serious damage to the reproductive capacity of *Calomys laucha*, and possibly to other species.

Conclusion

Exposure to atrazine, even at low dosages, showed significant harmful effects regarding the functionality of *Calomys laucha* male sperm cells.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure legends

Figure 1. Total and progressive motility percentage of mice sperm cells, after exposure to different dosages of atrazine. Values represent mean ± standard deviation (n=8). Means were compared using the Kruskal-Wallis test with a significance level of 0.05. Different letters indicate differences between treatments.

Figure 2. Percentage of mice sperm cells with intact and fluid membranes, after exposure to different dosages of atrazine. Values represent mean ± standard deviation (n=8). Means were compared using the Kruskal-Wallis test with a significance level of 0.05. Different letters indicate differences between treatments.

Figure 3. Percentage of mice sperm cells with functional mitochondria, after exposure to different dosages of atrazine. Values represent mean ± standard deviation (n=8). Means were compared using the Kruskal-Wallis test with a significance level of 0.05. Different letters indicate differences between treatments.

Figure 4. Percentage of mice sperm cells with acrosome reaction, after exposure to different dosages of atrazine. Values represent mean ± standard deviation (n=8). Means were compared using the Kruskal-Wallis test with a significance level of 0.05. Different letters indicate differences between treatments.

Figures

Figure 1

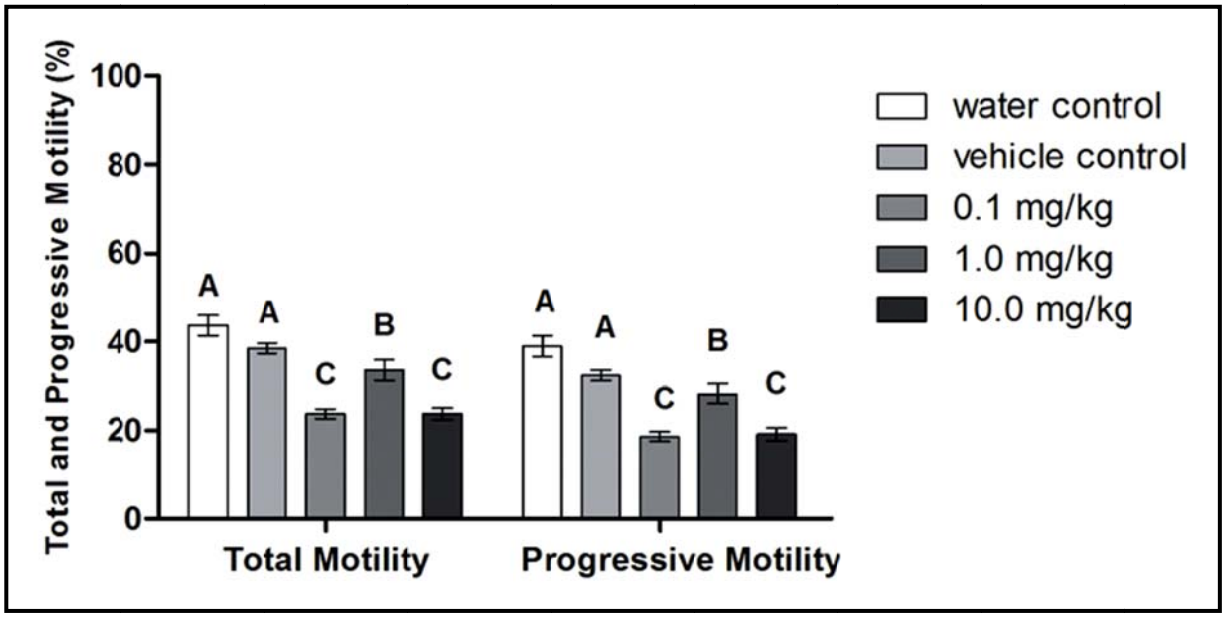
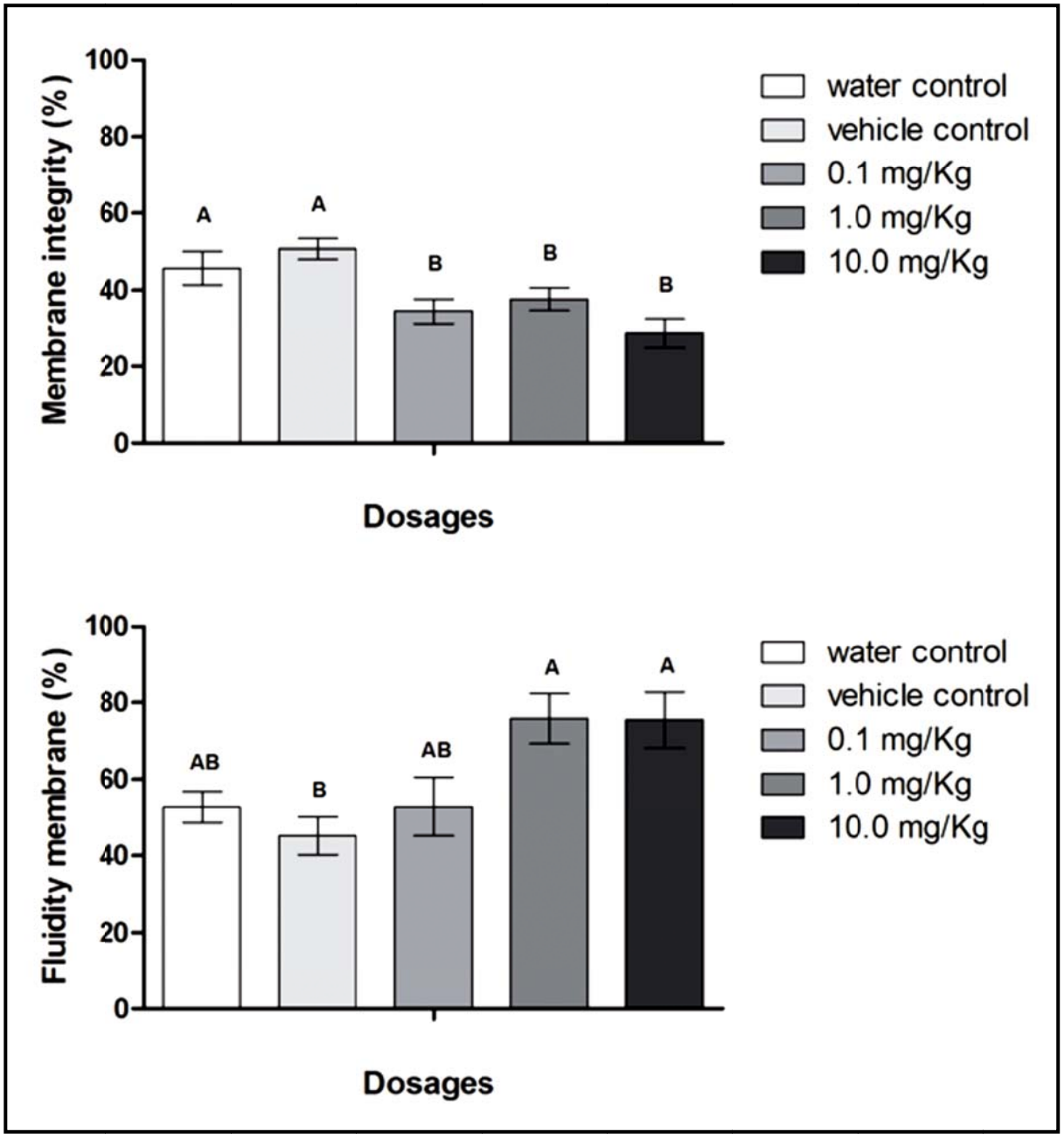


