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Desfechos reprodutivos intergeracionais da exposição oral de ratas Wistar ao dimetanossulfonato de etano durante a prenhez e lactação

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1. Ratos Wistar - Reprodução. 2. Controle da população. 3. Fertilidade. 4. Hormônios esteroidianos. 5. Prenhez. 6. Lactação.

Palavras-chave: Animais sinantrópicos; Controle populacional; Dimetanossulfonato de etano; Fertilidade; Rato.

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Sumário

LISTA DE ABREVIATURAS E SIGLAS DA INTRODUÇÃO

17β-HSD	17-β-hidroxiesteroide desidrogenase
AR	Receptor androgênico
cAMP	Adenosina 3',5'-monofosfato cíclico
CLA	Células de Leydig adultas
CLF	Células de Leydig fetais
DG	Dia gestacional
DPN	Dia pós-natal
EDS	Dimetanossulfonato de etano
FSH	Hormônio folículo estimulante
GnRH	Hormônio liberador de gonadotrofina
HHG	Hipotálamo-hipófise-gônada
INSL3	Peptídeo 3 semelhante à insulina
LH	Hormônio luteinizante
TNF-α	Fator de necrose tumoral alfa
VCD	Diepóxido de 4-vinilcicloexeno
VIP	Peptídeo vasoativo intestinal

LISTA DE ABREVIATURAS E SIGLAS DOS MANUSCRITOS

3β-HSD	3β-Hydroxysteroid Dehydrogenase
AGD	Anogenital distance
DSP	Daily sperm production
EDS	Ethane dimethanesulfonate
FSH	Follicle-stimulating hormone
GD	Gestational day
HE	Hematoxylin and eosin
HPG	Hypothalamic-pituitary-gonadal
ITT	Intratesticular testosterone
LH	Luteinizing hormone
mDF	Modified Davidson's fluid
PND	Postnatal day
SEM	Standard error of mean
StAR	Steroidogenic acute regulatory protein
VCD	4-vinylcyclohexene diepoxide

Resumo

Hormônios esteroides são fundamentais para a diferenciação sexual, instalação da puberdade e manutenção da fertilidade em animais adultos. Em machos, a esteroidogênese se inicia no período fetal e auxilia no processo de masculinização. Em fêmeas, a esteroidogênese tem início apenas no período pós-natal. Um dos principais hormônios esteroides responsáveis por estes processos é a testosterona, produzida pelas células de Leydig em machos e pelas células da teca em fêmeas. Compostos que comprometem a concentração plasmática de testosterona têm influência direta sobre a fertilidade. O dimetanossulfonato de etano (EDS) é um agente citotóxico para as células de Leydig, causando sua destruição nos 3 dias subsequentes à administração de uma dose única de 75mg/kg de peso corpóreo via injeção intraperitoneal em ratos, com consequente queda da produção de testosterona. Neste trabalho, foram investigados os efeitos da exposição oral materna ao EDS durante a gestação e lactação de ratas Wistar, do dia gestacional (DG) 12 ao 21º dia pós-natal (DPN), sobre as matrizes (F0) e sobre o desenvolvimento e os parâmetros reprodutivos das proles F1 (masculina e feminina) e F2 (masculina). A geração F1 foi avaliada quanto a parâmetros de desenvolvimento físico, sensorial e sexual. Houve atraso na abertura de olhos de fêmeas F1 do grupo EDS. Tanto F0 quanto F1 do grupo EDS apresentaram ingestão de ração e peso corpóreo reduzidos em relação ao controle. Na prole masculina observou-se redução da distância anogenital, peso de testículo e epidídimo, produção espermática diária e redução na inseminação de fêmeas receptivas. Os machos foram acasalados com fêmeas sem tratamento e deram origem à F2. A F2 apresentou redução do peso absoluto do testículo apenas no DPN 21. Na prole feminina da F1, observou-se atraso na instalação da puberdade, redução do peso do útero (DPN 90) e diminuição da contribuição do endométrio para a altura total do epitélio uterino. Essas fêmeas foram acasaladas com machos sem tratamento e eutanasiadas no DG 20 para avaliação de parâmetros de fertilidade. Foi observada a redução do número de corpos lúteos e implantações e aumento no número de reabsorções. Em suma, o tratamento ocasionou redução de peso corpóreo e ingestão alimentar total em F0 e F1, redução do peso de órgãos do sistema genital na F1 masculina (testículo e epidídimo) e feminina (útero) e de parâmetros de fertilidade, bem como redução do peso testicular no início do desenvolvimento da F2. Conclui-se que a combinação das alterações intergeracionais causadas pela ingestão de EDS adicionado ao alimento sólido nas condições acima referidas, leva à redução da fertilidade em ratos, e apresenta um método novo e promissor para o controle populacional de animais sinantrópicos.

Abstract

Steroid hormones are essential for sexual differentiation, puberty onset and maintenance of fertility in adult animals. In males, steroidogenesis begins in the fetal period and contributes to the masculinization process. In females, steroidogenesis starts only in the postnatal period. One of the main steroid hormones responsible for these processes is testosterone, produced by Leydig cells in males and by theca cells in females. Compounds that compromise testosterone plasmatic levels have a direct influence on fertility. Ethane dimethanesulfonate (EDS) is a cytotoxic agent for Leydig cells, causing its destruction in the 3 days following the administration of a single dose of 75mg / kg body weight via intraperitoneal injection in rats, with a consequent drop in testosterone production. In the present study we investigated the effects of maternal exposure to EDS during pregnancy and lactation of Wistar rats, from gestational day (GD) 12 to postnatal day (PND) 21, on the dams (F0) and on the development and reproductive parameters of the F1 and F2 offspring. F1 was evaluated for parameters of physical, sensorial, and sexual development. Eye opening was delayed on F1 females from EDS group. Both F0 and F1 of the EDS-treated group had reduced food intake and body weight compared to the control group. In the male offspring, there was a reduction in anogenital distance, testis and epididymis weight, daily sperm production and insemination rate. Males were mated with untreated females to obtain the second generation (F2). F2 had reduced absolute testis weight only on PND 21. In F1 female offspring, there was a delay in the onset of puberty, a reduction of the weight of the uterus (PND 90) and of the contribution of the endometrium to the total uterine epithelium height. These females were mated with untreated males and euthanized on GD 20 to assess fertility parameters. To summarize, the treatment caused a reduction in body weight and total food intake in F0 and F1, a reduction in the weight of organs of the genital system in male (testis and epididymis) and female (uterus) F1 and in fertility parameters, as well as reduced testicular weight at the beginning of F2 development. It was observed a reduction in the number of corpora lutea and implantation sites and an increase in the number of resorbed fetuses. We conclude that the combination of intergenerational alterations caused by EDS added to the solid food intake, in the specified conditions, leads to reduced fertility in rats, and presents a new and promising method for populational control of synanthropic animals.

Introdução

1. A formação do sistema genital

O desenvolvimento inicial do sistema genital em machos e fêmeas de mamíferos (Figura 1) segue intrinsicamente ligado à formação do sistema urinário, uma vez que determinadas estruturas são compartilhadas por ambos os sistemas (SCHOENWOLF et al., 2015). Inicialmente, de ambos os lados do embrião, surge uma crista genital. Esta crista genital primordialmente é constituída por células somáticas. Por volta do dia gestacional (DG) 10 em ratos, as células germinativas primordiais migram da parede do saco vitelínico para a região da crista genital na então gônada bipotencial (CUPP; SKINNER, 2005).

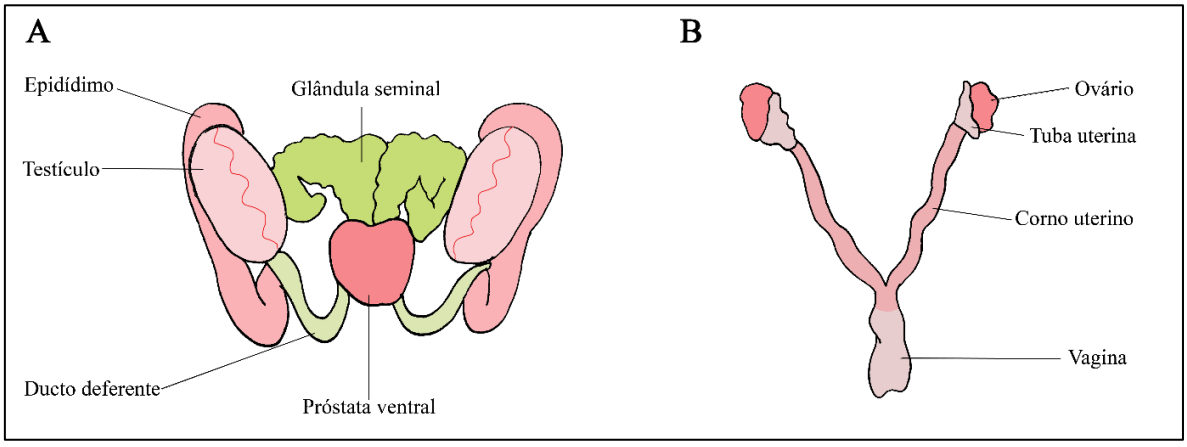


Figura 1. Parte do sistema genital masculino (A) e feminino (B) de ratos. Fonte: Elaborada pela autora.

A determinação gonadal se inicia em machos com a expressão do gene *Sry*, o fator de determinação testicular (Figura 2). Este é um gene associado ao braço curto do cromossomo Y e que é responsável por ativar vias de diferenciação que conduzem a gônada indiferenciada a se transformar em testículo. Em fêmeas, a ausência do gene *Sry* conduz para a diferenciação da gônada em ovário. Em ratos, a expressão deste gene pode ser detectada a partir do DG 10,5 por células de suporte presentes na gônada indiferenciada (SCHMAHL et al., 2000; CUPP; SKINNER, 2005).

Em sequência ao início da expressão do *Sry*, a população de células de Sertoli começa a se diferenciar e proliferar. Os agregados de precursores das células de Sertoli e de células germinativas primordiais formam cordões seminíferos, os quais futuramente formarão os túbulos seminíferos durante a instalação da puberdade. Diferentemente dos cordões

seminíferos, os túbulos seminíferos contém um epitélio germinativo com células em diversas fases da espermatogênese e são dotados de uma luz onde os espermatozoides serão liberados (CUPP; SKINNER, 2005; OJEDA; SKINNER, 2006). As células de Sertoli secretam Hormônio Anti-Mülleriano, fundamental para inibir o crescimento dos ductos de Müller. No desenvolvimento das fêmeas, a ausência desta inibição permite que os ductos de Müller originem a cérvix, útero, tuba uterina e região anterior da vagina (CUPP; SKINNER, 2005).

As células de Leydig fetais (CLF), em ratos, iniciam seu desenvolvimento e começam uma efetiva produção androgênica no DG 15 (CUPP; SKINNER, 2005; WEN; CHENG; LIU, 2016). Estes andrógenos promovem a manutenção do ducto de Wolff, o qual é o precursor do epidídimo, ducto deferente, glândula seminal e ducto ejaculatório no macho (PATRÃO; SILVA; AVELLAR, 2009).

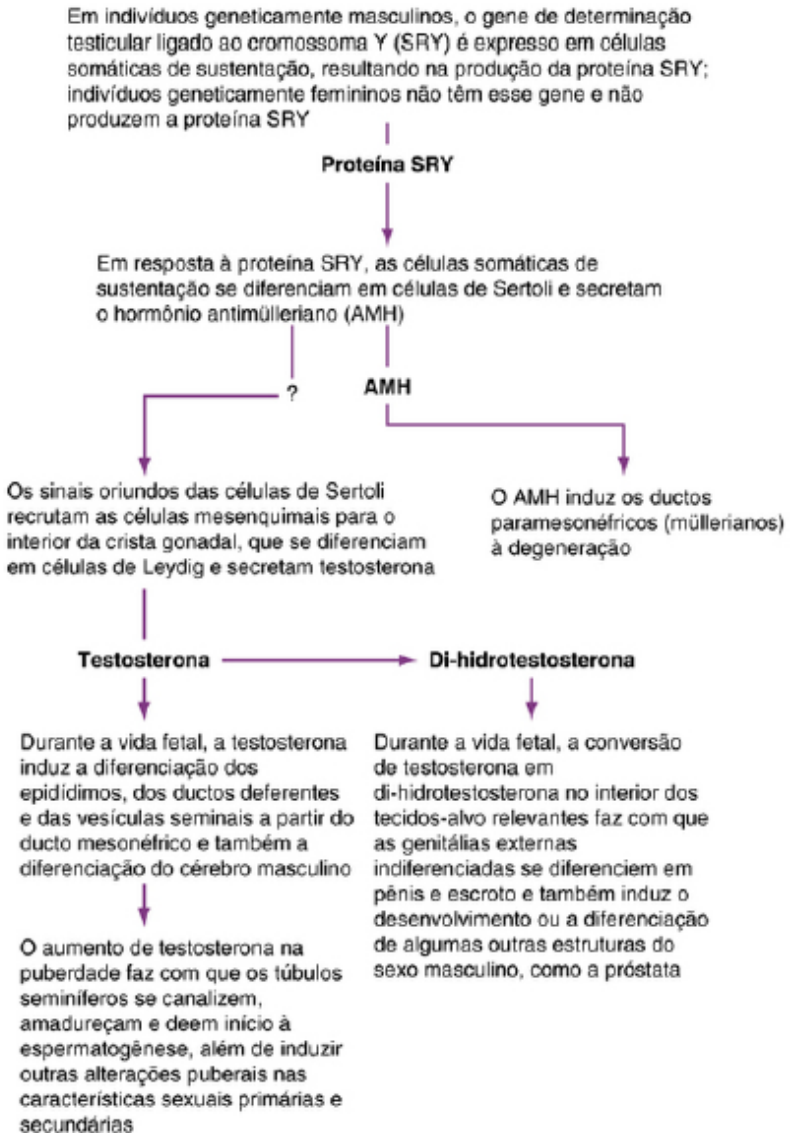


Figura 2. Resumo da cascata de diferenciação do desenvolvimento do sistema genital masculino. Fonte: Schoenwolf et al., 2015.

Hormônios tireoidianos maternos e fetais influenciam na proliferação e diferenciação de células de Sertoli. Sabe-se que a população total de células de Sertoli determina o tamanho final do testículo, bem como sua capacidade de produção espermática (CUPP; SKINNER, 2005). Os hormônios gonadotróficos, por outro lado, parecem exercer pouca influência neste momento do desenvolvimento gonadal, embora haja expressão de receptores para Hormônio Luteinizante (LH) e Hormônio Folículo Estimulante (FSH) nas gônadas fetais (OJEDA; SKINNER, 2006).

O desenvolvimento ovariano tem início a partir da determinação sexual e depende da expressão de DAX-1 e Wnt-4 (VAINIO et al., 1999). As células germinativas proliferam e formam ninhos com agregados de células germinativas envoltas por camadas de células epiteliais (Figura 3). Apenas após o nascimento estes ninhos são desfeitos e cada célula germinativa individual é envolvida por sua própria camada de células epiteliais, formando folículos ovarianos (OJEDA; SKINNER, 2006). Em contraste com o desenvolvimento do testículo, os ovários não são esteroidogênicos neste período.

O desenvolvimento folicular inicial é independente de gonadotrofinas e tem princípio nos primeiros dias após o nascimento. A progesterona materna protege os ovócitos da apoptose durante a vida fetal (OJEDA; SKINNER, 2006). No entanto, após o nascimento, a queda na concentração deste hormônio permite que essas células germinativas sejam expostas ao Fator de Necrose Tumoral alfa (TNF-α), o que causa a apoptose em massa de ovócitos. Dessa forma, ocorre um rearranjo e cada ovócito fica envolto por sua própria camada de células foliculares planas, originando os folículos primordiais (MORRISON; MARCINKIEWICZ, 2002).

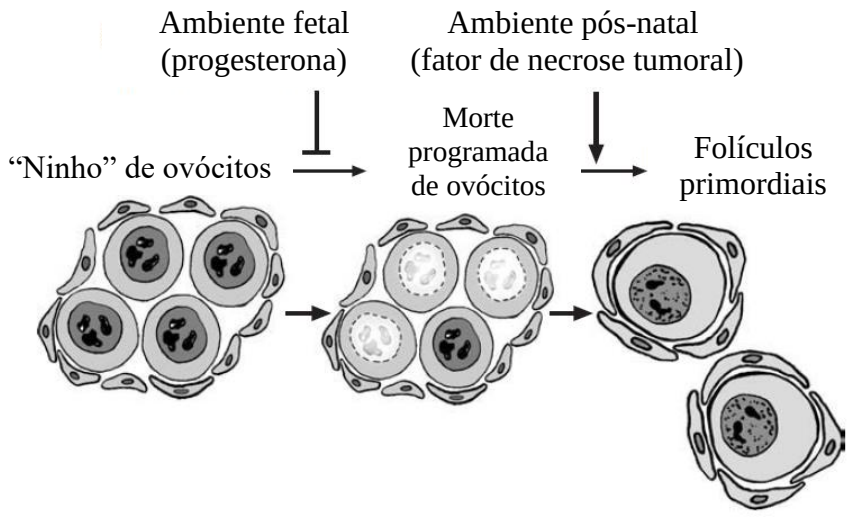


Figura 3. Apoptose de ovócitos. Adaptado de Ojeda e Skinner, 2006.