Figure 2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80
60
40
20
0
A
A
B
B
B
Dosages
water control
vehicle control
0.1 mg/Kg
1.0 mg/Kg
10.0 mg/Kg
Fluidity membrane (%)
100
80
60
40
20
0
AB
B
AB
A
A
Dosages
water control
vehicle control
0.1 mg/Kg
1.0 mg/Kg
10.0 mg/Kg

Figure 3

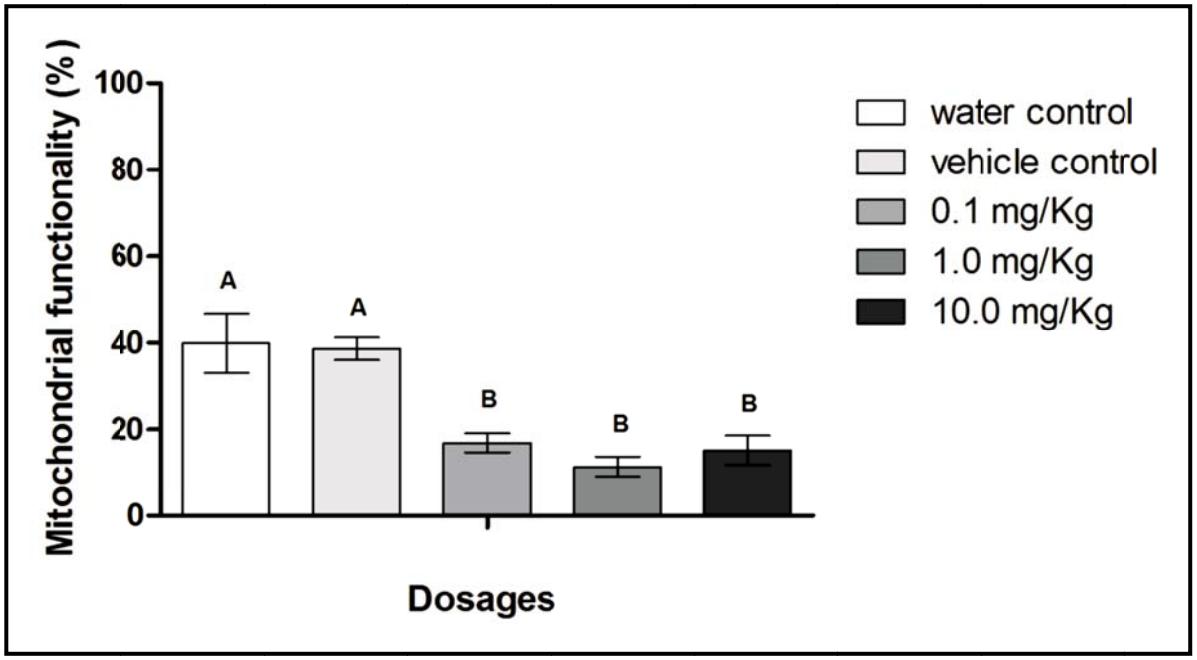
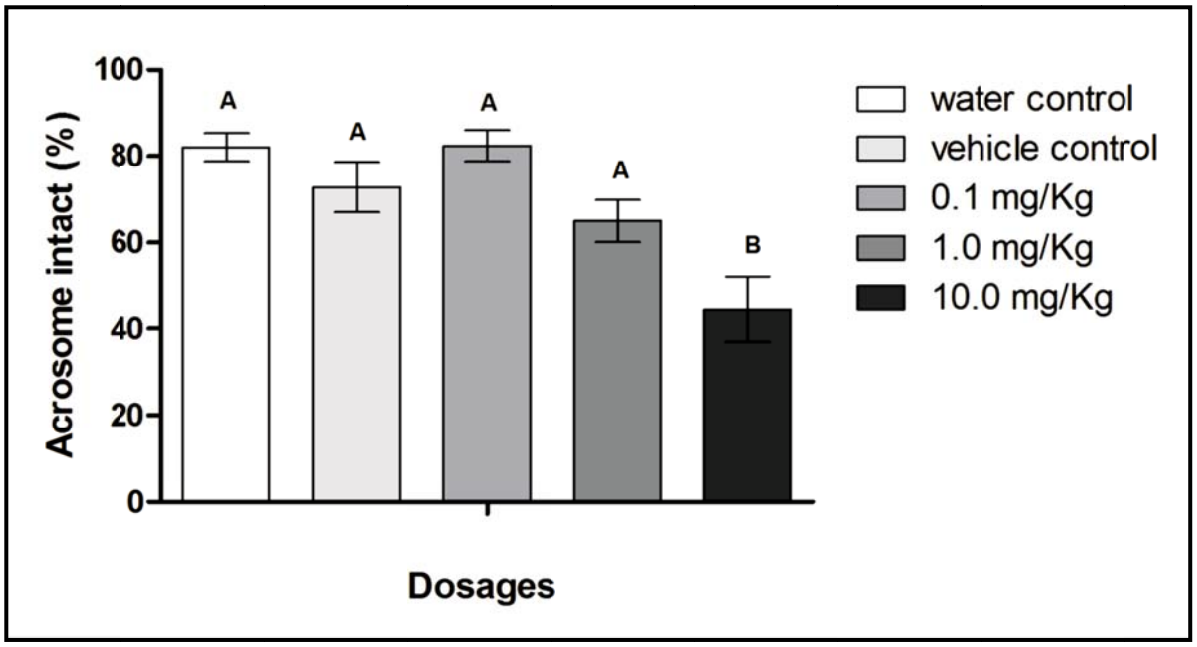


Figure 4



Manuscrito 2:

Low atrazine dosages received during prenatal or breastfeeding affect developmental *Calomys laucha* sperm cells

Será submetido à revista Reproduction

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Low atrazine dosages received during prenatal or breastfeeding affect development *Calomys laucha* sperm cells

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Short Tittle: Atrazine affects *Calomys laucha* sperm quality

Abstract

This study evaluates the effects of low doses of atrazine administered during gestation or breastfeeding on sperm capacity of wild rodent *Calomys laucha*. Ten groups of adult females received different doses of atrazine (water and vehicle controls; 0.1; 1 and 10 mg / kg), trough gavage, during gestational or lactation periods, and at three months of age their puppies were evaluated. The spermatic parameters analyzed were: sperm motility, integrity, fluidity and rupture of membrane, mitochondrial functionality, DNA damage, sperm concentration, lipid peroxidation and production of reactive oxygen species (ROS). Total and progressive motility reduced drastically in male sperm cells, at all dosages and both exposure periods. Plasma membrane integrity decreased at all dosages of atrazine in sperm cells of adult males exposed during breastfeeding, while membrane fluidity increased at all tested dosages in these cells. Sperm mitochondrial functionality reduced at all dosages of atrazine in both exposure periods. Sperm DNA damage was higher in male exposed to the highest dosage (1 mg/kg) during prenatal period, and in animals exposed to the lowest dosage of atrazine (0.1 mg/kg) during breastfeeding. Sperm concentration reduced at the highest dose of atrazine (1 mg/kg) and there was a decrease in the amount of ROS at all evaluated dosages in male exposed during prenatal period. These results suggest that even low doses of atrazine, when administered at critical periods of development, as in prenatal and breastfeeding periods, may cause permanent changes in the spermatogenesis process of the adult male mice, reducing the sperm quality of these animals.