Os ovócitos prosseguem em crescimento estimulados por fatores parácrinos produzidos pelas células da granulosa ou pelo próprio ovócito até o estágio primário. Tanto a norepinefrina quanto o peptídeo vasoativo intestinal (VIP) sinalizam por vias responsivas ao cAMP e promovem a atividade da enzima aromatase, iniciando a síntese hormonal (OJEDA; SKINNER, 2006).

2. O eixo hipotálamo-hipófise-gônada

2.1. Período pré-natal

A função reprodutiva dos mamíferos é regulada principalmente pela liberação de hormônios do eixo hipotálamo-hipófise-gônada (HHG). O hipotálamo produz e secreta o Hormônio Liberador de Gonadotrofina (GnRH), que interage com seus receptores na hipófise anterior e estimula a liberação dos hormônios gonadotróficos Luteinizante (LH) e Folículo-Estimulante (FSH). Estes hormônios estimulam a gametogênese e esteroidogênese nas gônadas (AUBERT et al., 1985; GUYTON; HALL, 2006). O eixo hipotálamo-hipófise-adrenal também merece atenção, uma vez que a glândula adrenal produz hormônios esteroides sexuais, corticosterona e hormônios adrenérgicos, os quais influenciam na função reprodutiva (JARZAB et al., 1986).

Em homogeneizados de cérebros de fetos de ratos machos e fêmeas, foi detectada a presença de GnRH a partir do DG 12 (OJEDA; SKINNER, 2006). A produção de GnRH por volta do DG 15 mostrou ser a responsável por iniciar a secreção de LH detectada em fetos machos no DG 17. A sinalização nos neurônios produtores de GnRH é mediada pelos receptores de kisspeptina. Uma vez que neste momento as células de Leydig passam a expressar receptores para LH, estes eventos influenciam no aumento da produção de andrógenos pelos testículos, culminando no pico de testosterona perinatal (AUBERT et al., 1985; CLARKSON; HERBISON, 2016). Essa observação indica que a esteroidogênese gonadal é influenciada desde o período pré-natal pelo eixo HHG, cujo estabelecimento é indispensável também para a instalação da puberdade e manutenção da fertilidade no adulto.

2.2. Instalação da puberdade

O eixo HHG opera mediante feedback negativo da testosterona sobre a secreção de gonadotrofinas em machos. Após o nascimento, a unidade hipotálamo-hipófise torna-se

progressivamente menos sensível à inibição da testosterona, o que permite aumento na secreção de gonadotrofinas e de andrógenos testiculares até o momento de instalação da puberdade (RAMIREZ; MCCANN, 1965; OJEDA; SKINNER, 2006). A diminuição da secreção de inibina também parece contribuir para este momento (SHETH et al., 1980).

Em relação às fêmeas, o padrão de secreção de GnRH e gonadotrofinas se altera momentos antes da instalação da puberdade. Poucos dias antes da abertura vaginal e do primeiro estro serem detectados em fêmeas, a concentração basal e a amplitude de pulso de LH no período da tarde aumenta. Este hormônio estimula a ativação ovariana peri-púbere, levando a um aumento da secreção de esteroides sexuais pelo ovário (OJEDA; SKINNER, 2006).

Além disso, aumenta-se a expressão ovariana de receptores para FSH e LH nas fases anteriores à primeira onda ovulatória. No período pré-púbere, o eixo HHG foi preparado para responder ao feedback negativo e positivo que o estradiol exerce sobre a secreção de gonadotrofinas. Devido a toda essa preparação inicial, a capacidade de produção crescente de estradiol pelos folículos ovarianos permite a alimentação do eixo HHG por tempo suficiente para haver o pico de LH necessário para a primeira ovulação (OJEDA; SKINNER, 2006).

Em ambos os gêneros, a instalação da puberdade é o período que estabelece a funcionalidade do eixo HHG e promove a produção dos primeiros gametas (Figura 4).

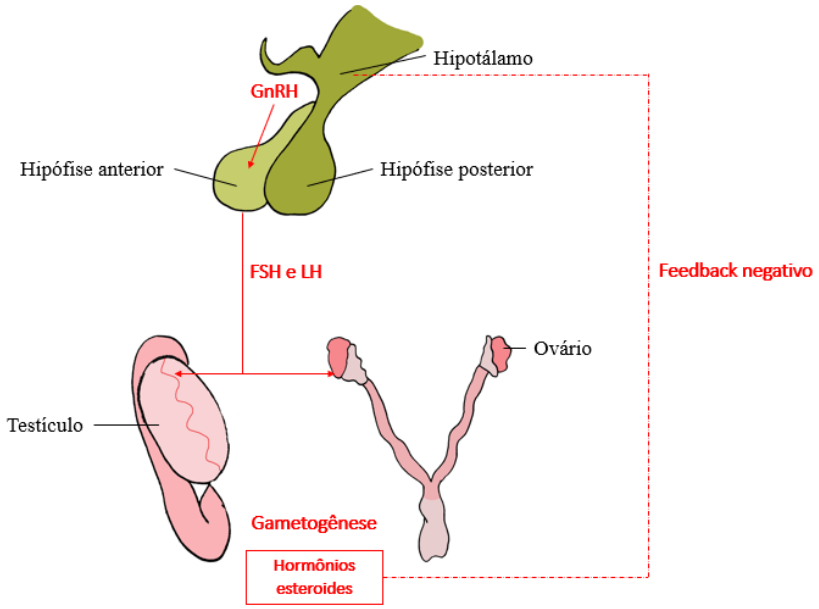


Figura 4. Esquema do eixo HHG. O hipotálamo produz GnRH que estimula a liberação de FSH e LH pela hipófise anterior. Estes hormônios agem nas gônadas e promovem a gametogênese e produção de hormônios esteroides. Por meio de feedback negativo, os hormônios esteroides regulam o eixo HHG. Fonte: Elaborada pela autora.

3. A diferenciação sexual do cérebro

Em mamíferos, a diferenciação sexual gonadal depende da presença do cromossomo Y, mais especificamente da região codificante do gene SRY/*Sry* (MCCARTHY; DE VRIES; FORGER, 2009; ROSENFELD, 2017). A diferenciação sexual cerebral, no entanto, será dependente de hormônios esteroides e auxiliada pela modulação da expressão do *Sry* em determinadas regiões cerebrais. Em machos, a produção de testosterona pelos testículos guiará a masculinização do cérebro e restante do organismo, enquanto em fêmeas a ausência deste hormônio conduzirá ao fenótipo feminino (MCCARTHY; DE VRIES; FORGER, 2009).

Estes picos hormonais ocorrem evidentemente em dois períodos distintos: inicialmente no período pré-natal, acompanhando o desenvolvimento gonadal, e tardiamente na vida adulta. A gônada em desenvolvimento secretará os hormônios que serão fundamentais para a diferenciação sexual do cérebro. Esta programação cerebral inicial é denominada período organizacional, o qual determinará a masculinização ou feminilização do cérebro (ROSENFELD, 2017). Além do auxílio desta determinação genética, muito das diferenças sexuais nos cérebros dos mamíferos e em seus comportamentos depende de regulação epigenética (FORGER, 2016).

No período pré-natal, dois processos distintos ocorrem na diferenciação sexual do cérebro masculino: a masculinização e a defeminização. Ambos são mediados por receptores estrogênicos. Dessa forma, a testosterona produzida no testículo fetal é convertida a estradiol pela enzima aromatase presente nos testículos e no cérebro, e este hormônio que efetivamente direciona a diferenciação sexual cerebral (KUDWA et al., 2006; MCCARTHY; DE VRIES; FORGER, 2009). A masculinização programará os comportamentos copulatórios e é mediada pelo receptor estrogênico α (ERα). A defeminização, por outro lado, é mediada pelo receptor estrogênico β (ERβ) e é responsável por reduzir a capacidade dos machos de reproduzir comportamentos femininos na vida adulta (KUDWA et al., 2006). Estes processos ocorrem em uma janela crítica de desenvolvimento que compreende o final da gestação e pouco após o nascimento de ratos (TOBET et al., 1985; KUDWA et al., 2006). Sendo assim, a redução da testosterona circulante neste período pode provocar alterações no comportamento sexual de animais adultos (WARD et al., 1999).

Após essa programação inicial, o macho necessita de exposição à testosterona na vida adulta para realizar o comportamento sexual masculino. Diferentemente da programação organizacional, esta é transitória e é chamada de ativacional (MCCARTHY; DE VRIES; FORGER, 2009). Para tanto, o animal exposto a determinado hormônio sexual deve ser capaz

de responder àquele estímulo e reproduzir o comportamento sexual, o que será possível se durante o período organizacional o seu cérebro foi programado para tal. Isso significa que machos adultos normais apresentarão o comportamento de cópula quando expostos à testosterona (PHOENIX et al., 1959). Por outro lado, a exposição a benzoato de estradiol pode levar ratos machos a realizar comportamento sexual feminino (YAMANOUCHI; ARIA, 1976).

Em animais geneticamente femininos, o cérebro é protegido da exposição ao estradiol na janela de diferenciação pré-natal masculina pela α-fetoproteína. A feminização, portanto, ocorre em um período pós-natal posterior, mediada pelo estradiol produzido pelos ovários quando a α-fetoproteína não possui mais um papel significativo por volta do dia pós-natal (DPN) 7 em ratos (BAKKER; BAUM, 2008). Conclui-se que tanto o desenvolvimento masculino quanto feminino dependem de estrógenos, no entanto, a janela de exposição a este hormônio no cérebro é crítica para a correta diferenciação sexual cerebral.

4. Andrógenos e células androgênicas

Andrógenos são hormônios esteroides produzidos a partir do colesterol, que possuem a capacidade de se ligar e ativar o receptor androgênico (AR), um receptor nuclear que regula a transcrição gênica (CAMPANA; PEZZI; RAINEY, 2015). Ao longo da vida do macho, a ativação do AR permite alterações fundamentais para o correto desenvolvimento e manutenção do sistema genital.

A produção androgênica é necessária para a formação do sistema genital masculino (testículos, epidídimos, próstata, glândulas seminais e pênis) e masculinização fetal durante a vida intrauterina (ROMMERTS, 1990; WELSH; SUZUKI; YAMADA, 2014; SCHOENWOLF et al., 2015), para a instalação da puberdade e o desenvolvimento dos caracteres sexuais secundários e para a contínua espermatogênese e manutenção da fertilidade na vida adulta (SCHOENWOLF et al., 2015).

Em machos, a testosterona é produzida essencialmente no interstício do testículo pelas células de Leydig e, em menor grau, pelo córtex da glândula adrenal. Células do cérebro também são capazes de sintetizar esse esteroide para contribuição fisiológica local, embora em quantidades insignificantes para o restante do organismo (ROMMERTS, 1990). Os andrógenos ativos mais relevantes são a testosterona (maior quantidade circulante) e a 5α-di-hidrotestosterona (maior potência de ativação do AR) (WATERMAN; KEENEY, 1992).

No período pré-natal, os andrógenos são produzidos pelas CLF. Estudos sugerem que estas células podem se originar a partir de diversas fontes, tais quais precursores

esteroidogênicos comuns das glândulas adrenais, células das cristas neurais e células vindas dos mesonefros (SVECHNIKOV et al., 2010; WEN; CHENG; LIU, 2016), e se encontram completamente diferenciadas, em ratos, por volta do DG 15, quando iniciam a síntese androgênica.

As CLF produzem androstenediona, porém, não possuem a enzima 17-β-hidroxiesteroide desidrogenase (17β-HSD), sendo, portanto, incapazes de converter este andrógeno à testosterona (WEN; CHENG; LIU, 2016). A conversão de androstenediona à testosterona é realizada pelas células de Sertoli fetais (WEN; CHENG; LIU, 2016) e essa é indispensável para a formação do sistema urogenital e determinação de padrões cerebrais de comportamento masculino, caracterizando a masculinização fetal (SVECHNIKOV et al., 2010; SCHOENWOLF et al., 2015; WEN; CHENG; LIU, 2016). As células de Sertoli secretam nesse período o Hormônio Anti-Mülleriano, glicoproteína que promove a regressão dos ductos paramesonéfricos que originariam partes do trato reprodutor feminino (SCHOENWOLF et al., 2015). Prejuízos na funcionalidade das CLF nesse período podem comprometer, portanto, a fertilidade e comportamento sexual do macho ao longo da vida (TARKA-LEEDS et al., 2003b).

Além da síntese androgênica, as CLF produzem peptídeo 3 semelhante à insulina (INSL3), proteína que participa da descida testicular (SVECHNIKOV et al., 2010) e exercem estimulação recíproca sobre as células de Sertoli durante o desenvolvimento intrauterino (WEN; CHENG; LIU, 2016).

As CLF possuem importância indiscutível durante o período perinatal. No entanto, a instalação da puberdade, desenvolvimento de caracteres sexuais secundários e regulação da espermatogênese devem-se às células de Leydig adultas (CLA) (Figura 5). Elas são de uma linhagem celular diferente das CLF e, no rato, seus precursores têm elevada proliferação entre os dias pós-natais 14 e 28 (CHEN; GE; ZIRKIN, 2009).

A partir do dia DPN 56, os andrógenos são predominantemente produzidos pelas CLA, sendo o principal deles a testosterona, uma vez que estas são dotadas de 17β-HSD. No animal adulto, comumente, não existe proliferação de CLA, embora possa haver a reposição de células danificadas ou destruídas (CHEN; GE; ZIRKIN, 2009; WEN; CHENG; LIU, 2016).

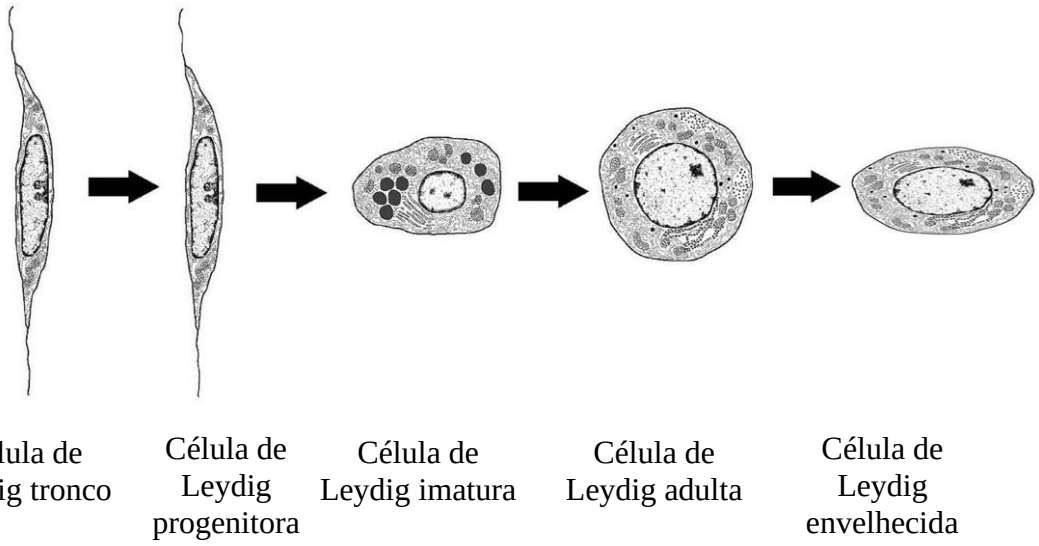


Figura 5. Estágios da diferenciação de CLA. Adaptado de Chen et al. 2009.

Em fêmeas, os andrógenos são predominantemente produzidos pelas células de teca presentes nos folículos ovarianos (HAVELOCK; RAINEY; CARR, 2004). Embora ainda incerto, estudos sugerem que as células da teca derivam de células progenitoras oriundas de duas fontes: do primórdio gonadal e de células que migraram dos mesonefros (LIU et al., 2020), assim como as células de Leydig (POTTER; KUMAR; DEFALCO, 2016; WEN; CHENG; LIU, 2016). Além de andrógenos, as células da teca também são produtoras de INSL3, cuja função no ovário é modular a síntese androgênica mediada por LH (SALILEW-WONDIM et al., 2015). O ovário fetal, no entanto, não produz hormônios. O suprimento androgênico nas fêmeas no período pré-natal é oriundo dos fetos masculinos da mesma ninhada (MARTINEZ-ARGUELLES et al., 2013).

Os andrógenos produzidos nos ovários regulam a expressão de receptores para FSH nas células foliculares, o que estimula o crescimento folicular nos estágios iniciais (RICHARDS et al., 2017). Estes hormônios também previnem a atresia folicular e auxiliam na manutenção do tempo de vida dos ovários de camundongas, de maneira que fêmeas knockout para o AR possuem redução da fecundidade em até 70% (ASTAPOVA; MINOR; HAMMES, 2019). Em relação ao útero, os andrógenos participam da regulação do ambiente trófico e citoarquitetura uterina de maneira diferente aos estrógenos, porém complementar (WEIHUA et al., 2002; NANTERMET et al., 2005).

Em suma, o comprometimento da ativação do AR durante o período perinatal pode prejudicar o desenvolvimento sexual e a fertilidade de machos e fêmeas com consequências

imediatas e tardias. Experimentalmente, este efeito pode ser obtido ao se antagonizar o AR ou por meio da redução de andrógenos circulantes.

5. O dimetanossulfonato de etano (EDS)

Uma das maneiras de se provocar a depleção androgênica no organismo é pela destruição de suas células produtoras, as células de Leydig. O EDS é um agente alquilante inicialmente estudado como potencial quimioterápico que causa depleção das CLA. Após a injeção intraperitoneal de uma única dose de EDS a 75mg/kg de peso corpóreo, a produção de testosterona dos ratos adultos diminui drasticamente nos 3 dias subsequentes e se mantém baixa por 21 dias (JACKSON et al., 1986), efeito observado em virtude do estresse oxidativo e apoptose das CLA mediados por receptores FAS e caspase-3 (TAYLOR et al., 1999; KIM; LUO; ZIRKIN, 2000; LEE et al., 2012)

Devido à queda drástica de testosterona intratesticular e circulante, órgãos acessórios dependentes de andrógenos têm redução em seus pesos, tais quais a próstata e glândula seminal, e a atividade espermatogênica é interrompida. Isso gera uma infertilidade transitória, uma vez que duas semanas após o tratamento com EDS, a população de CLA começa a se regenerar (KERR; DONACHIE; ROMMERTS, 1985). A repopulação é oriunda da diferenciação de células mesenquimais do interstício que são precursoras das CLA e não são destruídas com a administração de EDS (KERR; DONACHIE; ROMMERTS, 1985; TEERDS et al., 1989). Em razão de sua toxicidade seletiva para as células de Leydig, o EDS tem sido empregado em muitos estudos acerca da diferenciação dessas células no testículo de ratos após o nascimento (ARIYARATNE et al., 2000).

O epidídimo é um órgão altamente dependente de andrógenos e é durante o trânsito por este ducto que o espermatozoide amadurece, adquire motilidade e a capacidade de fertilizar o ovócito (BEDFORD, 1975; ROBAIRE, 1988). A administração de EDS intraperitoneal a diferentes doses causa a formação de granulomas de espermatozoides na região da cabeça e alterações morfológicas na cauda epididimária (KLINEFELTER et al., 1990, 1994; DUTTA et al., 2019).

O EDS também é efetivo quando administrado via oral, seja por gavagem ou adicionado ao alimento sólido (ração para roedores) dos animais, como foi demonstrado em estudos recentes realizados por nosso laboratório. Tanto na administração via gavagem quanto na ração, observa-se redução dos pesos de testículo e epidídimo, queda da testosterona circulante e intratesticular e impacto negativo sobre os parâmetros de fertilidade de ratos adultos. Além

disso, os animais tratados com EDS reduzem o consumo de alimento (dados ainda não publicados devido à tramitação de solicitação de patente).

A própria redução da testosterona no organismo causa a redução na ingestão de alimento. Em ratos machos gonadectomizados, a diminuição da disponibilidade de andrógenos a longo prazo causou redução na ingestão de alimento, a qual também está relacionada à queda do peso corpóreo total. Com a reposição hormonal de testosterona, a ingestão de comida foi igual a de ratos não gonadectomizados (GENTRY; WADE, 1976).

Embora grande parte dos estudos com EDS sejam voltados para a avaliação da depleção e regeneração de células de Leydig adultas, alguns pesquisadores expuseram os animais via injeção intraperitoneal no período perinatal. Dentre as alterações observadas, destacam-se a redução da distância anogenital, do peso de testículos e órgãos sexuais acessórios, das reservas espermáticas, prejuízos na espermatogênese, depleção de células de Leydig fetais, redução do crescimento e ganho de peso, entre outras (SMART et al., 1990; TARKA-LEEDS et al., 2003a, 2003b; SU et al., 2018). Mesmo após a regeneração da população de células de Leydig, as CLA dos animais tratados com EDS permanecem menos eficazes na produção de testosterona que as de animais sem tratamento (SU et al., 2018). Esses resultados sugerem potencial programação fetal, com consequências prolongadas do tratamento de EDS mesmo após a repopulação por CLA.

A programação fetal consiste em alterações em estrutura ou função de órgãos (ou ambos) a longo-prazo devido a fatores que afetam o desenvolvimento ou crescimento fetal, sendo a função reprodutiva uma das potenciais afetadas (REYNOLDS et al., 2019). Alguns efeitos podem possuir caráter intergeracional, quando a exposição em matrizes F0 leva a alterações nas gerações F1 e F2 (SALES; FERGUSON-SMITH; PATTI, 2017).

6. Controle populacional de animais sinantrópicos e EDS

Tendo em vista a importância da regulação hormonal para o correto funcionamento do sistema genital e a capacidade do EDS de interferir diretamente sobre o eixo HHG, surge a oportunidade de estudar uma abordagem de castração química utilizando este composto. A castração é a eliminação ou supressão da função gonadal e a castração química implica a utilização de um composto químico para atingir este objetivo (LOPES; SILVA, 2014).

A castração química tem como vantagens a alta eficácia, a ausência da necessidade de cuidados pré e pós-operatórios e tem a capacidade de alcançar um elevado número de animais em um curto período (LEVY et al., 2008). Atualmente, a castração é amplamente utilizada em

animais domésticos, porém, não abrange os animais sinantrópicos (MIHALCA; SÁNDOR, 2013; BERMÚDEZ et al., 2017).

Os animais sinantrópicos são animais silvestres que se adaptam ao convívio em ambientes habitados por humanos, os quais são diferentes de seu habitat natural (MORAIS, 2007). Dos animais sinantrópicos mais comuns, os roedores são de grande relevância, uma vez que podem atuar como agentes transmissores de zoonoses bacterianas como leptospirose, peste bubônica, tifo endêmica, além de outras doenças virais e parasitárias (HIMSWORTH et al., 2013; ALONSO et al., 2020).

Em regiões de precariedade no saneamento básico, associado a fatores socioeconômicos e culturais, a população de ratos têm crescido e o custo em saúde associado é estimado em 23 bilhões de euros por ano no mundo (JACOB; BUCKLE, 2018). As espécies mais recorrentes de roedores no Brasil são *Rattus norvegicus* (ratazana ou rato de esgoto), *Rattus rattus* (rato de telhado ou rato preto) e *Mus musculus* (camundongo) (MINISTÉRIO DA SAÚDE, 2016).

O controle populacional destes ratos visa reduzir a transmissão de doenças associadas a estes animais e os prejuízos econômicos advindos da perda de alimentos e danificação de máquinas e equipamentos. Os métodos empregados atualmente consistem na aplicação de iscas ou substâncias tóxicas nos ambientes infestados por ratos (MINISTÉRIO DA SAÚDE, 2016). Estes métodos raticidas causam uma morte lenta e dolorosa e podem levar a contaminações ambientais, prejudicando outros ecossistemas (BERNY et al., 2015).

Os primeiros rodenticidas desenvolvidos eram altamente tóxicos inclusive para outros animais que ingerissem a isca. Atualmente, existem diferentes classes de rodenticidas. Os venenos comuns geralmente são consumidos enquanto fêmeas estão preparando o ninho para os filhotes. Sendo assim, após a morte da mãe, os filhotes dependentes são deixados para morrer de fome e desidratação (MASON; LITTIN, 2003).

Os anticoagulantes são um dos tipos de veneno rodenticida, cujo mecanismo de ação consiste em causar hemorragia interna pela inibição de produção de fatores de coagulação pelo fígado, levando o animal à morte após 5 a 15 dias consumindo a isca (MEERBURG; BROM; KIJLSTRA, 2008). Além disso, o acúmulo de sangue na hemorragia causa dor moderada a severa que pode levar à paresia e paralisia dois dias antes da morte do animal. Outro exemplo de veneno é o fosfeto de zinco, um raticida de ação aguda que leva à morte geralmente por falência cardíaca e pulmonar. Com o avanço do envenenamento, surge uma dor ardente e severa na região abdominal ou retroesternal. Não bastasse o sofrimento dos animais, ainda existe a permanência das carcaças decorrentes do envenenamento, o que pode continuar a propagação de doenças transmitidas pelos ratos (MASON; LITTIN, 2003).

Métodos diferenciados de controle de animais sinantrópicos estão ganhando destaque atualmente. O Contrapest (SENESTECH, 2020) é um produto comercializado que promove o controle de ratos por meio do controle de fertilidade ao administrar uma isca na forma líquida contendo diepóxido de 4-vinilciclohexeno (VCD) e triptolide. Esta isca foi ofertada livremente a ratos selvagens capturados e ocasionou a diminuição no número de filhotes gerados por ninhada, mesmo se acasalados com animais não tratados (WITMER et al., 2017).

O VCD é um subproduto industrial da síntese de borracha que causa depleção de folículos primordiais e primários (HOYER et al., 2001). Uma vez que todo o reservatório folicular disponível para as fêmeas de mamíferos é formado no período pré-natal, a exposição a este composto pode levar à esterilidade (SPRINGER et al., 1996). Em humanos, a exposição ocupacional ao VCD em mulheres pode ocasionar menopausa precoce e consequente encurtamento da vida reprodutiva (HOYER et al., 2001; KAPPELER; HOYER, 2012).

O triptolide é um componente ativo isolado da videira de deus do trovão (*Tripterygium wilfordii*) que interfere em parâmetros reprodutivos tanto de machos quanto de fêmeas. Em ratos machos, o triptolide leva a alterações na espermatogênese, morfologia espermática, função epididimária, e os danos não são completamente recuperados mesmo após 60 dias da interrupção do tratamento (HUYNH et al., 2000; SINGLA et al., 2013). Em fêmeas, a exposição ao triptolide causa alterações no ciclo estral, concentração sérica de hormônios hipofisários e gonadais, e redução do peso de órgãos do sistema genital (LIU et al., 2011).

Neste âmbito, uma proposta mais humanitária e segura que reduza a população de ratos de maneira indolor, que não cause um desequilíbrio ecológico e que não ofereça perigo à saúde de outros animais se faz necessária. O EDS é um excelente candidato para este fim, visto que causa infertilidade transitória por atuar seletivamente sobre as células de Leydig. Nosso laboratório tem desenvolvido um método de administração de EDS por via oral, adicionado ao alimento sólido (ração), com a finalidade de promover controle populacional destes animais sinantrópicos. Os estudos conduzidos no laboratório até então avaliaram a exposição de ratos Wistar adultos e pré-púberes, havendo uma necessidade de avaliação da exposição durante a prenhez e lactação.

Dessa forma, este estudo avaliou os efeitos da exposição durante a prenhez e lactação ao EDS em ratos Wistar por meio da oferta do composto adicionado ao alimento sólido, utilizando-se assim de uma abordagem inovadora. Foram investigadas as consequências sobre a geração parental (F0), bem como as repercussões imediatas e tardias sobre a prole masculina (F1 e F2) e feminina (F1) dessa exposição, a fim de identificar potenciais efeitos deletérios sobre o desenvolvimento sexual e a fertilidade, assim como a potencial recuperação da

capacidade fértil após a interrupção do tratamento. Ademais, buscamos validar a administração de EDS pelo alimento sólido como um método de controle populacional de roedores, além de instigar a investigação desta metodologia em outros animais de interesse.

Justificativa

O presente trabalho justifica-se por:

a) se inserir em um projeto guarda-chuva para investigar, em ratos, os efeitos sobre a reprodução e a fertilidade após a exposição oral ao EDS, por intermédio de uma nova via de administração do composto químico: o alimento sólido. Os resultados obtidos em ratos adultos e jovens já estão em processo de patenteamento. A presente investigação complementa o projeto, ao abordar os possíveis efeitos da exposição durante a prenhez e lactação;
b) uma vez que os achados poderão contribuir com conhecimentos acerca do desenvolvimento sexual e fertilidade da prole de ratos exposta a um agente químico citotóxico para células de Leydig pela via materno-fetal e consequente depleção androgênica;
c) pela ausência na literatura especializada de estudos utilizando EDS para a avaliação de parâmetros de fertilidade feminina;
d) pela necessidade de uma abordagem mais eficaz e humanitária no controle de animais sinantrópicos.

Objetivos

Objetivos gerais

Avaliar, em ratos, os efeitos da exposição oral, pelo alimento sólido, ao EDS durante parte da prenhez e lactação sobre parâmetros de desenvolvimento e fertilidade das gerações F0, F1 e F2.

Avaliar o possível desenvolvimento de um método para o controle de animais sinantrópicos.

Objetivos específicos

Nas gerações F0, F1 e F2 de ratos Wistar:

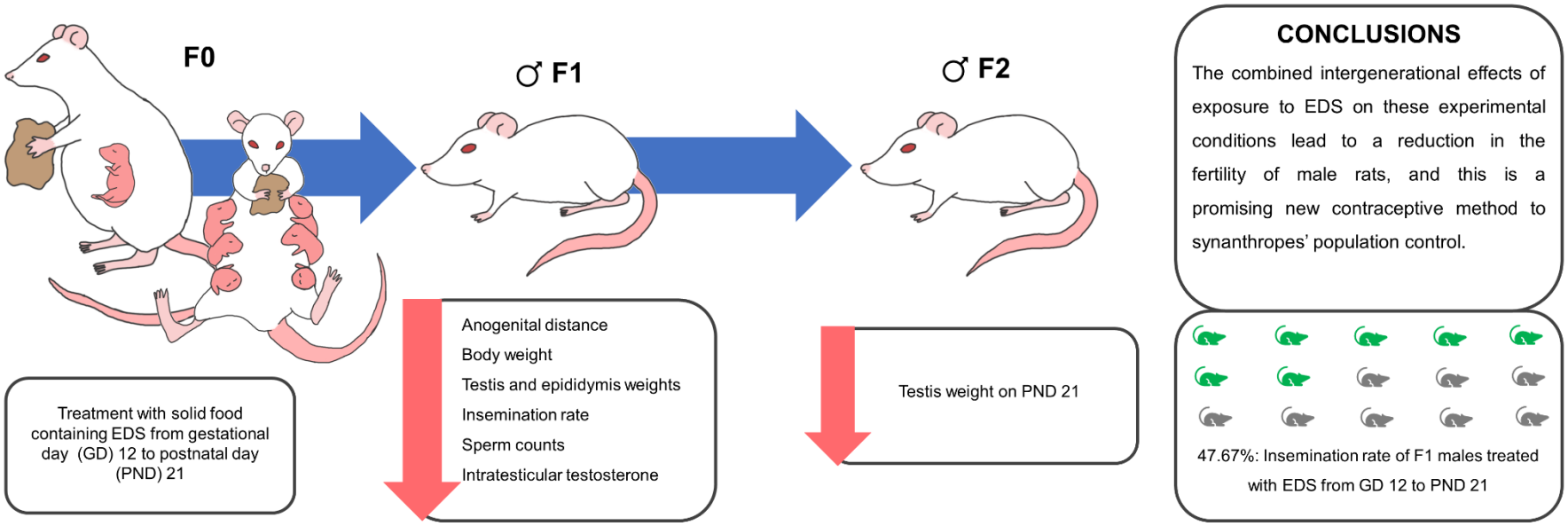
- Avaliar a ingestão de alimento, o ganho de peso e potenciais indicativos de toxicidade sistêmica nas matrizes;
- Investigar possíveis alterações no desenvolvimento intrauterino dos fetos;
- Acompanhar o desenvolvimento sexual dos animais, avaliando a distância anogenital ao nascimento e determinando a instalação da puberdade;
- Avaliar potenciais efeitos deletérios especialmente sobre os órgãos do sistema genital masculino, como o testículo e epidídimo, e se houve recuperação após a interrupção do tratamento, por meio da análise histopatológica e de parâmetros de qualidade espermática;
- Avaliar potenciais efeitos deletérios especialmente sobre os órgãos do sistema genital feminino, como útero e ovários, por meio da análise histopatológica;
- Verificar o potencial fértil dos animais, por meio da realização de avaliação do comportamento sexual e teste de fertilidade natural.
- Determinar se a segunda geração de machos também foi afetada por meio da avaliação da distância anogenital, instalação da puberdade e parâmetros de qualidade espermática.
- Avaliar a viabilidade de utilizar o alimento sólido com EDS como método para controle de animais sinantrópicos.

Capítulo 1

Este trabalho deu origem a dois manuscritos. O primeiro reune os resultados obtidos a partir dos machos F1 e F2 e foi intitulado *"Intergenerational impairment of sexual development and fertility of male rats after midgestational and lactational exposure to a new diet formulation containing ethane dimethanesulfonate (EDS)*". Este manuscrito será submetido a um periódico de impacto na área da Toxicologia e Reprodução. O manuscrito apresentado abaixo seguiu as normas da revista "Food and Chemical Toxicology" (Fator de impacto: 4.6).

Graphical abstract

Intergenerational impairment of sexual development and fertility of male rats after midgestational and lactational exposure to a new diet formulation containing ethane dimethanesulfonate (EDS)



Intergenerational impairment of sexual development and fertility of male rats after midgestational and lactational exposure to a new diet formulation containing ethane dimethanesulfonate (EDS)

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ABSTRACT

Synanthropes cause great economical and health damages per year. Currently, pest population control relies on death or complete sterilization of exposed animals. This leads to contamination of the environment and pesticide resistance. We propose a dietary formulation containing ethane dimethanesulfonate (EDS) to reduce rat fertility and in the long term reduce pest populations without developing pesticide resistance. For this, two studies were performed. On the first one, male Wistar rats (F1) were exposed to a dietary formulation containing EDS from gestational day 12 to postnatal day 21. The control group received commercial chow (Presence®). F1 males were mated with untreated females for obtention of F2. Males from both generations were evaluated on sexual development and fertility parameters. On the second study, female pregnant Wistar rats were exposed to EDS as in study 1, and the serum of the male offspring was collected for hormonal assays. Both F1 and F2 male rats had reduced reproductive organ weights. Also, F1 EDS-exposed animals had decreased daily sperm production, lower intratesticular testosterone levels, and reduction of the rate of insemination of receptive females. This method of EDS exposure compromises sexual development and fertility intergenerationally and is promising as a new pest population control since it is safer, more humanitarian, and less likely to cause pesticide resistance.