Key words: atrazine, puppies, prenatal, breastfeeding, *Calomys laucha*, sperm quality

Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is an herbicide widely used in agriculture, mainly in maize, sorghum and sugarcane crops, being also used for selective control of weeds (Bridges, 2008). The use of atrazine has been banned in the European Union since 2001, but it is still legal in many countries, including United States of America, China, Mexico, Argentina and Brazil (LeBaron *et al.*, 2008). Atrazine is a contaminant commonly found in drinking and shallow waters (Jablonowski *et al.,* 2011), as well as in food (Garcia *et al.*, 2012). Accordingly to Brazilian National Health Protection Agency - ANVISA, the maximum permitted residue limits for pineapple and sorghum is 0.02 mg/kg and for maize and sugar cane is 0.25 mg/Kg (ANVISA, 2003). While the Brazil National Environmental Council (CONAMA) establishes the maximum acceptable concentration at 2µg/L for drinking water (CONAMA, 2005). However, concentrations above these are often found in surface water, groundwater and precipitation (Nwani, 2010; Moreira *et al.*, 2012).

Atrazine is considered a potent contaminant due to its chemical characteristics, which include low hydrolysis, low to moderate solubility in water, and greater solubility in organic matter and adipose tissue (Ross *et al.*, 2009). In addition, atrazine and its metabolites have been detected in plasma, urine, other animal tissues (such as brain, liver and kidneys) of exposed rats (Fraites *et al.*, 2011; Ross *et al.*, 2009, Peighambarzadeh *et al.*, 2011), fetuses and milk (Fraites *et al.*, 2011), as well as in human umbilical cord samples (Whyatt *et al.*, 2003).

Recent research has been performed focusing on the effects of atrazine on mammalian reproductive system. Studies with male rats have shown damage in the spermatogenesis process, causing a decrease in the amount and motility of spermatozoa in the epididymis, morphological changes in Sertoli and Leydig cells (Kniewald *et al.*, 2000), induction of

74 oxidative stress and increased lipid peroxidation in the testes and epididymis (Abarikwu *et al.*,
75 2010; Adesiyan *et al.,* 2011).

76 Effects of atrazine on puppies exposed during prenatal and breastfeeding periods are
77 also reported in the literature. In males, the effects of atrazine exposure prenatal period were
78 delays in the preputial separation (Rosemberg *et al.*, 2008; Stanko *et al.*, 2010), decrease in
79 anogenital distance and decrease in testosterone levels (Rosemberg*et al.*, 2008). Rats exposed
80 during breastfeeding period showed reductions in body weight and reproductive organs (Jin *et*
81 *al.*, 2013, Pogmic-Majkic *et al.*, 2010), reduction in testosterone levels (Jin *et al.*, 2013,
82 Pogmic *et al.*, 2009), increase in estrogen levels (Jin *et al.,* 2013), as well as a decrease in
83 expression of steroidogenic genes (Jin *et al.*, 2010; Pogmic *et al.*, 2009). These studies
84 demonstrate that atrazine strongly impairs the reproduction of rodents, which are highly
85 vulnerable during prenatal and breastfeeding periods.

86 *Calomys laucha* is a small wild rodent that lives in areas of southern Bolivia, southern
87 Brazil, Uruguay and central Argentina where it is responsible for damages in agriculture and
88 also for the transmission of the virus Junin, causes of Argentine hemorrhagic fever (Alche and
89 Coto, 1993). This species is usually found in pastures, agricultural areas and around cereal
90 fields, mainly corn and wheat (Mills *et al.,* 1992).

91 Many studies have shown damage to reproductive organs and changes in hormone
92 levels of puppies exposed to atrazine during prenatal and breastfeeding periods. However,
93 there are still no reports in the literature about the effects of low atrazine dosages on sperm
94 cells of wild rodents exposed to this contaminant during prentatal and breastfeeding periods.
95 Since these rodents live in areas close to agricultural crops and are therefore subject to
96 atrazine contamination, *Calomys laucha* becomes susceptible to reproductive injury caused by
97 this contaminant, which may lead to a decrease in the populations of this species, and possibly
98 from other mammals. Thus, this study evaluated the effects of low atrazine dosages on sperm

parameters of males *Calomys laucha* species exposed during prenatal and breastfeeding periods.

Material and Methods

Animal Handling

Specimens were obtained from the non-conventional animal's vivarium of the Federal University of Rio Grande (FURG), Rio Grande do Sul, Brazil. The capture and maintenance of these specimens was approved by the Brazilian Institute for Environmental Protection (IBAMA) (SISBIO - permit number 14174). Specimens were kept in plastic boxes (35x20x13cm) under controlled temperature (20ºC ± 2ºC), photoperiod of 12h light / 12h dark and received water and food *ad libitun*. Animals selected for this study are from the F5 generation maintained in the vivarium. The experimental procedures of this study were approved by the Ethics Committee of FURG (CEUA - report number P031/2013).

Experimental Design

Fifty females and 25 males of the *Calomys laucha* were used in this experiment. In each plastic box (35 x 20 x 13 cm) two females and one male were kept for 5 days. Thereafter, males were removed. Twenty five females were randomly selected for gestational exposure and the other 25 for lactational exposure.

The females exposed in the gestational period were allocated into 5 groups (5 individuals each) and received atrazine daily by gavage in dosages of 0.1 mg/kg, 1 mg/kg and 10 mg/kg. Atrazine was diluted in 1% carboxymethylcellulose and given daily in the volume of 100 µl for each female. Control females received water (control) or the vehicle carboxymethylcellulose (vehicle control). Females were exposed to atrazine dosages during

all gestational period, until the birth of the pups. After birth, puppies were kept with their mother until the end of the breastfeeding period, so they were sexed and the males kept apart for further analysis.

The females exposed in the lactational period were also allocated into 5 groups (5 individuals for each group), and on the day of the birth of their puppies, started a daily exposure by gavage to controls (water and vehicle) and atrazine (0.1, 1 and 10 mg/kg) during all breastfeeding period (21 days). Then this period, the puppies were sexed and the males were separated and kept under the laboratory conditions until they completed 3 months, when the analyses were performed.

When male puppies exposed during prenatal (mother's gestation) and breastfeeding periods completed 3 months of age they were sacrificed by cervical dislocation. The gonads were collected by opening of abdominal cavity. The cauda of both the epididymis and part of the vas deferens were removed, ruptured with a needle and placed in a Petri dish containing Beltsville Thawing Solution (BTS) for sperm dilution. Sperm quality analyses were performed at the Laboratory of Animal Reproduction of the Veterinary Faculty at the Federal University of Pelotas (UFPel).