Keywords: male rat, contraception, pest population control, ethane dimethanesulfonate, fertility, intergeneration.

1. INTRODUCTION

Synanthropes are wild animals frequently considered as pests that live near and benefit from the association with human beings (Morais, 2007). From these animals, various species of rodents are known to spread numerous zoonoses such as leptospirosis, bubonic plague, and endemic typhus (Himsworth et al., 2013; Strand et al., 2019). Furthermore, rodents worldwide annual estimated financial costs from agricultural losses, infrastructural and health damages is over 23 billion Euros. To overcome this economic and health issue, pest control using anticoagulant rodenticides is the most frequent approach. This method causes hemorrhages and suffering, besides contaminating the environment and being a potential threat to other animals (Jacob and Buckle, 2018). Also, there has been an increase to pesticide resistance that leads to more expenses and less efficacy (Gould et al., 2018).

Other possible less cruel and more sustainable approach is to reduce synanthropes reproduction. Castration is not feasible once the currently available methods require interaction with every individual animal, which would be impractical in large scale. Fertility reduction methods using liquid baits are already being used in the United States of America, such as the ContraPest ® (SenesTech, 2020). Regarding this possibility, it is interesting to explore alternative ways to reduce the reproduction of rats using ingestible baits.

Ethane dimethanesulfonate (EDS) is an alkylating agent that promotes adult Leydig cells depletion after a single intraperitoneal injection of 75mg / kg of body weight in rats. Consequently, testosterone production decreases significantly on the three subsequent days and remains low for 21 days (Jackson et al., 1986) due to oxidative stress and apoptosis of adult Leydig cells (Kim et al., 2000; Lee et al., 2012; Taylor et al., 1999). As a result of testosterone decrease, testis, epididymis and accessory sexual organs weights are reduced, and spermatogenesis is interrupted (Dutta et al., 2019; Kerr et al., 1985; Klinefelter et al., 1990). Precursor Leydig cells differentiate after the end of EDS treatment and repopulate the testis, thus leading to a transitory infertility (Kerr et al., 1985).

EDS is also cytotoxic to fetal Leydig cells, and perinatal exposure to this compound leads to short and long-term impairment in sexual development and fertility of male rats (Smart et al., 1990; SU et al., 2018; Tarka-Leeds et al., 2003a, 2003b). Given the urge to develop a more humanitarian and sustainable method to synanthropes population control, our research group evaluated the effects of the midgestational and lactational exposure to a solid food

preparation containing EDS on the sexual development and fertility of male rats on the first and second generations.

2. MATERIAL AND METHODS

2.1. Animals

Untreated adult Wistar rats (70 - 80 days old) were obtained from the Central Biotherium of Botucatu and maintained under controlled conditions (22 °C, 30% air humidity, 12 / 12-h light / dark cycle) with food and water available *ad libitum* before the beginning of the experimental period. They were housed in polypropylene cages bedded with wood shavings.

All experimental procedures in this study were approved by the local Ethics Committee for the Use of Experimental Animals of Sao Paulo State University (1090-CEUA) in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

Euthanasia was performed by decapitation following CO_2 asphyxiation.

2.2. Experimental design

2.3. Study 1: Early and late development, sperm quality and fertility

Untreated animals were mated by placing two females on the cage of a male on the dark cycle of the photoperiod (Figure 1). Gestational day (GD) 0 was determined by the detection of sperm on the vaginal smear of female rats in natural estrus on the subsequent morning.

Dams (F0) were kept in individual cages and pseudo-randomly allocated into two experimental groups (n=8 / group): control, which received commercial chow, and EDS, which received solid food formulated containing EDS. Other compounds and nutrients of the formulated solid food were the same as the commercial chow (Presence®) offered to the control group. Treatment began on GD 12, which corresponds to the beginning of fetal Leydig cell differentiation (Wen et al., 2016), and ended on weaning, postnatal day (PND) 21.

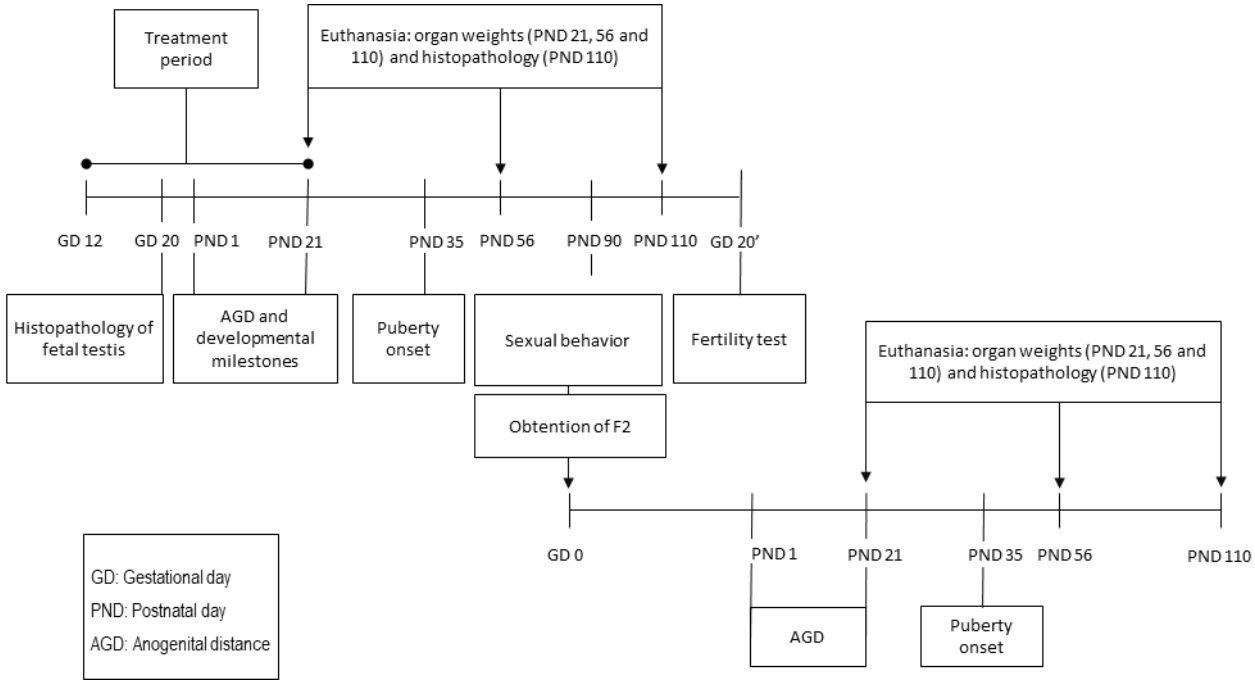

Figure 1. Experimental design.

After birth, pups were randomly selected to obtain four males and four female littermates (F1) per lactating female to maintain a similar pattern of food distribution between pups. After weaning, rats were given commercial chow *ad libitum* and food consumption and body weight were recorded every four days on one male per litter until PND 90.

On PND 90, F1 males (n=8 / group) were mated with untreated adult females to originate the second generation of control (n=6) and EDS (n=5) groups. Male offspring from both generations was evaluated on the following parameters.

2.3.1. Early development

Physical development and developmental reflexes were evaluated as described by Heyser (2004) only on F1. Early sexual development parameters were evaluated regarding experimental toxicological studies (Gallavan et al., 1999; Gray et al., 1999a; Hood, 2016).

Physical development milestones

The day of upper and lower incisor eruption was observed daily from PND 6 and the day of complete eye opening was observed from PND 12 on one male pup per litter.

Developmental reflexes

Developmental reflexes were evaluated daily from PND 1 on one male pup per litter until three identical consecutive results were observed.

Rats were given 30 seconds on the rightening reflex test and result was considered positive when they were able to turn from their back on their belly with all their legs resting freely on the table.

The grasp reflex was evaluated by gently stroking the paw of the rat with a paper clip and the day this reflex disappeared was recorded.

Sexual development

Four male rat pups per litter were weighted and their anogenital distance (AGD) was measured at PND 1 and 21. AGD measure was corrected by body weight as described by Gallavan et al. (1999). Puberty onset was determined by total manual retraction of the preputial skin in three male pups per litter.

2.3.2. Body and organ weights

On PND 21, 56 and 110, one male per litter was weighted and euthanized. Pituitary, thyroids, adrenals, testis, epididymis, seminal gland, and ventral prostate were dissected and weighted. The right testis and epididymis (PND 110) were fixed in modified Davidson's fluid (mDF) (Latendresse et al., 2002) for histology.

On PND 110, the left testis was decapsulated, the testicular fluid was removed after centrifugation (3000 rpm) for 30 min at 4ºC and the remaining parenchyma was frozen at −20ºC for determining sperm counts. The left proximal cauda epididymis was used for sperm collection for sperm quality parameters. The remaining tissue was frozen and used for sperm counts, as was the caput / corpus epididymis.

2.3.3. Sperm quality

Sperm motility

The left proximal cauda epididymis was gently poked to release sperm. The sperm was transferred to 1 ml of Human Tubal Fluid (HTF) at 34ºC and an aliquot of 10 µl was immediately pipetted to a Makler chamber and maintained at 34ºC. Using a phase-contrast microscope (400×magnification), 100 sperm were counted and classified as Type A (mobile

with progressive movement), Type B (mobile without progressive movement), and Type C (immobile). The number of cells on the sperm suspension was counted and posteriorly added to cauda epididymis sperm count.

Sperm morphology

An aliquot of 100 μL of the previous sample was added to 900 μL of formol saline (810 μL saline + 90 μL formol). Smears were prepared on histological slides and left to settle and 100 spermatozoa per animal were analyzed under a phase-contrast microscope (400× magnification). Morphological parameters were grouped as head abnormalities (isolated head and head without characteristic curvature), tail abnormalities (broken and twisted into a loop), and presence or absence of cytoplasmatic droplet.

Sperm counts and transit time

Homogenization-resistant spermatids (stage 19 of spermiogenesis) were counted as described previously (Borges et al., 2017). Testes (n=6 / group) were decapsulated, weighed, and homogenized in 5 ml of NaCl 0.9% containing Triton X 100 0.5%, followed by sonication for 30s. After a 10-fold dilution, one sample was transferred to Neubauer chambers (4 fields per animal), and mature spermatids were counted. To calculate the daily sperm production (DSP), the number of spermatids at stage 19 was divided by 6.1,which is the number of days of the seminiferous cycle during which these spermatids are present in the seminiferous epithelium (Robb et al., 1978). The relative DSP was also obtained by dividing the daily sperm production by testis weight. The caput / corpus and cauda epididymis were cut into small fragments with scissors and homogenized followed by sonication. Sperm were counted as described for the testis. The sperm reservoir in the cauda region of the epididymis was calculated by combining the number of cells in the sperm suspension used in the motility assay and the sperm count in the homogenized tissue. The sperm transit time through the epididymis was determined by dividing the number of sperm in each region of the epididymis by the DSP (Robb et al., 1978).

2.3.4 Histological evaluation

Testes (GD 20 and PND 110) and epididymides (PND 110) were evaluated. Fetal testes (n=4 / group), right testes and epididymides of adult animals (n=6 / group) were fixed by immersion in mDF, dehydrated and embedded in Paraplast Plus®(P3683, Sigma-Aldrich). Tissues were sectioned (5 μm), mounted on glass slides and stained with hematoxylin and eosin (HE) to evaluate testicular and epididymal morphology. Histopathology was analyzed in a blind assay

using a Leica DMLB microscope mounted with a digital camera and analysis software (LAS V4.12).

Fetal testis

All seminiferous cords were evaluated in a section of the testis. Sertoli cells and gonocytes were counted, and their ratio was determined. The general aspect of the interstitium and other histopathological alterations were also observed as described previously (Barlow and Foster, 2003; Borch et al., 2006).

Adult testis

Briefly, three non-serial testicular sections were taken for each animal to evaluate 100 seminiferous tubules in adult rats (Borges et al., 2017). Leydig cell nuclear volume was also determined in adult rats using the program ImageJ by measuring 50 circular or elliptical Leydig nuclei per animal (n=6 / group). Their diameters (D) were measured, and the volume obtained using the formula: $V=[D\times\pi] / 6$.

The diameter of the seminiferous tubules (stage IX of spermatogenesis) and thickness of germinal epithelium were evaluated in 10 sections of seminiferous tubules per animal using Image J. The dynamics of spermatogenesis was estimated by the relative frequency of stages: I-VI (two generations of spermatids), VII-VIII (mature spermatids), IX-XIII (only one generation of spermatids), XIV (secondary spermatocyte) in 100 cross-sections of seminiferous tubules per animal. The relative frequency of stages estimates the rate or duration of the spermatogenic process (Clermont and Harvey, 1965).

Adult epididymis

The epididymides were evaluated qualitatively on the regions of the initial segment, caput, corpus, and cauda for morphological alterations (Kempinas and Klinefelter, 2014).

2.3.5. Sexual behavior

Adult males (n=8) were placed on a cage for 10 min prior the introduction of a sexually receptive female. Female receptivity was determined by the detection of natural estrus on vaginal smears and confirmed by lordosis behavior when placed with a vasectomized rat. Paired test animals were observed during the dark cycle of the photoperiod. Male sexual behavior was evaluated for 40 min and included the following parameters: latency to the first mount; latency to the first intromission; latency to the first ejaculation; number of intromissions until the first

ejaculation; latency of the first post-ejaculatory intromission; number of post-ejaculatory intromissions; and total number of ejaculations. Animals were given two chances on the sexual behavior test. If they did not mount the female on the first 10 min on both tries, they were excluded from the test and considered sexually inactive.

2.3.6. Obtention of the second generation

After the sexual behavior test, one more female was placed on the cage of the male to obtain the second generation (F2). The percentage of pregnant females from both sexual behavior test and obtention of second generation was named insemination test. Pregnancy was confirmed by the detection of sperm on the vaginal smear of female rats in estrus on the subsequent morning. Sperm-positive females were preferably selected to obtain F2 and the remaining rats were destined to the fertility test.

2.3.7. Fertility test

Females from the sexual behavior test were euthanized on DG 20 to evaluate fertility. After collection of the uterus and ovaries the numbers of corpora lutea, implants and reabsorptions were recorded, and the following endpoints determined: pregnancy rate: pregnant females / sperm-positive females; fertility potential (efficiency of implantation): implantation sites / corpora lutea × 100; rate of pre-implantation loss: (number of corpora lutea - number of implantations / number of corpora lutea) × 100; rate of post-implantation loss: (number of implantations - number of live fetuses) / number of implantations × 100 (Borges et al., 2017).

2.4. Study 2 - Hormonal assays

Untreated animals were mated and kept under controlled conditions as described in study 1. Dams were kept in individual cages and pseudo-randomly allocated into two experimental groups (n=5): control, which received commercial chow with bacon flavoring, and EDS, which received solid food formulated containing EDS. Other compounds and nutrients of the formulated solid food were the same as the commercial chow (Presence®) offered to the control group. Treatment began on GD 12 and ended on PND 21. Male offspring was euthanized on PND 1, 21, 56 and 90 for blood sample collection. Those blood samples (n=5) were allowed to coagulate, and the serum obtained by centrifugation (2400 rpm) for 20 min at 4◦C. Serum

samples were subsequently stored at −20◦C. Serum testosterone, FSH and LH levels were determined by radioimmunoassay. Serum levels of FSH and LH were measured using a double-antibody radioimmunoassay with specific kits provided by the National Hormone and Peptide Program (Harbor-University of California at Los Angeles). Serum and intratesticular levels of testosterone were measured using a double-antibody radioimmunoassay with specific kits supplied by MP Biomedicals (ImmuChem™Double Anti-body, Testosterone: 07814891. Orangeburg,NY, USA). All samples were measured in the same assay to avoid inter-assay errors. Using the hormone values, Leydig cell function was determined by measuring intratesticular testosterone levels (ITT) / LH levels (IT ratio) as described previously (Chang et al., 2015).

2.5. Statistical analyses

Data are presented as mean ± standard error of mean (SEM) or median and interquartile range. Student's t-test was used for comparison of parametric variables. Non-parametric variables were compared using Mann-Whitney test. Percentage data was compared using the Chi-Square test. Results were considered significantly different when $p \leq 0.05$. Statistical analyses were performed using the GraphPad InStat software (version 6; La Jolla, CA).

3. RESULTS

3.1. Study 1 - Early and late development, sperm quality and fertility

Evident signs of general toxicity and stress (mortality and morbidity, weakness, lethargy, excessive weight gain/loss, presence of bristly hair, and abnormal behavior) derived from the treatment were not observed throughout the experimental procedures.

3.1.1. Early development

Early physic and reflex developmental milestones were not different between control and EDS groups (Table 1).

Table 1. Physical and reflexological developmental milestones of F1 males (n=8 / group; 1 / litter).