Sperm analysis:

Motility

Total and progressive motility were evaluated using an automatic sperm analysis program: Computer Assisted Sperm Analysis (CASA) - Automatic system of sperm analysis by computer, Sperm Vision©. The software was adjusted to pre-existing movement patterns of rodent sperm. 3µl of sperm samples were placed in a standard count analysis chamber, and

146 then incubated at 37 °C. For the analysis, 10 automated fields were considered, with a
147 minimum total of 1000 cells.

148

149 **Flow cytometry**

150 Flow cytometry was performed using the Attune Acoustic Focusing Cytometer®
151 (Applied Biosystems). We analyzed the results using the Attune Cytometric Software v2.1.
152 For detection of cell populations in all analysis, cells were stained with Hoechst 33342 and
153 the population was detected by the photo detector VL1 (filter 450/40). The green fluorescence
154 of H_2DCFDA (ROS), carboxyfluorescein diacetate (plasma membrane integrity and rupture),
155 rhodamine 123 (mitochondria functionality) were read with a BL1 photodetector
156 (filter530/30), the orange fluorescence of the merocyanine 540 (plasma membrane fluidity)
157 was read with BL2 photodetector (filter 574/26). The red fluorescence of propidium iodide
158 (membrane integrity) was read with the photodetector BL3 (640LP filter).Ten thousand
159 events were analyzed per sample at a flow rate of 200 cells/s. The non-spermatozoa debris
160 were eliminated based on scatter plots (Piehler *et al.,* 2005).

161 Integrity and rupture of plasma membrane were realized combining fluorescent dyes:
162 carboxifluorescein diacetato (CFD) (27 μM) and propidium iodide (PI) (7.3 μM). The sperm
163 cells were classified as not injured (CFD+ and PI-), and injured (CFD+ and PI+ or CFD- and
164 PI+). Membrane disruption was classified as PI+ (ruptured membranes) and PI- (intact
165 membranes). (Fernández-Gago *et al.,* 2013). Results were expressed as the total of intact or
166 not injured membranes / total cells * 100.

167 Plasma membrane fluidity was evaluated using the fluorescent dye merocyanine 540
168 (2.7 μM) and YO-PRO-1 (0.01 μM). Samples were mixed and incubated in the dark for 10
169 minutes at 37°C. Only live sperm (YO-PRO negative) were selected and classified into high

fluidity cells (high M540 concentration) or low fluidity cells (low M540 concentration) (Fernández-Gago *et al.* 2013). Results were expressed as percentage of cells with high fluidity.

Mitochondrial functionality was performed using the fluorescent dye Rhodamine 123 (13 μM) and propidium iodide (PI) (7.3 μM). Only intact sperm (PI-) were selected and classified either as those with more active mitochondria (high fluorescence, higher accumulation of rhodamine) and less active (low fluorescence, lower accumulation of rhodamine) (Silva *et al.* 2016). Results were expressed as a percentage of cells with high mitochondrial functionality.

DNA integrity was evaluated by the sperm chromatin structure assay (SCSA). The fluorescent dye orange acridine was used. Spermatozoa were classified with as integrate DNA (green fluorescence) or fragmented DNA (orange/red fluorescence). DNA fragmentation index was calculated by the number of spermatozoa with fragmented DNA / total number of spermatozoa * 100.

For this analysis production of reactive intracellular oxygen species (ROS) was used the fluorescent dye 2'7' dichlorofluoresceindiacetate (H_2DCF-DA) (1mM) and propidim iodide (PI) (7.3 μM). Median green fluorescence intensity of the living sperm (PI-) was used to represent these measurements (Domínguez-Rebolledo *et al.,* 2011).

Lipid peroxidation (LPO) was evaluated using the fluorescent dye BODIPY 581/591 C11 (0.002 mM). The samples were mixed and incubated in the dark at 37°C during two distinct intervals, 5 (background) and 60 minutes. Then, they were washed by centrifugation at 600 g for 5 minutes to remove unbound dye. Sperm cells were measured as a percentage of lipid peroxidation by red and green median fluorescence intensity. The lipidic peroxidation percentage was calculated with green fluorescence / (green + red fluorescence) * 100 (Balao da Silva *et al.,* 2013).

For the determination of the sperm concentration, 900 μL of saline formol was added in 100 μL sperm aliquot. The suspension was mixed and charged into Neubauer's counting chamber. The total sperm count in 5 squares of 1 mm^2 each was determined and multiplied by 5×10^4 to express the number of spermatozoa by epididymis.

Data analysis

Descriptive data (mean and standard error mean) were generated for the dependent variables. For all dependent variables, normality was tested using the Shapiro-Wilk test. Following, the Kruskal Wallis test was used for non-parametric data. The software Statistix® version 2009 was used for the analysis.

Results

No significant differences ($p > 0.05$) were observed between the water and vehicle control in any of the sperm parameters analyzed in both exposure periods (prenatal and breastfeeding).

Prenatal exposures

Females exposed to atrazine at the highest dosage (10 mg/kg) during gestational period presente either abortions or stillborn puppies. Thus, there was no sperm analysis at the highest dosage of atrazine in the prenatal period.

Total motility of the spermatozoa of males exposed in the prenatal period to different dosages of atrazine were significantly reduced ($p \leq 0.05$), when compared to the vehicle control (Figure 1). Progressive motility presented the same reduction tendency of motile cells, when compared to the vehicle control (Figure 1).

A reduction tendency in the percentage of cells with intact membranes was verified in all evaluated atrazine dosages in the males exposed during prenatal period. However the fluidity and rupture membrane did not present significant differences when compared to vehicle control (Figure 3).

Mitochondrial functionality was significantly reduced ($p \leq 0.05$) in all atrazine dosages analyzed in the sperm cells of males exposed during prenatal period, comparing with vehicle control. ROS were reduced to the highest dosage tested (1 mg/kg) in sperm cells. Still, lipid peroxidation of sperm cells showed no significant differences in comparison with vehicle control (Figure 4).

Sperm DNA fragmentation index was elevated at the highest dosage of atrazine evaluated (1mg/kg), while the sperm concentration was significantly reduced in the highest dosage of atrazine (1mg/kg) in males exposed in prenatal period, comparing with vehicle control (Table 1).

Breastfeeding exposures

Sperm cells of the puppies exposed to different doses of atrazine during breastfeeding had a significant reduction of their Total and Progressive Motility ($p \leq 0.05$) to values of about 18.4% and 14.3%, respectively, when compared to vehicle control groups (57.6% and 55.1%) (Figure 2).

Membrane integrity was significantly reduced, while the membrane fluidity increased significantly ($p \leq 0.05$) in all tested dosages of atrazine, in comparison to vehicle control. The membrane rupture showed an increasing tendency for the two higher dosages, 1 and 10 mg/kg, when compared to vehicle control (Figure 3).

Mitochondrial functionality was significantly reduced ($p \leq 0.05$) in all dosages analyzed of atrazine, in sperm cells of males exposed during the breastfeeding period, when compared to the vehicle control. Similarly, ROS were reduced at all dosages of atrazine. While lipid peroxidation in sperm cells did not show significant differences in comparison to vehicle control (Figure 4).

DNA fragmentation index increased at the lowest dosage (0.1 mg/kg) in sperm cells of males exposed during the breastfeeding period, when compared to the vehicle control. Sperm concentration did not change in any of the dosages evaluated (Table 1).