Parameters (days)	Control	EDS
Physical development		
Eye opening	14.00 ± 0.27	14.75 ± 0.31
Incisor eruption	9.88 ± 0.23	9.50 ± 0.38
Developmental reflexes		
Grasp reflex	8.50 ± 0.33	8.25 ± 0.36
Rightening reflex	2.63 ± 0.46	2.38 ± 0.26

Values expressed as mean ± SEM. Student's test. $p > 0.05$.

Body weight and AGD at PND 1 and 21 were reduced on EDS group on F1. On F2, only body weight at PND 1 was reduced (Table 2).

Table 2. Body weight and AGD on PND 1 and 21 of F1 and F2 males. Numbers in parenthesis represent the number of litters at PND 1 or animals per group at PND 21.

Parameters	PND 1		PND 21	
	Control	EDS	Control	EDS
F1				
Body weight (g)	7.04 ± 0.13 (8)	6.48 ± 0.16** (8)	49.54 ± 2.74 (8)	34.40 ± 1.19*** (8)
AGD (mm / g¹ / ³)	1.96 ± 0.03 (8)	1.84 ± 0.03** (8)	4.21 ± 0.10 (8)	3.67 ± 0.24* (8)
F2				
Body weight (g)	7.40 ± 0.11 (6)	6.72 ± 0.08**** (5)	52.94 ± 0.96 (6)	52.04 ± 0.81 (5)
AGD (mm /g¹ / ³)	1.83 ± 0.03 (6)	1.83 ± 0.04 (5)	4.61 ± 0.10 (6)	4.41 ± 0.17 (5)

Values expressed as mean ± SEM. Student's test. * $p \leq 0.05$. ** $p \leq 0.01$. *** $p \leq 0.001$. **** $p \leq 0.0001$.

Puberty onset was not altered on both generations, but body weight at puberty onset was reduced on F1 EDS group (Figure 2B).

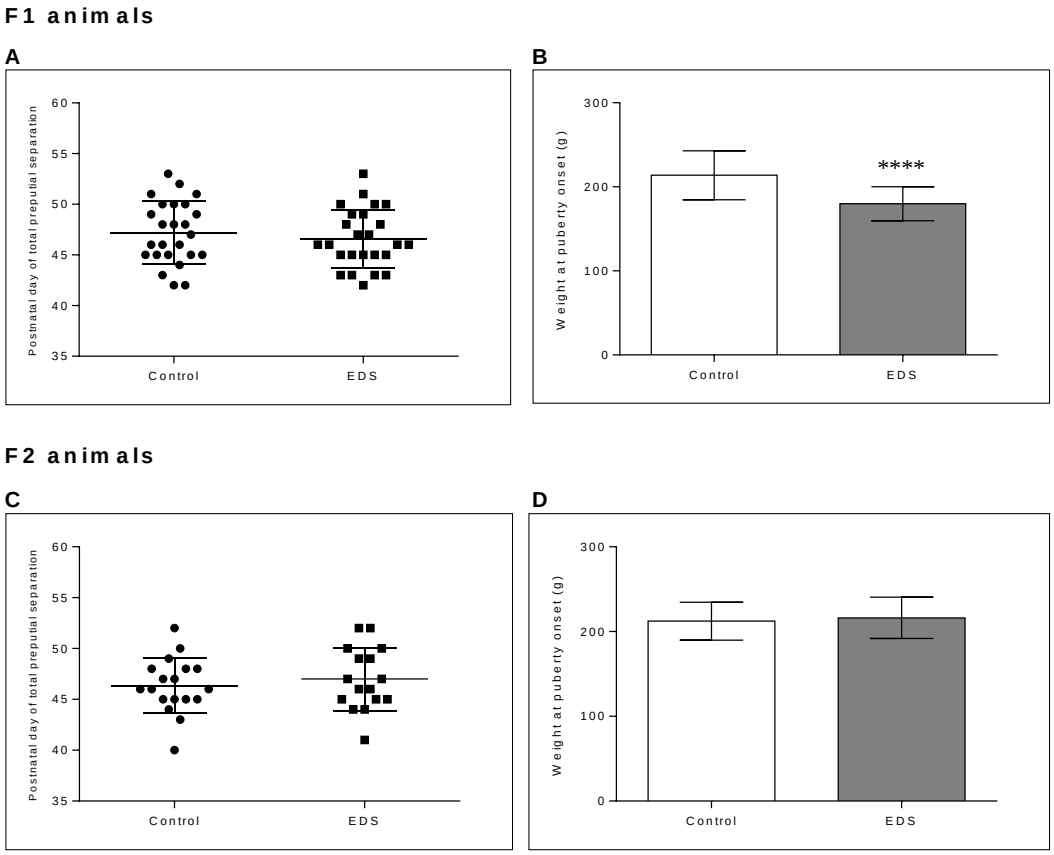

Figure 2. Postnatal day of total preputial separation on F1 (A) and F2 (C), and respective body weight at puberty onset (B and D). Values expressed as mean ± SEM. Student's t-test. **** $p \leq 0.0001$.

3.1.2. Food intake and body weight gain

Mean food intake and body weight gain from PND 21 to PND 90 were similar between control and EDS groups (Figure 3B and D) on F1 animals. The food intake per body weight, however, was increased on EDS group on PND 21, 25, 29, 33, 41, 49, and 61 (Figure 3A). Additionally, body weight was reduced on EDS group throughout the experimental period, except on PND 45, 53, 73 and 77 (Figure 3C).

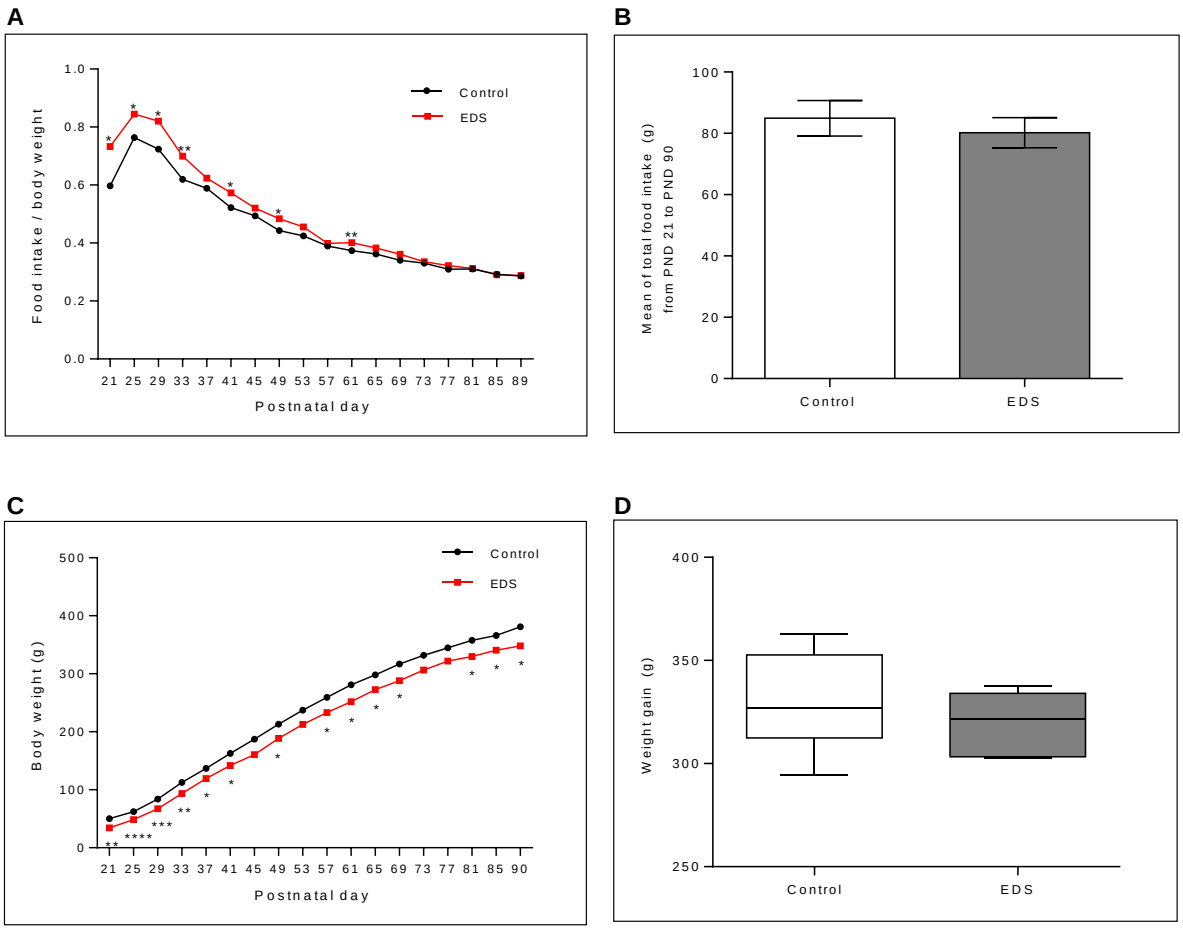

Figure 3. Food intake / body weight (A), mean of total food intake (B), body weight evolution (C) and total body weight gain (D) of F1 males from PND 21 to PND 90. Values expressed as mean ± SEM. Student's test. * $p \leq 0.05$. ** $p \leq 0.01$.*** $p \leq 0.001$. **** $p \leq 0.0001$.

3.1.3. Body and organ weights

Body weight was reduced on PND 21, 56 and 110 on EDS group on F1 animals (Table 3), but not on F2 (Table 4).

Regarding the first-generation animals, absolute testis weight was reduced on EDS group on PND 21, 56 and 110. Epididymis was reduced on PND 56 and 110. Also, relative seminal gland weight was non-significantly increased on PND 110 (p=0.069). Furthermore, pituitary (PND 56) and adrenals (PND 110) relative weights were increased (Table 3).

On second-generation animals, absolute testis weight was reduced on PND 21, and adrenals relative weight was reduced on PND 56 on EDS group (Table 4).

Table 3. F1 organ weights on PND 21, 56 and 110.

Parameters	PND 21		PND 56		PND 110	
	Control (n=8)	EDS (n=6)	Control (n=8)	EDS (n=8)	Control (n=8)	EDS (n=8)
Body weight (g)	49.54 ± 2.74	34.40 ± 1.19***	284.40 ± 10.28	247.70 ± 4.32**	426.20 ± 12.20	397.10 ± 5.52*
Testis (mg)	118.20 ± 5.91	79.03 ± 5.67**	1434.00 ± 43.62	1149.00 ± 25.72****	1733.00 ± 54.58	1419.00 ± 37.30***
Epididymis (mg)	16.94 ± 1.11	15.23 ± 0.84	255.40 ± 11.60	209.60 ± 9.08**	635.60 ± 19.55	546.20 ± 15.52**
Ventral prostate (mg / 100g BW)	-	-	81.09 ± 5.48	72.23 ± 4.51	97.93 ± 7.90	99.02 ± 10.58
Seminal gland (mg / 100g BW)	-	-	129.20 ± 11.99	153.20 ± 10.87	267.50 ± 14.68	301.30 ± 8.88 (p=0.069)
Seminal gland without fluid (mg / 100g BW)	-	-	59.28 ± 7.89	60.23 ± 4.90	79.57 ± 2.81	89.31 ± 4.90
Pituitary (mg / 100g BW)	3.65 ± 0.31	5.11 ± 0.86	3.09 ± 0.15	3.62 ± 0.11*	4.15 ± 0.41	3.87 ± 0.19
Thyroids (mg / 100g BW)	10.44 ± 1.14	11.97 ± 1.60	6.45 ± 0.42	6.48 ± 0.20	6.67 ± 0.51	5.91 ± 0.61
Adrenals (mg / 100g BW)	31.30 ± 1.48	34.60 ± 1.39	20.89 ± 0.80	20.83 ± 1.63	16.38 ± 0.90	21.26 ± 1.48*

Values expressed as mean ± SEM. Student's test. * $p \leq 0.05$. ** $p \leq 0.01$.*** $p \leq 0.001$. **** $p \leq 0.0001$. BW: Body weight.

Table 4. F2 organ weights on PND 21, 56 and 110.

Parameters	PND 21		PND 56		PND 110	
	Control (n=6)	EDS (n=5)	Control (n=6)	EDS (n=5)	Control (n=6)	EDS (n=5)
Body weight (g)	52.94 ± 0.96	52.04 ± 0.81	268.7 ± 10.20	288.0 ± 8.68	428.6 ± 18.09	391.4 ± 28.23
Testis (mg)	127.50 ± 4.54	114.10 ± 3.03*	1372.00 ± 45.26	1304.00 ± 76.26	1724.00 ± 40.83	1618.00 ± 39.20 (p=0.10)
Epididymis (mg)	19.43 ± 1.53	19.10 ± 0.80	245.30 ± 5.63	228.40 ± 15.55	599.70 ± 15.22	598.40 ± 14.99
Ventral prostate (mg / 100g BW)	-	-	59.34 ± 5.92	60.26 ± 3.58	91.00 ± 9.21	120.50 ± 19.19
Seminal gland (mg / 100g BW)	-	-	122.90 ± 12.58	132.90 ± 16.39	214.60 ± 14.14	292.20 ± 51.81
Seminal gland without fluid (mg / 100g BW)	-	-	56.33 ± 4.40	56.03 ± 4.82	97.26 ± 8.90	112.70 ± 10.89
Pituitary (mg / 100g BW)	5.64 ± 0.70	6.47 ± 1.31	3.82 ± 0.28	3.39 ± 0.33	2.80 ± 0.08	2.81 ± 0.24
Thyroids (mg / 100g BW)	11.92 ± 1.40	15.50 ± 3.58	6.25 ± 0.57	6.40 ± 0.62	4.66 ± 0.38	4.75 ± 0.51
Adrenals (mg / 100g BW)	29.03 ± 2.43	29.08 ± 1.28	22.86 ± 1.58	18.88 ± 0.49*	18.19 ± 1.46	20.55 ± 2.38

Values expressed as mean ± SEM. Student's test. *$p \leq 0.05$. BW: Body weight.

3.1.3. Sperm quality

No differences were observed on sperm motility (Figure 4), nor on sperm morphology (Table 5) between control and EDS groups on both generations. Spermatid number, daily sperm production, and sperm number in caput / corpus were reduced on EDS group only on F1 males (Table 6).

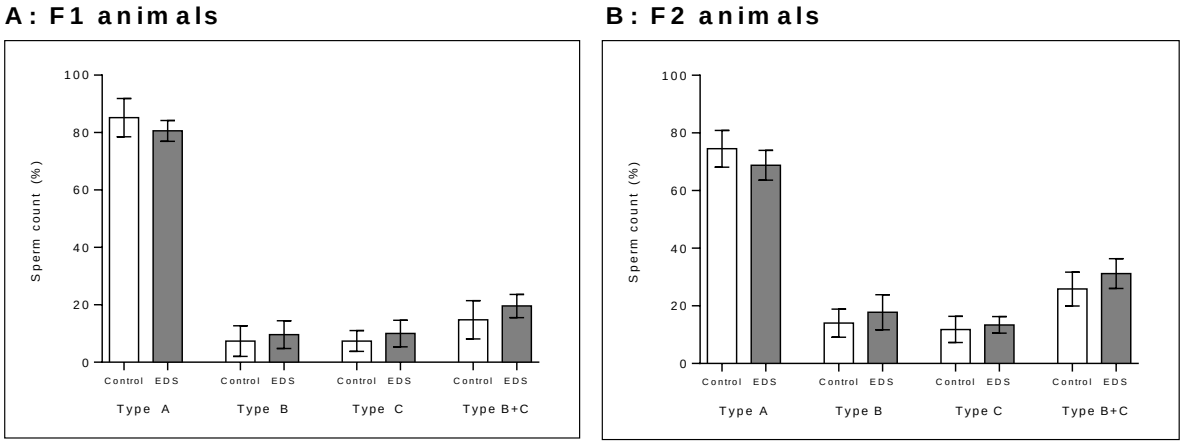

Figure 4. Sperm motility of F1 (A) and F2 (B) males on PND 110. Mann-Whitney's test (n=6 / group). Values expressed as mean ± SEM. $p > 0.05$.

Table 5. Sperm morphology of F1 and F2 males on PND 110.

Parameters	F1		F2	
	Control (n=6)	EDS (n=6)	Control (n=6)	EDS (n=5)
Normal sperm	93.00 (90.00 - 95.50)	93.50 (91.75 - 96.50)	95.50 (93.25 - 97.25)	96.00 (91.00 - 97.00)
Head abnormalities	2.50 (1.50 - 3.50)	4.00 (0.75 - 5.00)	1.50 (0.75 - 2.50)	3.00 (0.50 - 4.50)
Tail abnormalities	3.00 (2.75 - 8.00)	3.00 (2.00 - 3.25)	3.50 (1.75 - 4.25)	3.00 (1.00 - 5.00)
Cytoplasmic droplet	57.00 (40.75 - 63.50)	53.50 (34.50 - 63.00)	48.00 (17.00 - 86.00)	31.00 (23.50 - 88.00)

Values expressed as median and interquartile intervals. $p > 0.05$. Mann-Whitney's test.

Table 6. Sperm count and sperm transit time on PND 110 of F1 and F2 males.