Discussion

In this study, *Calomys laucha* females were exposed to low dosages of atrazine in gestation or breastfeeding period, so their puppies were exposed indirectly to atrazine during critical periods of their development. Thus, we observed damages on the sperm cells of these males that were possibly caused by changes in their testicles, which resulted in sperm damage persist in adult life These results show that atrazine is transmitted from the mother's blood circulation to the developing fetus, and can be also transferred through breast milk. It happens because highly lipophilic substances, such as atrazine, can easily overpass the placenta and reach the fetus during pregnancy (Breslin *et al.*, 1996; Breitzka *et al.*, 1997). In addition, atrazine has a low molecular weight which, combined with its high lipophilicity, confers easily diffusion and transferred from the mother to the puppies through breast milk (Breitzka *et al.*, 1997). The transfer of atrazine and its metabolites through breast milk has been proven by Stoker and Cooper (2007), who verified the presence of ^{14}C-atrazine and its ^{14}C-CITRI residues in the stomach and brain of puppies exposed during breastfeeding. In addition, Fraites *et al.* (2011) verified the presence of atrazine and its metabolites in the tissues of

mothers, fetuses and neonates after exposures performed during gestation and breastfeeding periods.

Therefore, the administration of atrazine in the prenatal and/or breastfeeding periods causes harmful effects on reproductive parameters of males, and these damages are noted late in the adult life. A number of studies report reductions in plasma and intratesticular levels of testosterone (Stoker *et al.*, 2000; Trentacoste *et al.*, 2001; Friedmann, 2002; Rosemberg *et al.*, 2008), delay in preputial separation (Stoker *et al.*, 2000; Stanko *et al.,* 2010), as well as a decrease in expression of steroidogenic genes (Pogrmic *et al.*, 2009; Jin *et al.*, 2013). However, there are few data about the effects of low dosages atrazine on sperm quality of adult male mice exposed during gestation and breastfeeding periods, and no studies with wild rodents.

Gestation period is quite susceptible to atrazine contamination, as demonstrated by Rosemberg *et al.* (2008) who observed a reduction in the survival of puppies mice from females exposed to atrazine during the gestation period, at dosages equal and greater than 10 mg/kg. Similarly, in this study, the higher dose of atrazine (10 mg/kg) given to females during gestation period resulted in abortions or stillborn puppies. This result agrees with the previous study, demonstrating this atrazine dosage is highly toxic to the fetuses.

Herein, we verified a drastic reduction in the percentage of total and progressive motility of the sperm cells of males exposed both in prenatal as breastfeeding periods. Sperm motility requires energy to occur, and mitochondria are the largest source of energy for the sperm cell (Copeland, 2002). In our study, the dosages of atrazine impair mitochondrial functionality of sperm cells, both in males exposed in prenatal as breastfeeding periods. Thus, the decrease in sperm motility verified may be directly related to the lack of energy from the lowest mitochondrial activity. In addition, the reduction of sperm motility may have been caused by damage to sperm membrane integrity. Sperm cells from puppies exposed during

291 breastfeeding period showed a reduction in membrane integrity at all dosages evaluated.
292 These damages are concomitant with the increased fluidity of the membrane observed in
293 sperm cells of these animals. Increased membrane fluidity may have been caused by the
294 reduction of cholesterol present in the plasma membrane. Singh *et al.* (2008) observed
295 reductions in total cholesterol and phospholipid in rat erythrocyte membranes after exposures
296 to 300 mg/kg of atrazine. The loss of cholesterol of the sperm membrane increases its fluidity
297 and as a consequence may cause a destabilization in the plasma membrane, reducing its
298 integrity. Integrity and fluidity membrane of sperm cells are important parameters for
299 fertilization, as well as crucial for the capacitation, acrosome reaction and the fusion of the
300 spermatozoid with the oocyte (Yanagimachi *et al*., 1994). Thus, exposure to atrazine during
301 breastfeeding period may impair the fertilization process of the spermatozoa of these animals,
302 impacting the reproductive capacity of this species. Thus, this study demonstrates that the
303 drastic reduction in sperm motility was caused by the reduction in mitochondrial
304 functionality, associated with increased membrane fluidity and consequent decrease in sperm
305 membrane integrity.

306 In addition to providing around 90% of cellular energy, mitochondria are also
307 responsible for most of ROS production (Copeland, 2002). Therefore the decrease in the
308 amount of ROS found in sperm cells may be due to low mitochondrial functionality
309 visualized in this work. ROS reduction and absence of lipid peroxidation in sperm cells
310 demonstrate that the damage found is not caused by an oxidative stress process in these cells,
311 but possibly by some permanent changes in gene expression.

312 Increase in the sperm DNA fragmentation index of males exposed to atrazine during
313 prenatal and breastfeeding periods was observed in this study. Similarly, DNA damage caused
314 by exposures to atrazine was verified in sperm cells of rats (Feyzi-Dehkhargani *et al.,* 2012)
315 and in human lymphocytes (Zeljezic *et al.,* 2006). According to Feyzi-Dehkhargani *et al*

316 (2012), atrazine exposure not only increases DNA damage but also affects the DNA packing
317 process. Sperm DNA fragmentation is often corrected by remodeling the chromatin in
318 spermatids. However, changes in the mechanisms of this remodeling may be responsible for
319 persistent damage to the DNA molecule, producing irreversible damage to the sperm
320 (Jasperses *et al.,* 2005). Thus, increased sperm DNA fragmentation seen in male mice may
321 impair spermatogenesis and consequently reduce the reproductive capacity of these animals in
322 adulthood.

323 Thereby, spermatic damages verified in this study are permanent, since the animals
324 were exposed during prenatal and breastfeeding periods, and were only evaluated when adult.
325 This shows that atrazine possibly caused some reproductive organs damage, since the periods
326 of exposure used in this work comprised critical periods of development of the reproductive
327 system of these animals. Therefore, damage to the development or maturation of the testes
328 may lead to failures in spermatogenesis process after puberty, resulting in sperm damage,
329 such as those verified in this study. Agreeing with this hypothesis, Mansour *et al.* (2014)
330 showed that males exposed to atrazine during prenatal and breastfeeding periods presented
331 degeneration in spermatogonia, thickening of the basal membrane and reduction of
332 seminiferous tubules diameter. In addition, a study by DeSesso et al. (2014) evaluating late
333 effects on the development of males exposed to atrazine during prenatal period, whose
334 pregnant rats were treated in the gestational day 6-21, found that males evaluated at 70 days
335 showed an increase in the number of abnormal spermatozoids in epididymis the treated group
336 with 125 mg/kg/day, while at the dose of 25 mg/kg.day this increase occurred in the sperm
337 cells of the animals evaluated at 170 days. In the same study, when atrazine exposure
338 occurred during breastfeeding, a reduction in testes and epididymal weight was observed in
339 males evaluated at 70 days, and in males with 170 days there was also an increase in the
340 number of abnormal spermatozoa of animals exposed to 125 mg/kg/day. Stoker *et al.* (1999)

observed an increase in prostate inflammation of adult males after they were exposed to atrazine in breastfeeding period through breast milk. Thus, in our study, low dosages of atrazine administered indirectly to male puppies through the maternal route, caused a reduction in sperm quality these animals, which may have occurred due to the damages found by these authors.

Another factor that may be changing the sperm parameters is the endocrine disruptor capacity of atrazine (Sanderson *et al.*, 2000, Victor-Costa *et al*., 2010, Breckenridge *et al*., 2015). Several studies have shown that males exposed to atrazine during the prenatal and breastfeeding periods present a reduction in intratesticular and plasmatic testosterone levels (Trentacoste *et al.*, 2001; Friedmann 2002, Rosemberg *et al*., 2008, Pogrmic *et al*., 2009, Jin *et al*., 2013). The decrease in the expression of steroidogenic genes in males exposed at atrazine in breastfeeding period has also been documented (Pogrmic *et al.*, 2009, Jin *et al*., 2013). Testosterone and its metabolite dihydrotestosterone affect the morphogenesis of reproductive organs, and also influence the activity of enzymes related to reproduction, which are expressed late (Forest, 1983). Thereby, reductions in the expression of steroidogenic genes and in androgen levels, occurring in critical moments of reproductive development, such as the embryonic period, impact the growth and development of reproductive organs, resulting in serious damages of spermatogenesis in adulthood. It can explain the reduction in sperm concentration observed in the animals exposed during prenatal period. In addition, possible reductions in androgen levels, caused by atrazine exposures in prenatal and breastfeeding periods, and seen in adulthood may also explain changes in animal's spermatozoa.

Therefore, this study showed that indirect exposure to atrazine of male puppies from *Calomys laucha* species, both during prenatal as breastfeeding periods, caused several damages in the spermatozoa of these individuals, drastically reducing their sperm quality. It should be noted that sperm damages were permanent, visualized in adulthood, indicating that

366 exposure in the early stages of development (prenatal and breastfeeding) are critical for
367 reproductive development, and that during these periods the animals are susceptible to
368 reproductive damage even exposed to low dosages of atrazine such as 0.1 mg / kg, allowed in
369 Brazil by regulatory agencies.

370

371 **Declaration of interest**

372 The authors declare that there is no conflict of interest.

373

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378

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Figure legends

Figure 1. Total and progressive motility of mice sperm cells, after exposure to different dosages of atrazine in prenatal period. Values represent mean ± standard deviation (n=8). Means were compared using the Kruskal-Wallis test with a significance level of 0.05. Different letters indicate differences between treatments.

Figure 2. Total and progressive motility of mice sperm cells, after exposure to different dosages of atrazine in breastfeeding period. Values represent mean ± standard deviation (n=8). Means were compared using the Kruskal-Wallis test with a significance level of 0.05. Different letters indicate differences between treatments.

Figure 3. Membrane Integrity (A), fluidity (B) and disruption (C), from sperm cells of males exposed to atrazine in prenatal period. Membrane Integrity (D), fluidity (E) and disruption (F), from sperm cells of males exposed to atrazine in breastfeeding period. Values represent mean ± standard deviation (n=8). Means were compared using the Kruskal-Wallis test with a significance level of 0.05. Different letters indicate differences between treatments.

Figure 4. Mitochondrial functionality (A), ROS (B) and lipid peroxidation (C), from sperm cells of males exposed to atrazine in prenatal period. Mitochondrial functionality (D), ROS (E) and lipid peroxidation (F), from sperm cells of males exposed to atrazine in breastfeeding period. Values represent mean ± standard deviation (n=8). Means were compared using the Kruskal-Wallis test with a significance level of 0.05. Different letters indicate differences between treatments.