Parameters	F1		F2	
	Control (n=6)	EDS (n=6)	Control (n=6)	EDS (n=5)
Testis				
Spermatid number ($x10^6$)	300.90 ± 20.94	235.80 ± 15.36*	267.90 ± 21.22	258.40 ± 14.02
Relative spermatid number ($x10^6$ / g)	180.40 ± 9.70	176.20 ± 7.00	201.5 ± 13.86	201.6 ± 13.99
Daily sperm production ($x10^6$ / day)	49.33 ± 3.43	38.66 ± 2.52*	43.92 ± 3.48	42.37 ± 2.30
Relative daily sperm production ($x10^6$ / g / day)	29.57 ± 1.59	28.89 ± 1.15	33.04 ± 2.27	33.05 ± 2.29
Epididymis				
Sperm number in caput / corpus ($x10^6$)	125.20 ± 10.86	91.00 ± 9.84*	109.90 ± 9.86	121.90 ± 2.09
Relative sperm number in caput / corpus ($x10^6$ / g)	364.60 ± 20.53	317.50 ± 25.57	320.30 ± 26.87	365.30 ± 6.13
Sperm number in cauda ($x10^6$)	259.30 ± 9.04	220.10 ± 22.96	239.50 ± 22.63	250.40 ± 22.84
Relative sperm number in cauda ($x10^6$ / g)	902.60 ± 37.33	863.90 ± 76.37	932.0 ± 67.20	958.7 ± 32.39
Sperm transit time in caput / corpus (days)	2.62 ± 0.31	2.37 ± 0.267	2.59 ± 0.36	2.90 ± 0.13
Sperm transit time in cauda (days)	5.40 ± 0.47	5.85 ± 0.77	4.97 ± 0.58	5.81 ± 0.58
Total sperm transit time (days)	8.02 ± 0.73	8.22 ± 0.74	7.45 ± 0.63	8.71 ± 0.76

Values expressed as mean ± SEM. Student's t test. * $p \leq 0.05$.

3.1.4. Histological evaluation

The morphology of the fetal testis and adult epididymis were similar between both experimental groups (data not shown). No differences in histopathology, morphometry of the adult testis, and spermatogenesis dynamics were observed between control and EDS groups on F1 males. On F2 EDS males, seminiferous tubule diameter was increased (Table 7).

Table 7. Histopathology and morphometry of the testis, and spermatogenesis dynamics on PND 110 of F1 and F2 males.

Parameters	F1		F2	
	Control (n=6)	EDS (n=5)	Control (n=5)	EDS (n=5)
[1]Normal seminiferous tubules (%)	93.50 (90.50 - 95.25)	92.00 (90.50 - 96.00)	97.00 (95.50 - 98.00)	94.00 (93.50 - 96.00)
[2]Seminiferous tubule diameter (μm)	310.5 ± 12.67	303.0 ± 7.38	271.60 ± 8.87	309.70 ± 5.45**
[2]Epithelial cell height (μm)	96.28 ± 3.62	93.30 ± 3.44	85.52 ± 3.61	91.17 ± 3.70
Leydig cell nuclear volume	174.80 ± 9.08	169.30 ± 6.13	179.50 ± 5.39	184.80 ± 12.95
Spermatogenesis dynamics				
[1]I-IV (%)	44.50 (41.75 - 48.50)	47.00 (45.00 - 53.00)	50.00 (46.50 - 54.50)	46.00 (42.00 - 47.50)
[1]VII-VIII (%)	16.00 (14.00 - 17.75)	14.00 (12.00 - 18.00)	16.00 (12.00 - 16.50)	13.00 (10.50 - 16.00)
[1]IX-XIII (%)	33.00 (29.50 - 35.50)	31.00 (31.00 - 34.00)	30.00 (27.00 - 33.50)	37.00 (33.00 - 39.00)
[1]XIV (%)	5.50 (4.75 - 7.00)	4.00 (3.50 - 5.50)	5.00 (3.50 - 6.00)	6.00 (2.50- 7.50)

[1]Values expressed as median and interquartile intervals (Mann-Whitney test). [2]Values expressed as mean ± SEM. Student's test. ** $p \leq 0.01$.

3.1.5. Sexual behavior

The number of intromissions until the first ejaculation was reduced on EDS group on the first-generation (Table 8). Other sexual behavior parameters were similar between control and EDS groups.

Table 8. Sexual behavior test of F1 males on PND 90.

Parameters	Control (n=8)	EDS (n=8)
Sexually active animals	7	7
[1]Latency to the first mount (s)	158.00 (45.00 - 254.00) (7)	52 (32.00 - 180.00) (7)
[1]Number of mounts until the first ejaculation	2.00 (2.00 - 9.00) (2)	6.00 (4.00 - 18.00) (2)
[1]Latency to the first intromission (s)	102.00 (38.50 - 206.00) (5)	195.00 (95.00 - 218.0) (7)
[1]Number of intromissions until the first ejaculation	27.00 (14.50 - 36.00) (2)	7.00 (3.00 - 15.00)* (2)
[2]Frequency of ejaculations (%)	25 (2)	25 (2)

[1]Values expressed as median and interquartile intervals (Mann-Whitney test). [2]Values expressed as percentage (Chi-Square test). *$p \leq 0.05$. Numbers in parenthesis represent the number of animals in the group that exhibited the evaluated behavior.

After sexual behavior, an insemination test was performed between animals in the control (n=7) and EDS (n=8) groups. Each of these animals was allocated with one or two receptive females (depending on availability) during the dark period of the photoperiod, totaling 12 females mated with animals from the control group and 15 females mated with animals from the EDS group. The morning after the test, a reduced number of females inseminated after mating with males from the EDS group (47.67%, * $p \leq 0.05$, Chi-Square Test) was found. In the control group, 11 females were inseminated (91.67%).

3.1.6. Fertility test

Of the total number of females in the insemination test, n=6 / group were destined for laparotomy (fertility test) and the rest were destined for obtention of F2. Of the animals destined for laparotomy, only 33.34% females (2 / 6) of the EDS group were sperm-positive and for that reason, were the only ones used for fertility parameters (Table 9). Pregnancy rate refers to the number of sperm-positive females that were pregnant when the fertility test was performed.

Table 9. Fertility results after natural mating of F1 males on PND 90.

Parameters	Control (n=6)	EDS (n=6)
Sperm-positive females (%)	83.33 (5 / 6)	33.33 (2 / 6)
[1]Pregnancy rate (%)	100.00 (5)	100.00 (2)
[2]Fertility potential (%)	100.00 (92.50 - 100.00)	96.67 (93.33 - 100.00)
[2]Pre-implantation loss (%)	0.00 (0.00 - 7.50)	3.33 (0.00 - 6.67)
[2]Post-implantation loss (%)	0.00 (0.00 - 9.09)	3.85 (0.0 - 7.69)
[3]Body weight of the dams (g)	358.70 ± 9.73	366.2 ± 17.60
[3]Uterus weight with fetuses (g)	77.51 ± 6.02	86.71 ± 0.64
[3]Corpora lutea number	12.60 ± 0.68	14.00 ± 1.00
[3]Implantation number	12.20 ± 0.58	13.50 ± 0.50
[3]Reabsorption number	0.40 ± 0.24	0.50 ± 0.50
[3]Fetus weight (g)	4.59 ± 0.06	4.66 ± 0.10
[3]Placental weight (g)	0.59 ± 0.02	0.67 ± 0.04

[1]Values expressed as percentage (Chi-Square test). [2]Values expressed as median and interquartile intervals (Mann-Whitney test). [3]Values expressed as mean ± SEM (Student's t-test). $p > 0.05$.

3.2. Study 2 - Hormonal assays

FSH was increased on PND 1 on EDS group; intratesticular testosterone (Figure 5) and ITT : LH ratio were reduced on EDS group (389.8 versus 106.0, * $p \leq 0.05$, Student's t test) on PND 90. Also, no signs of general toxicity were observed.

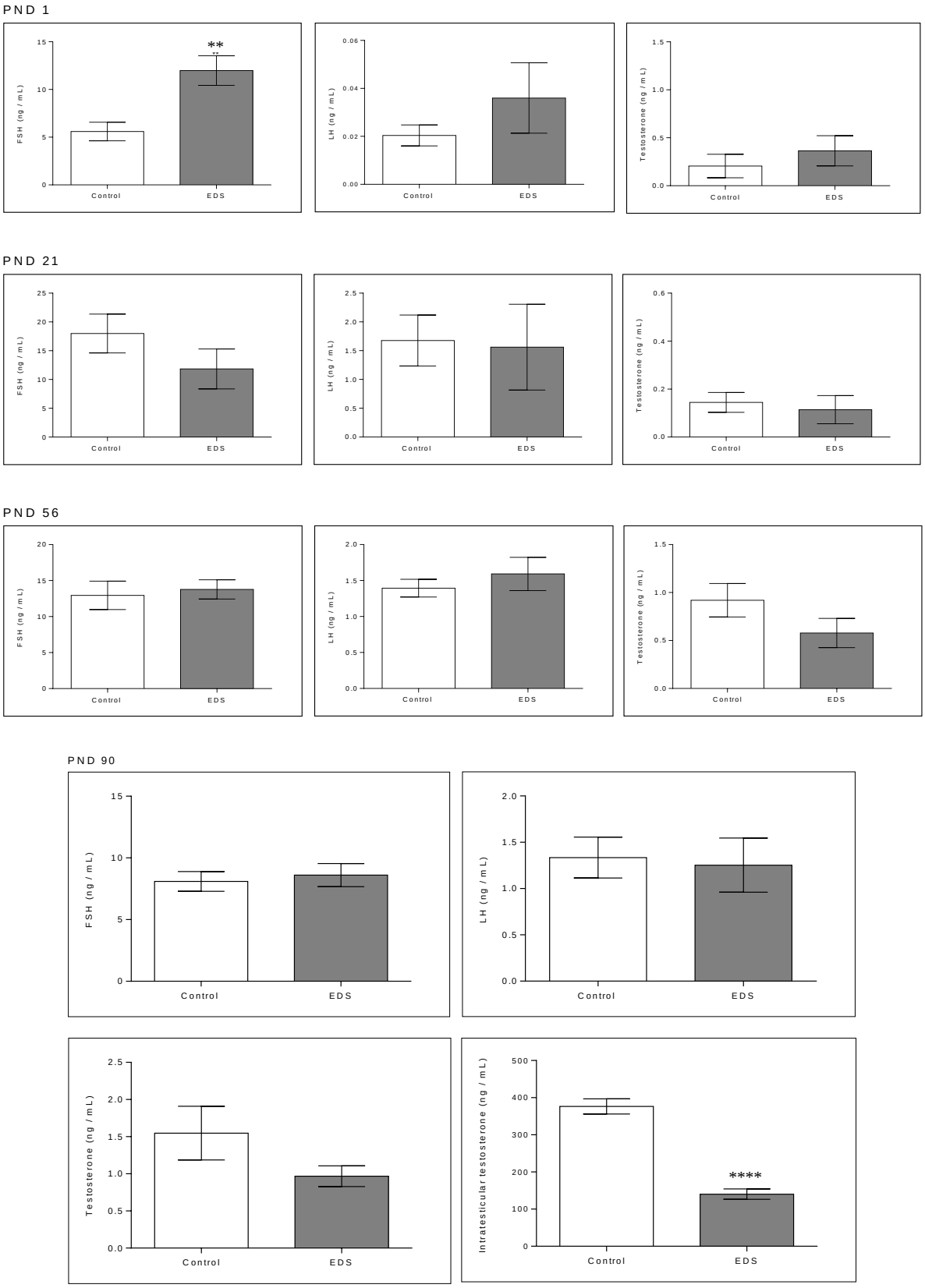

Figure 5. Hormone levels of male rats at PND 1, 21, 56 and 90. Values expressed as mean ± SEM. **p ≤ 0.01. ****p ≤ 0.0001.

4. DISCUSSION

In this study, we treated pregnant female Wistar rats from GD 12 to the end of lactation with a solid food formulation containing EDS and observed the outcomes on the first and second male generations. To summarize, both generations had impaired sexual development and fertility parameters such as reduction of genital organ weights (F1 and F2), decreased daily sperm production, lower intratesticular testosterone levels, and reduction of the rate of insemination of receptive females (F1).

The increase in pest control resistance is related to higher economical and health damages. Pesticides that kill or sterilize synanthropes eventually select non-responsive individuals, which leads to an increase in overall pesticide resistance in that population (Hawkins et al., 2019; Ishizuka et al., 2008). Contraceptive methods that reduce fertility could be more effective at controlling pest populations and slowing the evolution of resistance to certain treatments (Shuster et al., 2018). Also, rodenticides currently available are responsible for various cases of wild and domestic animals primary and secondary poisoning, as well as accidental poisoning of children, which could lead to death (Bertero et al., 2020; Durão and Machado, 2016; Rodrigues Mendonça et al., 2016; Sánchez-Barbudo et al., 2012). In such way, our new contraceptive method using EDS in a solid food formulation shows promising results in reducing fertility in first and second-generation of male rats, without the observation of physical signs of general toxicity. These results were observed by alteration in early sexual development and fertility parameters.

The perinatal period is critical for early and late development, and any sort of stress may cause long-lasting impairment in health and fertility (Tamashiro and Moran, 2010). Pups intraperitonially exposed to 50 mg / kg of EDS from PND 4 to 15 reduced food consumption and body weight (Smart et al., 1990). In the present work, we observed reduction of body weight but not on mean food consumption on F1. In addition, there were no alterations on physical development and developmental milestones, which was also observed by Smart et al. (1990).

Regarding sexual development, AGD was reduced on F1 males on PND 1 and 21. Males exposed to antiandrogens have reduced AGD, may present nipple retention, hypospadias, and epididymal agenesis; the severity of the outcomes depends on the dosage (Gray et al., 2001; Tarka-Leeds et al., 2003). Reduction of weight at birth and AGD are indicators of fetal programming (Hanson and Gluckman, 2008), which could be caused by lower testosterone levels (Gray et al., 2001, 1999b). Intratesticular testosterone was reduced on PND 90, as well

as ITT : LH ratio. These results indicate a deficiency in Leydig cell function, leading to decreased androgenic production (Zirkin and Chen, 2000).

Fetal Leydig cells are the main source of androgens prenatally. After birth, fetal Leydig cells population decreases rapidly and adult Leydig cells proliferate from stem Leydig cells. Adult Leydig cells are well-stablished at the rat testis at PND 56 and are the main androgen producers that contribute to puberty onset (Chen et al., 2009). Puberty onset was not altered in this study, although body weight at puberty onset was reduced on F1 males from EDS group.

After Leydig cell ablation, adult Leydig cells cannot proliferate, but their population can be restored by differentiation of stem Leydig cells (Chen et al., 2009; Wen et al., 2016). Differentiation and self-renewal of stem Leydig cells is mediated by growth factors and activin A (Liu et al., 2021). Even after Leydig cell repopulation following EDS treatment, adult Leydig cells are still less effective in producing testosterone than in untreated animals (SU et al., 2018). In males from EDS group, Leydig cells were observed in both fetal and adult testis, meaning they were not completely ablated by the treatment. A limitation of this is study is the lack of molecular analysis that could better evaluated those Leydig population function and determine the proportion of differentiated and undifferentiated Leydig cells in the adult testis.

Androgens are essential for genital system formation and maintenance (Svechnikov et al., 2010; Wen et al., 2016), and decreased androgenic levels caused by perinatal exposure to EDS leads to reduction of AGD, testis and accessory sexual organ weights, decrease in sperm production and insemination rate (Smart et al., 1990; SU et al., 2018; Tarka-Leeds et al., 2003a, 2003b). Likewise, our results on F1 males are similar to those in previous studies. In F2 males we also observed reduction in sexual organ weights, potentially caused by epigenetic regulation. Prenatal exposure to endocrine disruptors leads to epigenetic modifications of 3β-HSD and StAR genes, altering intergenerationally testicular development and testosterone production in mice (Alamdar et al., 2017; Hong et al., 2016).

Sexual desire and behavior are androgen-dependent, which promotes brain masculinization and posterior activation of male sexual behavior (Charlier et al., 2013; McCarthy et al., 2009; Rosenfeld, 2017; Welsh et al., 2014). Prenatally, testosterone is converted to estrogen in the male brain and promotes masculinization by activation of estrogen receptors α (McCarthy et al., 2009). The medial preoptic area is one of the most important in the integration of male sexual behavior, and copulation depends on dopamine and testosterone activation in adult males (Kudwa et al., 2006; Putnam et al., 2001). In this study, we observed a reduction in sperm-positive females after the sexual behavior test. Mice prenatally exposed to

160 mg EDS / kg from GD 11 to 17 had a reduction in mating ratio, fertility ratio and the size of F2 litters (Tarka-Leeds et al., 2003b).

Even if androgen is administered in adulthood in rats that were gonadectomized neonatally, sexual behavior is impaired. The frequency of intromissions and ejaculations is reduced, which indicates that neonatal testosterone is related to ejaculatory behavior in adulthood (Bloch and Mills, 1995). Even though there was no difference in the number of ejaculations between the experimental groups, the reduction in the number of sperm-positive females mated with EDS males indicates a reduction in mating ratio during the night. Hence, there was potentially an impairment in brain sexual differentiation or activation on F1 EDS males, associated with decreased androgenic concentration, which led to a reduction in sexual desire in adulthood. Also, rats with a major reduction in sperm production are still able to mate, which means that a reduction in mating ratio could contribute even more to overall fertility reduction than sperm counts itself.