Figure 1

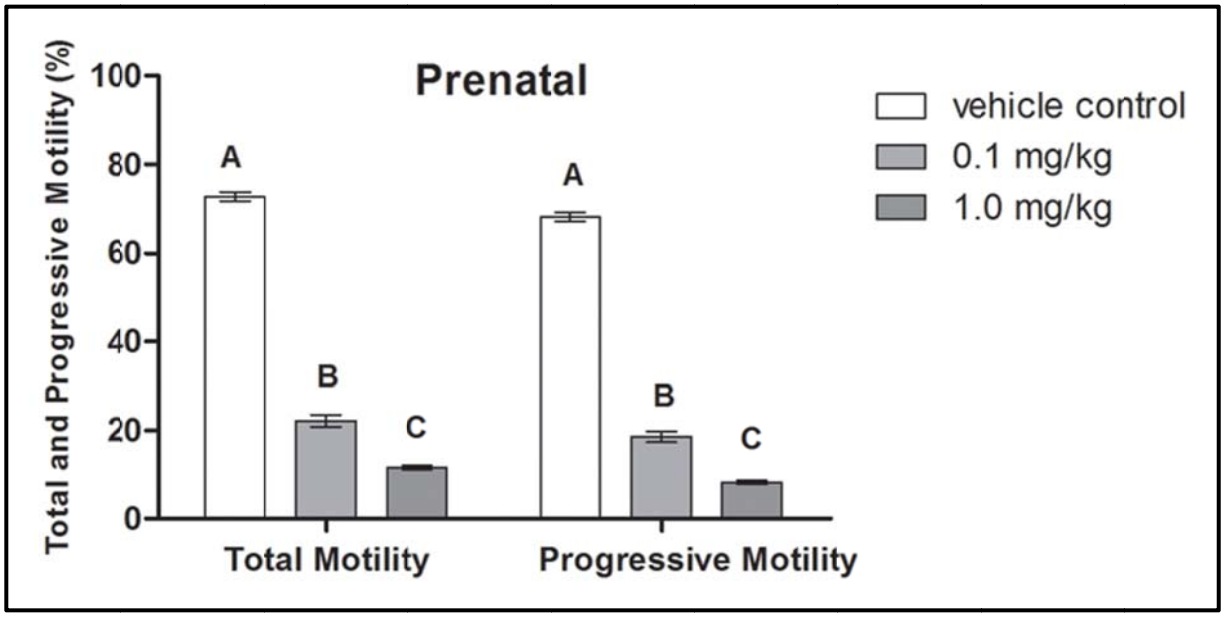

Figure 2

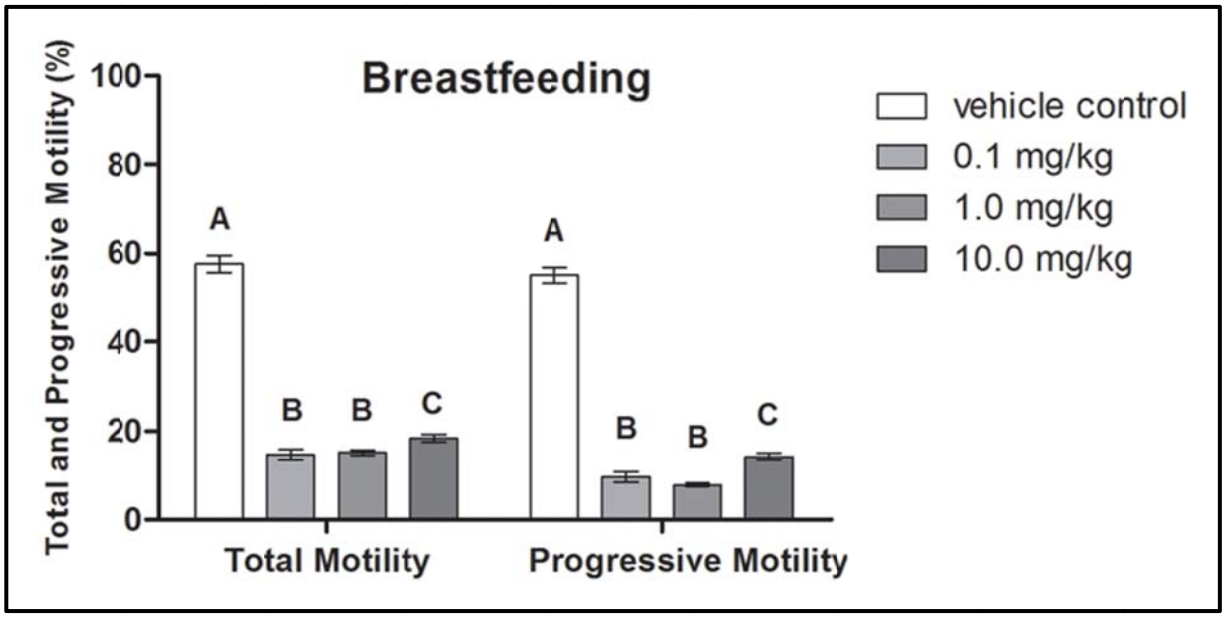

Figure 3

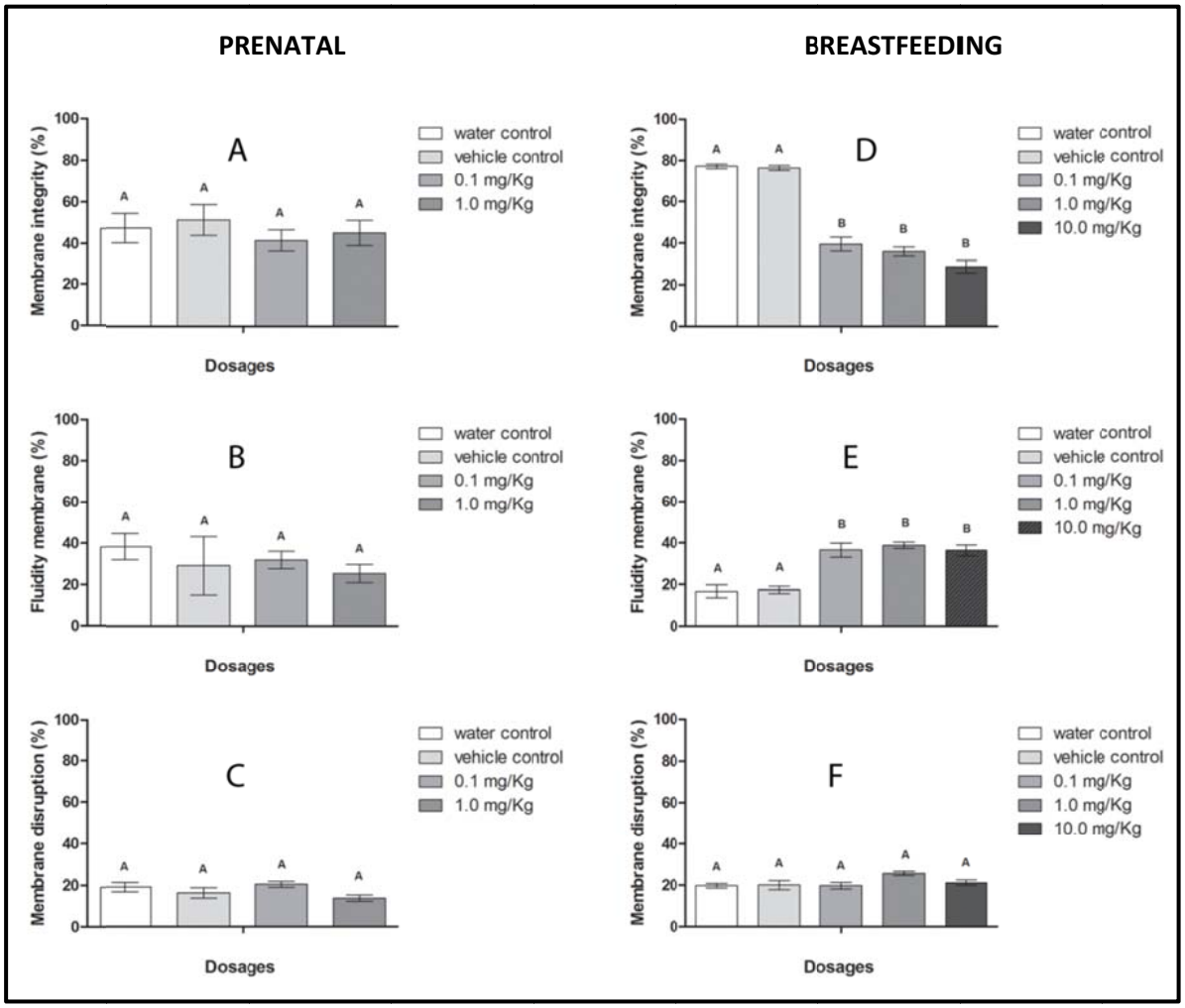
PRENATAL
BREASTFEEDING
A
B
C
D
E
F
Membrane integrity (%)
Fluidity membrane (%)
Membrane disruption (%)
Dosages
water control
vehicle control
0.1 mg/Kg
1.0 mg/Kg
10.0 mg/Kg

Figure 4

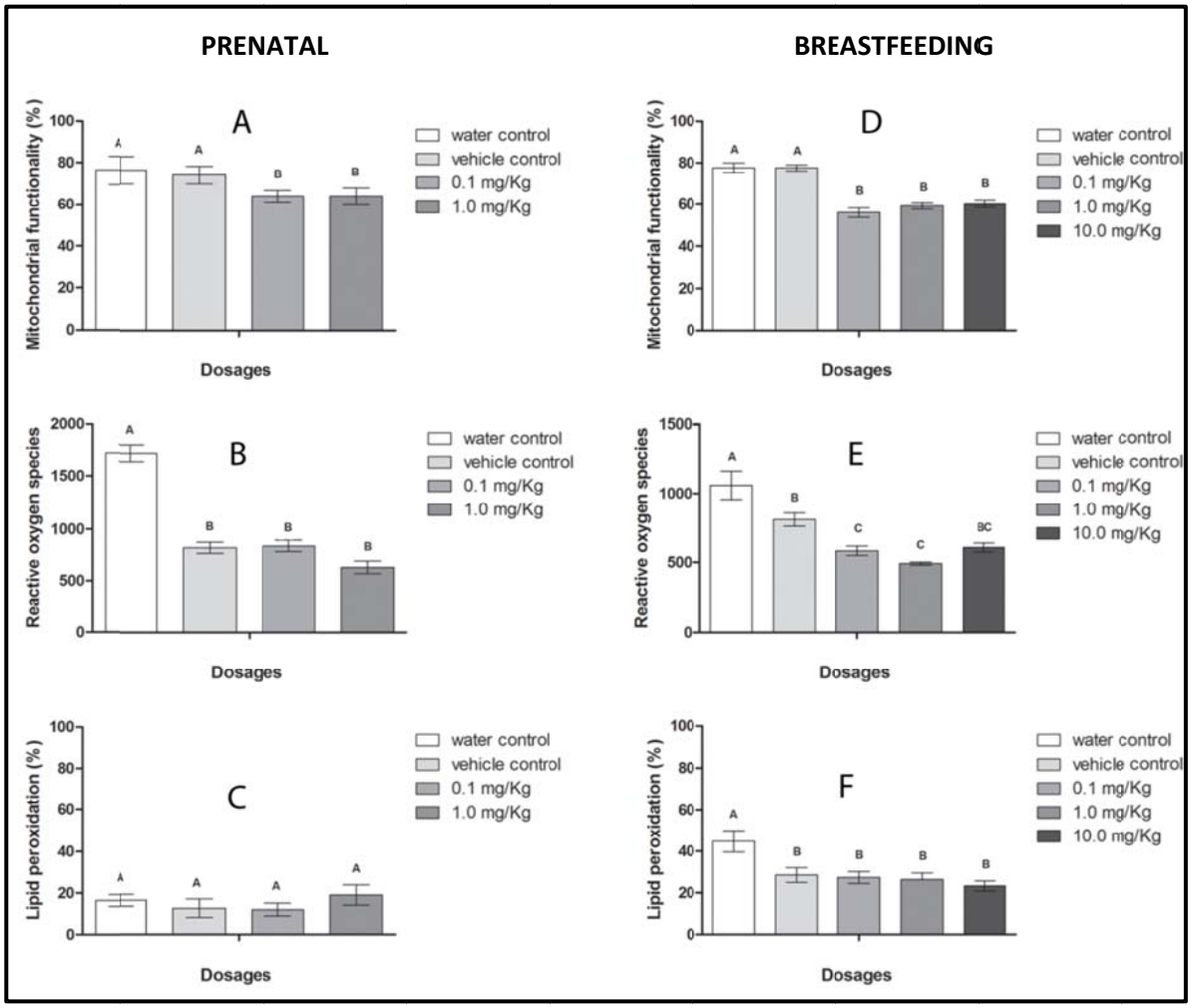
PRENATAL
BREASTFEEDING
A
B
C
D
E
F
Mitochondrial functionality (%)
Reactive oxygen species
Lipid peroxidation (%)
Dosages
water control
vehicle control
0.1 mg/Kg
1.0 mg/Kg
10.0 mg/Kg

Table legends

Table 1. Sperm concentration and DNA fragmentation index in sperm cells of *Calomys laucha* males exposed to different dosages of atrazine in prenatal or breastfeeding periods.

Table 1

	DNA fragmentation Index		Concentration	
	Prenatal	Breastfeeding	Prenatal	Breastfeeding
Vehicle control	2.66 ± 0.30^{A}	2.22 ± 0.09^{A}	$224060,0 \pm 109206.0^{A}$	$214108,0 \pm 16723,0^{A}$
0.1 mg/kg	3.03 ± 0.20^{AB}	2.74 ± 0.10^{B}	$258024,0 \pm 47714.0^{A}$	$204605,0 \pm 16019,0^{A}$
1 mg/Kg	3.51 ± 0.30^{B}	2.43 ± 0.07^{AB}	$87525,0 \pm 12947.0^{B}$	$244412,0 \pm 20617,0^{A}$
10 mg/kg	-	2.43 ± 0.08^{AB}	-	$236937,0 \pm 27858,0^{A}$

Values represent mean ± standard deviation (n=8). Means were compared using the Kruskal-Wallis test with a significance level of 0.05. Different letters in the same column indicate differences between treatments.

Considerações finais

No presente trabalho foi demonstrado que a atrazina pode não apenas causar danos imediatos às células espermáticas de roedores da espécie *Calomys laucha* expostos diretamente, mas também em longo prazo quando a exposição ao contaminante ocorre de forma indireta através do leite materno e também pela placenta.

Os machos adultos expostos à atrazina apresentaram diversos danos em suas células espermáticas, tais como a redução da funcionalidade mitocondrial capaz de reduzir a motilidade espermática, aumento na fluidez e consequente diminuição na integridade da membrana plasmática e do acrossomo. Da mesma forma, os filhotes, expostos indiretamente à atrazina através da exposição das ratas mães, durante os períodos gestacional e lactacional, apresentaram os mesmos danos espermáticos vistos nos machos adultos expostos diretamente, além do aumento do índice de fragmentação do DNA e da redução na concentração espermática (nos filhotes expostos durante a gestação).

Assim, é possível concluir que a exposição à atrazina provocou uma desestruturação na membrana espermática e no acrossoma, além de afetar o funcionamento da mitocôndria, o que reduziu a motilidade espermática. Aliado a isso, a atrazina pode ter afetado também o funcionamento das células de Sertoli e de Leydig, prejudicando a síntese hormonal e consequentemente o processo de maturação espermática. Similarmente os danos verificados nos filhotes machos, expostos indiretamente, podem ter sido causados pelas mesmas alterações nas células testiculares ligadas ao processo de espermatogênese, bem como possíveis alterações nos níveis de andrógenos e estrógenos, dessa forma, aliado ao fato de que os períodos de exposição (gestacional e lactacional) são críticos para o desenvolvimento e maturação do testículo, os danos provocados pela atrazina nesses animais foram permanentes.

Tendo em vista que as dosagens de atrazina utilizadas nesse estudo são relativamente baixas, sendo uma delas inferior aos limites aceitos na legislação brasileira (ANVISA), os resultados demonstram que a atrazina pode causar sérios danos à qualidade espermática do roedor *Calomys laucha*. Esse trabalho, assim como outros que já foram realizados com espécies similares, podem e devem ser utilizados como balizadores das legislações vigentes, tanto para definir limites aceitáveis do contaminante no meio ambiente quanto para decretar sua proibição em determinados casos.

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Anexo

PARECER DA COMISSÃO DE ÉTICA EM USO ANIMAL Nº P036/2013.

COMISSÃO DE ÉTICA EM USO ANIMAL
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CE A

PARECER Nº P036/2013

PROCESSO Nº	23116.004714/2013-18
CEUA Nº	Pq012/2013
UNIDADE	ICB
TÍTULO DO PROJETO	Efeitos da exposição à atrazina em parâmetros reprodutivos da espécie *Calomys laucha* OLFERS, 1818 (Mammalia: Rodentia)
NÚMERO DE ANIMAIS E VIGÊNCIA	320 (*Calomys laucha*) – 01/08/2016
ENVIO DO RELATÓRIO FINAL	Agosto de 2016
PROFESSOR RESPONSÁVEL	Elton Pinto Colares

PARECER DA CEUA:

Após a análise do conteúdo do processo, o protocolo experimental utilizando animais foi considerado APROVADO, pois cumpre o disposto na Lei no 11.794, nas demais normas aplicáveis e nas Resoluções Normativas e Diretrizes do Conselho Nacional de Controle de Experimentação Animal (CONCEA). A CEUA lembra aos pesquisadores que qualquer alteração no protocolo experimental ou na equipe deve ser encaminhada à comissão para avaliação e aprovação. Um relatório final deve ser enviado à CEUA no término da vigência do seu projeto.

Rio Grande, 13/09/2013.

Profa. Dra. Ana Paula Horn
Coordenadora da CEUA-FURG