Added to a reduction in mating ratio, males from EDS group had reduced sperm counts, which were also associated with a reduction on genital organ weights. Reduced sperm counts associated with EDS treatment were also observed by other authors, as well as the formation of sperm granulomas and other morphological changes on the epididymis of rats (Dutta et al., 2019; Klinefelter et al., 1990). Even with a reduction on epididymis weight, we did not observe morphological changes on this organ, nor on sperm motility and morphology of males from EDS group. Also, sperm counts were similar between the experimental groups when corrected by the organ weights, which suggests that the general efficacy of sperm production by the testis was not altered. This result is supported by the fact that histopathological changes were not observed in the testis.

Besides regulating genital system function, steroid hormones interfere in other organs as the pituitary and the adrenals. In this study, pituitary (PND 56) and adrenals (PND 110) weights were increased on F1, and adrenals (PND 56) weight was decreased on F2. EDS has no direct deleterious effects on the production of gonadotropins by the pituitary (Rommerts et al., 1985), however, LH secretion increases significantly in the first days after EDS administration in response to the drop in testosterone concentration (Dong and Handelsman, 1991; Gromoll et al., 1993). Additionally, exposure to antiandrogens in the fetal period causes changes in the pituitary secretion pattern (Rossi et al., 1991).

As for the adrenals, the administration of intraperitoneal EDS to adult males causes weight reduction of this organ (Plećas et al., 2001) and atrophy of the adrenocortical zones

(Plećaš et al., 1997). Rats exposed in the prenatal period to high doses of the antiandrogen di-butyl phthalate (DBP) showed an increase in adrenal weight in adulthood (Jiang et al., 2007). Other phthalates also have an influence on adrenal secretory activity. In addition, aldosterone stimulates androgen production by Leydig cells, and some antiandrogens are responsible for reducing the expression of receptors for aldosterone in these cells (Martinez-Arguelles et al., 2013).

In conclusion, these combined intergenerational results promote a reduction in the fertility of male rats without causing complete sterilization or death. As EDS caused infertility is transitory, this new contraceptive method is promising for pest populations control, as it also does not select pesticides resistant individuals. In addition, lower fertility effects could be observed in multiple generations, leading to a gradual decrease in pest populations even after the period of exposure to this treatment. This method is also not invasive, safer, and more practical than already available methods. Further studies could evaluate this new contraceptive method in other synanthropes.

5. CONFLICT OF INTEREST

The authors Lethícia Valencise, Patrícia Villela e Silva, Ana Flávia Quiarato Lozano, Cibele dos Santos Borges, Jorge Willian Franco de Barros, Gary Robert Klinefelter and Wilma De Grava Kempinas are developing a pest control method of EDS oral exposure (Patent Protocol 18CI097).

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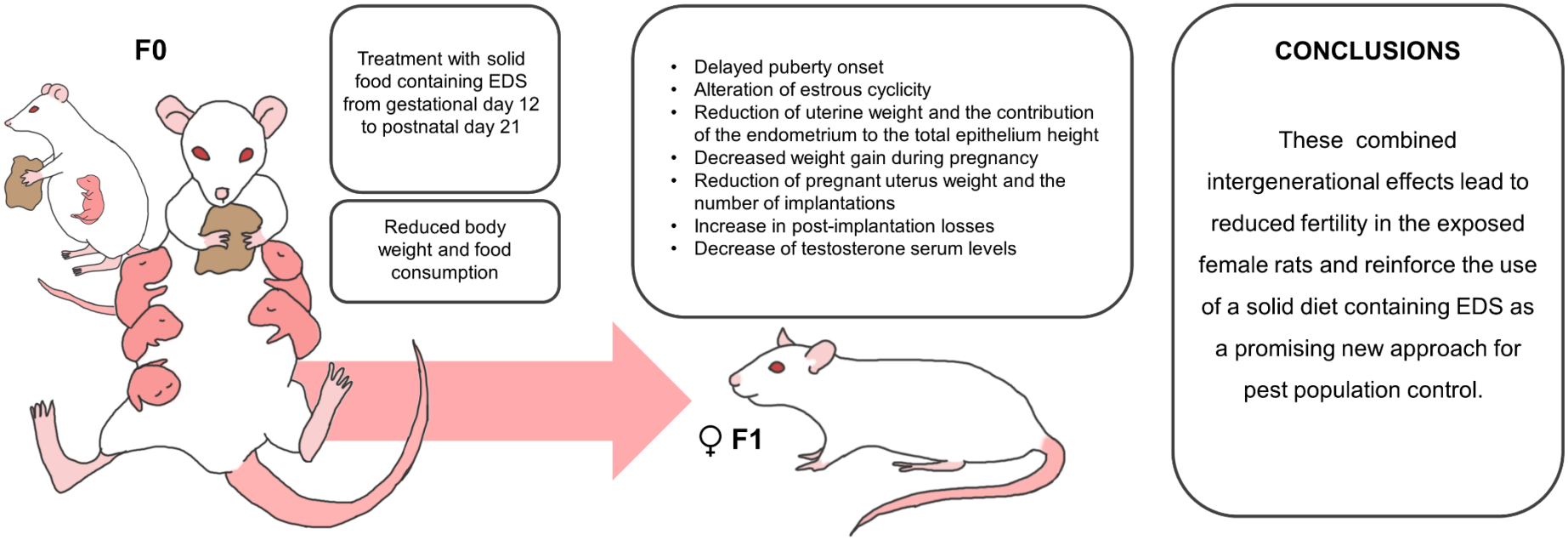
Capítulo 2

O segundo manuscrito reune os resultados obtidos a partir das fêmeas F0 e F1 e foi intitulado *"Exposure from midgestation to weaning to an ethane dimethanesulfonate (EDS) dietary formulation delays sexual development and impairs fertility of F1 female Wistar rats"*. Este manuscrito será submetido a um periódico de impacto na área da Toxicologia e Reprodução. O manuscrito apresentado abaixo seguiu as normas da revista "Food and Chemical Toxicology" (Fator de impacto: 4.6).

Graphical abstract

Exposure from midgestation to weaning to an ethane dimethanesulfonate (EDS) dietary formulation delays sexual development and impairs fertility of F1 female Wistar rats



Exposure from midgestation to weaning to an ethane dimethanesulfonate (EDS) dietary formulation delays sexual development and impairs fertility of F1 female Wistar rats

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ABSTRACT

A couple of rats could originate 15,000 descendants in one year. This high reproduction rate associated with the transmission of zoonotic diseases are responsible for great economical and health impacts. The control of synanthropes population using anticoagulant methods causes suffering, leads to environment contamination, and are potentially dangerous to wildlife and humans accidentally exposed. Here, we introduce a new approach in pest population control that reduces the fertility of the animals and gradually decreases the size of their populations. For this, we propose a dietary formulation containing ethane dimethanesulfonate (EDS) for fertility control of synanthropes. Two studies were performed. On the first study, female Wistar rats (F0) were exposed to a dietary formulation containing EDS from gestational day 12 to the end of lactation. The control group received commercial chow (Presence®). The female offspring (F1) was evaluated on sexual development and fertility parameters. On the second study, female pregnant Wistar rats were exposed to EDS as in study 1, and the serum of the female offspring was collected for hormonal assays. F1 females from the exposed group had delayed puberty onset, alteration of estrous cyclicity, reduction of uterine weight and the contribution of its functional layer (endometrium) to the total epithelium height, decreased weight gain during pregnancy, reduction of pregnant uterus weight and the number of implantations and increase in post-implantation losses. Additionally, testosterone levels at adulthood were reduced. These combined intergenerational effects lead to reduced fertility in the exposed female rats and reinforce the use of a solid diet containing EDS as a promising new approach for pest population control.

Keywords: female rat, contraception, pest population control, ethane dimethanesulfonate, fertility, sexual development.

1. INTRODUCTION

Female rats produce 4-5 litters on their lifespan with an average of 8.5 pups per litter and a gestation period of 21-23 days (King, 1924; Saksena and Datta, 1970). In a year, one single rat couple can produce 15,000 descendants. Given their fertility and adaptability to human environment, rats are considered synanthropes that can represent serious health and economic issues. Regarding human health, rats can carry hematophagous ectoparasites that are vectors of various zoonotic bacterial diseases (Alonso et al., 2020). Minimizing those damages caused by rats is essential, and the current option is pest control. Anticoagulant rodenticides have been the chosen approach for years, but they are prone to induce pesticide resistance (Gould et al., 2018). Additionally, this method causes suffering and is a potential threat to other animals (Jacob and Buckle, 2018), including accidental poisoning of humans that can cause death (Bhat and Kenchetty, 2015).

Recent studies have shown that fertility control is effective in reducing synanthropes population (Shuster et al., 2018). Captured wild rats free fed a liquid bait containing 4-vinylcyclohexene diepoxide (VCD) and triptolide had reduced litter sizes even if mated with untreated animals (Witmer et al., 2017). Besides, this method is considered less toxic than other available control methods (National Toxicology Program, 1986). However, the mechanism of action of VCD can cause permanent sterility since it depletes smalls follicles , and females are born with limited follicle reservoir (Springer et al., 1996). In humans occupationally exposed to VCD, this can lead to early menopause (Hoyer et al., 2001; Kappeler and Hoyer, 2012).

Unlike VCD, ethane dimethanesulfonate (EDS) causes transitory infertility in male rats, given the selective depletion of Leydig cells with repopulation after the interruption of the treatment (Kerr et al., 1985). The exposure of male rats to EDS is widely studied (Ariyaratne et al., 2000; Dutta et al., 2019; Gromoll et al., 1993; Kerr et al., 1985; Kim et al., 2000; Plećaš et al., 1997), but the exposure of female rats is restricted to the perinatal period (Smart et al., 1990; Tarka-Leeds et al., 2003). The androgen-producing cell in females is the theca cell, and it shares the same embryonic origin with Leydig cells (Potter et al., 2016; Rotgers et al., 2018).

Given the common origin of steroidogenic cells in both males and females, and the urge to develop a more humanitarian and sustainable method to pest control, our research group evaluated the effects of the exposure from midgestation to weaning to a dietary formulation with EDS on the sexual development and fertility of female rats.

2. MATERIAL AND METHODS

2.1. Animals

Wistar rats (70 - 80 days old) were obtained from the Central Biotherium of Botucatu and housed in polypropylene cages bedded with wood shavings under controlled conditions (22 °C, 30% air humidity, 12 / 12h light/dark cycle). Food and water were available *ad libitum* before the beginning of the experimental period.

All experimental procedures in this study were approved by the local Ethics Committee for the Use of Experimental Animals of São Paulo State University (1090-CEUA) in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

Euthanasia was performed by decapitation following CO_2 asphyxiation.

2.2. Experimental design

2.3. Study 1: Early and late development and fertility

Two females per male were placed together on the dark cycle of the photoperiod for mating (Figure 1). On the subsequent morning, vaginal smears were collected from the mated females for the detection of sperm. If those females were sperm-positive, gestational day (GD) 0 was determined.

Dams (F0) were kept in individual cages and pseudo-randomly allocated into two experimental groups (n=13 / group): control, which received regular chow, and EDS, which received solid food formulated with EDS. Other compounds and nutrients of the formulated solid food were the same as the commercial chow (Presence®) offered to the control group. Treatment began on GD 12, and ended on weaning, postnatal day (PND) 21. Food consumption was monitored daily from GD 0 to PND 21, whereas body weight was monitored every three days. On GD 20, five dams per group (F0) were euthanized to access fertility.

After birth, the pups were randomly selected to obtain 4 males and 4 female littermates (F1) per lactating female to maintain a similar pattern of food distribution between pups. After weaning, rats were given regular chow (Presence®) *ad libitum*.

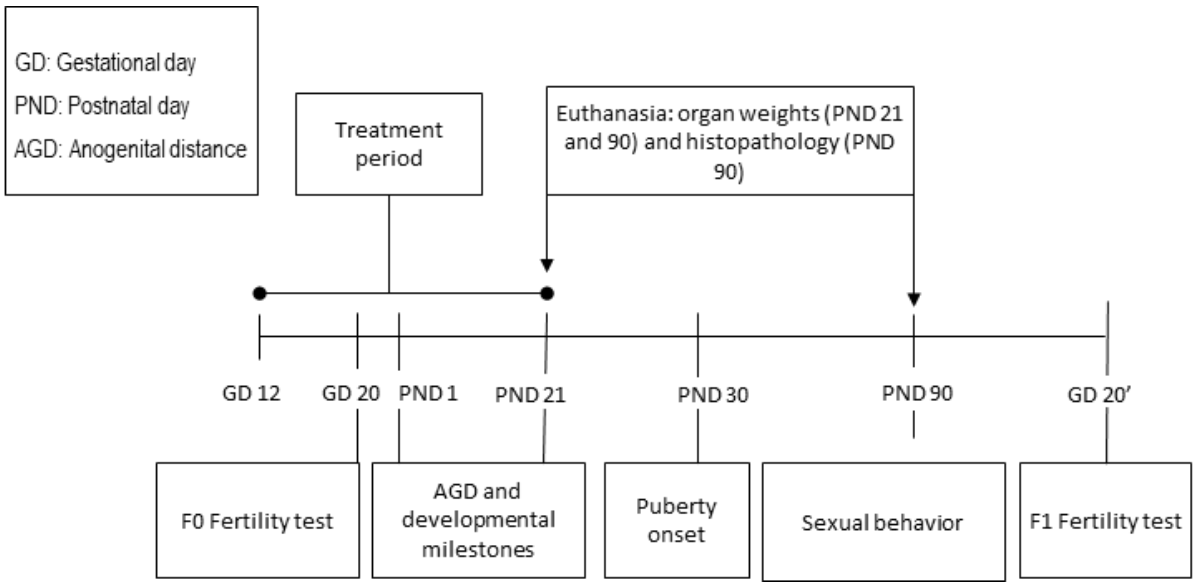

Figure 6. Experimental design.

2.3.1. Early development

Physical development and developmental reflexes were evaluated as described by Heyser (2004). Early sexual development parameters were evaluated regarding experimental toxicological studies (Gallavan et al., 1999; Gray et al., 1999; Hood, 2016).

Physical development milestones

The day of upper and lower incisor eruption was observed daily from PND 6 and the day of complete eye opening was observed from PND 12 on one female pup per litter.

Developmental reflexes

Developmental reflexes were evaluated daily from PND 1 on one female pup per litter until three identical consecutive results were observed.

Rats were given 30 seconds on the rightening reflex test and result was considered positive when they were able to turn from their back on their belly with all their legs resting freely on the table.

The grasp reflex was evaluated by gently stroking the paw of the rat with a paper clip and the day this reflex disappeared was recorded.

Sexual development

Four female rat pups per litter were weighted and their anogenital distance (AGD) was measured at PND 1. AGD measure was corrected by body weight as described by Gallavan et al. (1999). On PND 13, the number of nipples were counted on four females per litter; female rats typically have 12 nipples (Gray et al., 2001). From PND 30, external signs of puberty onset were observed and recorded: the day of vaginal opening and first estrus. The first estrus was determined by collection of vaginal lavages described below.

2.3.2. Estrous cyclicity

The estrous cycle is divided into four phases in the rat, proestrus, estrus, metestrus and diestrus, and each phase can be identified by the observation of the proportions of cellular types on vaginal lavages (e Silva et al., 2016). From PND 60 to 75, 10µl of saline solution was gently introduced into the rat vagina with an automatic pipettor and the fluid aspired was placed on clean histological slides for analysis under a light microscope. The phase of the estrous cycle observed was recorded and used to calculate the frequencies of the phases, cycle length and number of cycles.

2.3.3. Body and organ weights

On PND 21 and 90 (on estrus), one female per litter was weighted and euthanized. Brain, pituitary, thyroids, adrenals, ovaries, and uterus (with fluid) were dissected and weighted. The ovaries and uteri of adult females (PND 90) in modified Davidson's fluid (mDF) (Latendresse et al., 2002) for histology.

2.3.4. Histological evaluation

The right ovary and horn of the uterus were fixed by immersion in mDF, dehydrated and embedded in Paraplast Plus®(P3683, Sigma-Aldrich). Tissues were sectioned (5 µm), mounted on glass slides and stained with hematoxylin and eosin (HE). Histopathology was analyzed in a blind assay using a Leica DMLB microscope mounted with a digital camera and analysis software (LAS V4.12). Ovarian follicles in different stages of follicular development and corpora lutea were counted, and the morphological aspect of this organ was observed as described by Talsness et al. (Talsness et al., 2005) Barros et al. (Barros et al., 2020) . The morphometry of the uterus was also evaluated. For that, the height of the luminal epithelium,

endometrium, myometrium and perimetrium were measured on the uterine sections as previously described by e Silva et al. (e Silva et al., 2016) with modifications. The mean heights of the uterine layers were combined, and a total height was noted. The percentage of the contribution of the height of each uterine layer to the total height was then determined.

2.3.5. Sexual behavior

Females on the first estrus phase around PND 90 were placed with a sexually experienced male rat on the dark cycle of the photoperiod, then allowed 10 mounts on the female while registering lordosis behavior. Results were expressed as the lordosis quotient (lordosis number / 10 mounts × 100) (Beach, 1976). After the test, females were left overnight in the cage of the male overnight and vaginal smears were collected on the next morning to confirm insemination. Inseminated females were kept for the fertility test. If no lordosis behavior was detected on two subsequent estruses, the female was considered sexually inactive and excluded from the test.

2.3.6. Fertility test

The fertility test was performed with F0 females and F1 females from the sexual behavior test. They were euthanized on GD 20 to evaluate fertility. After collection of the uterus and ovaries the numbers of corpora lutea, implants and reabsorptions were recorded, and the following endpoints determined: pregnancy rate: pregnant females / sperm-positive females; fertility potential (efficiency of implantation): implantation sites / corpora lutea × 100; rate of pre-implantation loss: (number of corpora lutea - number of implantations / number of corpora lutea) × 100; rate of post-implantation loss: (number of implantations - number of live fetuses) / number of implantations × 100 (Borges et al., 2017).

2.4. Study 2 - Hormonal assays

Untreated animals were mated and kept under controlled conditions as described in study 1. Dams were kept in individual cages and pseudo-randomly allocated into two experimental groups (n=7 / group): control, which received regular chow, and EDS, which received solid food formulated with EDS. Treatment began on GD 12 and ended on PND 21. Female offspring was euthanized on the first estrus around PND 90 for blood sample collection. Those blood samples (n=7 / group) were allowed to coagulate, and the serum was obtained by centrifugation

(2400 rpm) for 20 min at 4◦C. Serum samples were subsequently stored at −20◦C. Serum levels of FSH, LH, and prolactin were measured using a double-antibody radioimmunoassay with specific kits provided by the National Hormone and Peptide Program (Harbor-University of California at Los Angeles). Serum levels of testosterone were measured using a double-antibody radioimmunoassay with specific kits supplied by MP Biomedicals (ImmuChem™Double Anti-body, Testosterone: 07814891. Orangeburg,NY, USA). All samples were measured in the same assay to avoid inter-assay errors.

2.5. Statistical analyses

Data are presented as mean ± standard error of mean (SEM) or median and interquartile range. Student's t-test was used for comparison of parametric variables. Non-parametric variables were compared using Mann-Whitney test. Percentage data was compared using the Chi-Square test. Results were considered significantly different when $p \leq 0.05$. Statistical analyses were performed using the GraphPad InStat software (version 6; La Jolla, CA).

3. RESULTS

3.1. Study 1 - Early and late development and fertility

Evident signs of general toxicity and stress (mortality and morbidity, weakness, lethargy, excessive weight gain/loss, presence of bristly hair, and abnormal behavior) derived from the treatment were not observed throughout the experimental procedures.

3.1.1. F0 food intake, weight gain and fertility test

Food intake of F0 females from EDS group was decreased throughout all treatment period (GD 12 – PND 21), except on PND 4 (Figure 2A and B). EDS-treated females consumed an average of 72% and of the total food control females consumed every day of the treatment period (Figure 3). Body weight was lower on PND 7 and13 (Figure 2D), and weight gain from GD 0 to 20 was reduced on EDS group (Figure 2C).

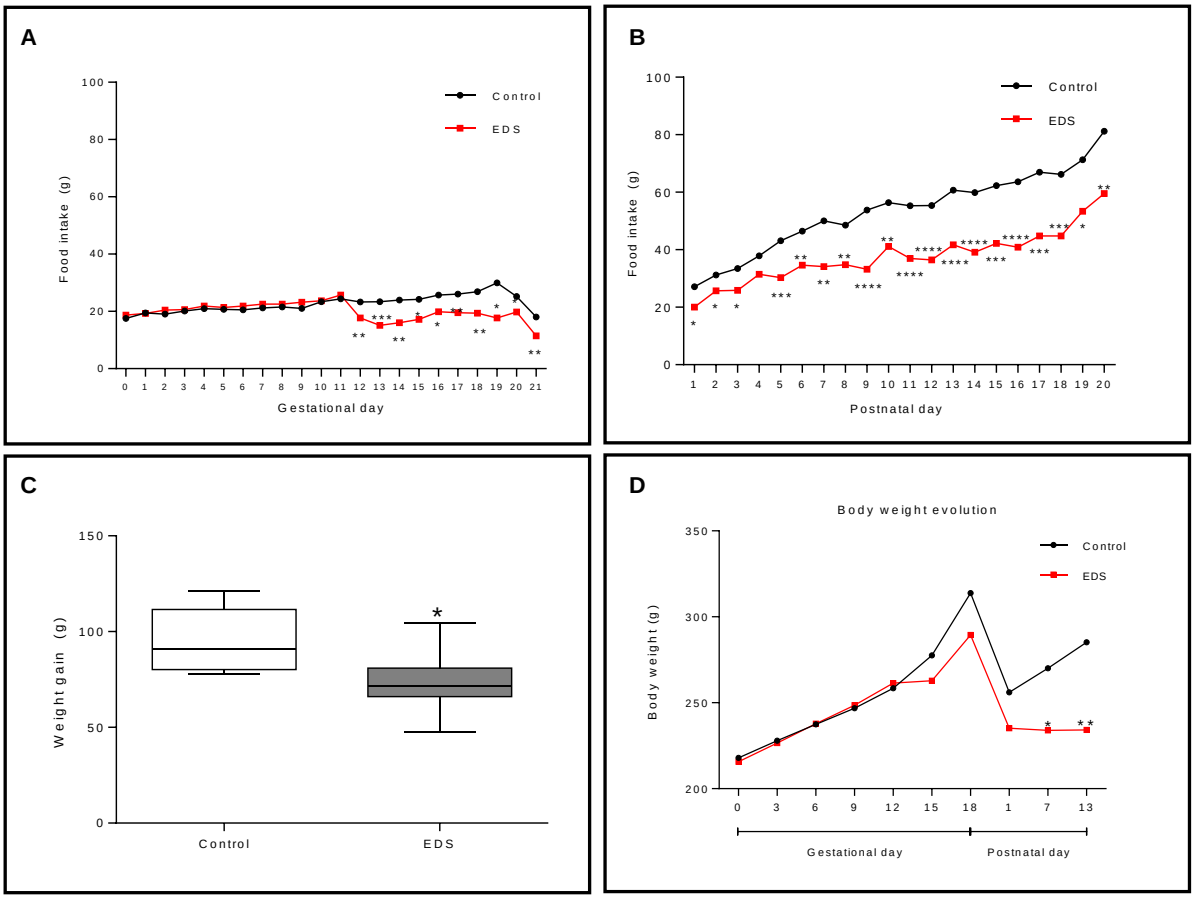

Figure 2. Food intake and weight gain of control and EDS F0 females. A: Food intake from GD 0 to 21 (n=13 / group). B: Food intake from PND 1 to 20 (n=8 / group). C: Weight gain from GD 0 to 20 (n=13 / group). D: Body weight evolution on gestational (n=13 / group) and postnatal (n=8 / group) days. Values expressed as mean ± SEM. Student's test. * $p \leq 0.05$. ** $p \leq 0.01$.*** $p \leq 0.001$. **** $p \leq 0.0001$.

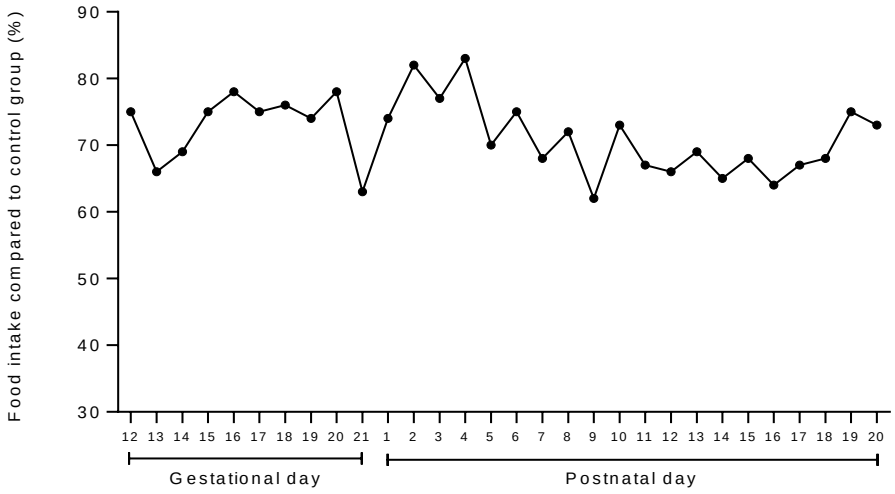

Figure 3. Food intake of EDS F0 females compared to the control group on the treatment period (GD 12 - PND 21). Mean food intake of EDS group on the treatment period was 72% of control group intake.

Regarding the fertility test of F0 females, the weight of the dams, the fetuses and the placentas were reduced on EDS group (Table 1). Other fertility parameters were not different between the experimental groups.

Table 1. Fertility test of F0 females on GD 20 (n=5 / group).

Parameters	Control	EDS
[1]Pregnancy rate (%)	100.00	100.00
[2]Fertility potential (%)	100.00 (95.45 - 100.00)	96.86 (92.31 - 96.67)
[2]Pre-implantation loss (%)	0.00 (0.00 - 4.55)	7.14 (3.33 - 7.69)
[2]Post-implantation loss (%)	0.00 (0.00 - 7.94)	0.00 (0.00 - 7.42)
[3]Body weight of the dams (g)	351.60 ± 15.55	307.00 ± 9.05*
[3]Uterus weight with fetuses (g)	78.43 ± 8.06	68.80 ± 2.11
[3]Corpora lutea number	13.00 ± 1.10	13.60 ± 0.40
[3]Implantation number	12.80 ± 1.20	12.80 ± 0.37
[3]Reabsorption number	0.40 ± 0.24	0.40 ± 0.24
[3]Fetus weight (g)	4.36 ± 0.04	3.79 ± 0.03****
[3]Placental weight (g)	0.57 ± 0.01	0.52 ± 0.01**

[1]Values expressed as percentage (Chi-Square test). [2]Values expressed as median and interquartile intervals (Mann-Whitney test). [3]Values expressed as mean ± SEM (Student's t-test). * p≤ 0.05. ** p≤ 0.01. ****p ≤ 0.0001.

3.1.2. F1 early development

Eye opening was delayed on EDS females from the first generation compared to the control group (Table 2). No other parameters from physical development and developmental reflexes evaluated were altered.

Table 2. Physical and reflexological developmental milestones of F1 females (n=8 / group; 1 / litter).

Parameters (days)	Control	EDS
Physical development		
Eye opening	13.88 ± 0.30	14.63 ± 0.18*
Incisor eruption	9.63 ± 0.32	9.50 ± 0.42
Developmental reflexes		
Grasp reflex	8.38 ± 0.38	8.00 ± 0.57
Rightening reflex	2.25 ± 0.31	2.00 ± 0.33

Values expressed as mean ± SEM. Student's t-test. * p≤ 0.05.

Body weight of F1 females was reduced on PND 1 and 21. Similarly, there was a trend towards a reduction in the anogenital distance of the EDS-treated group (p=0.06) (Table 3). The number of nipples were similar between experimental groups on PND 13 (data not shown).

Table 3. Body weight and AGD on PND 1 and 21 of F1 females. Numbers in parenthesis represent the number of litters at PND 1 or animals per group at PND 21.

Parameters	PND 1		PND 21	
	Control	EDS	Control	EDS
Body weight (g)	6.62 ± 0.12 (8)	6.23 ± 0.13* (8)	47.70 ± 2.23 (7)	33.54 ± 1.15*** (7)
AGD (mm/g$^{1/3}$)	1.03 ± 0.03 (8)	0.96 ± 0.03 (8)	2.79 ± 0.13 (7)	2.48 ± 0.09 (7) (p=0.06)

Values expressed as mean ± SEM. Student's t-test. * $p \leq 0.05$. ***$p \leq 0.001$.

As regards to puberty onset, both external indicators were delayed on EDS group (Figure 4A and C), but the respective body weight was not altered (Figure 4B and D).

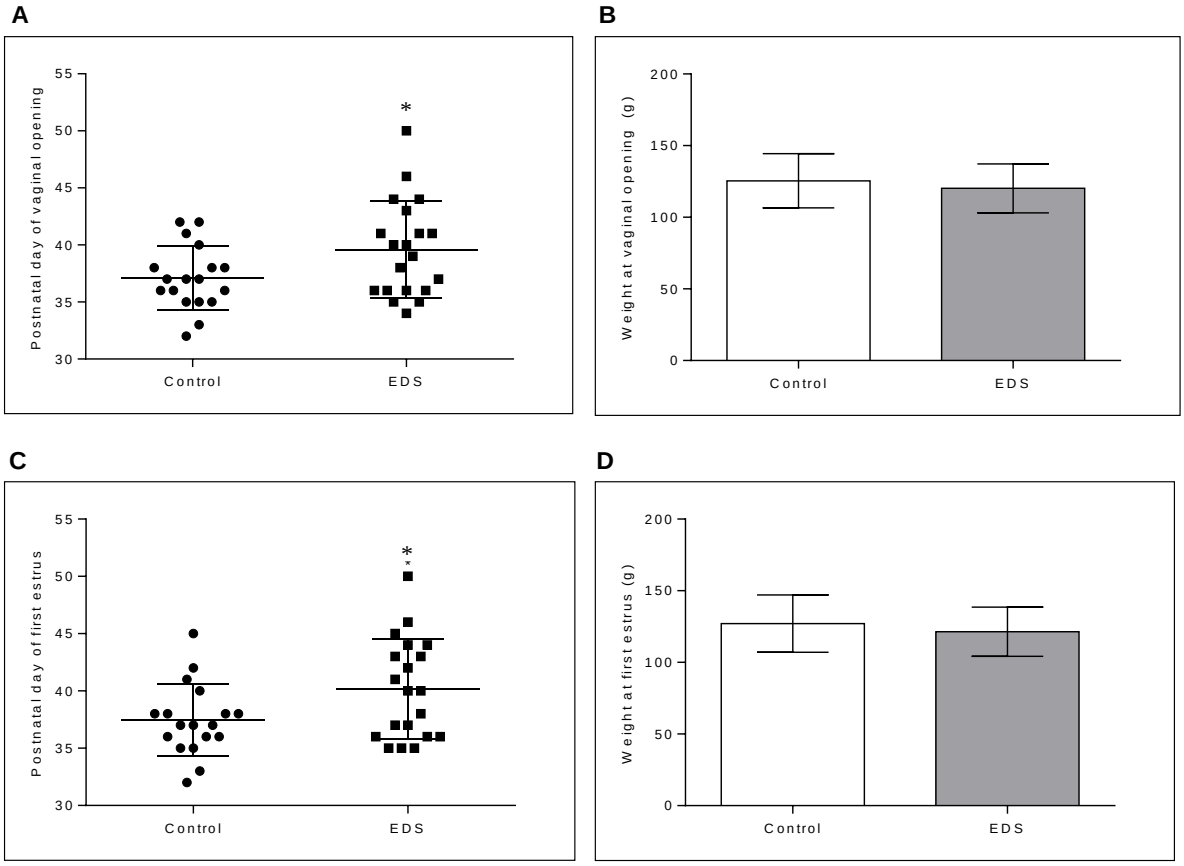

Figure 4. Puberty onset on F1 females. Postnatal day of vaginal opening (A) and first estrus (C), and body weight at the respective puberty indicator (B and D). Values expressed as mean ± SEM. Student's t- test. * $p \leq 0.05$.

3.1.3. Estrous cyclicity

Estrous cyclicity was altered in F1 females from the EDS group by the increase of the frequency of diestrus and decrease of the frequency of metestrus (Figure 5). The duration and quantity of cycles were similar between both groups (data not shown).

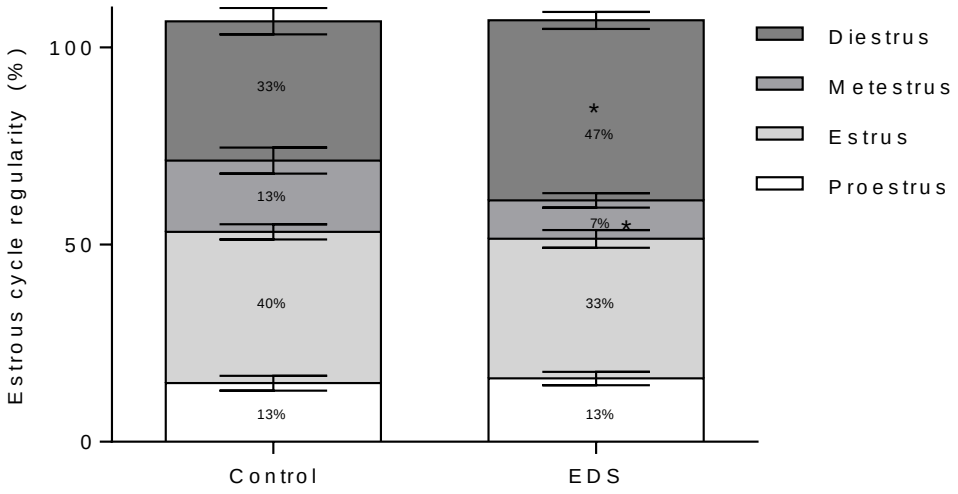

Figure 5. Frequency of each phase of the estrous cycle of F1 females. Values expressed as mean ± SEM. Mann-Whitney's test. *p ≤ 0.05.

3.1.4. Body and organ weights

Body weight of F1 females was reduced on EDS group on PND 21, but not on PND 90. Uterus absolute weight was also decreased on that group, but on PND 90. Pituitary and brain relative weights, however, were increased on EDS group on PND 21 (Table 4).

Table 4. F1 organ weights on PND 21 and 90.

Parameters	PND 21		PND 90	
	Control	EDS	Control	EDS
Body weight (g)	47.70 ± 2.23	33.54 ± 1.15****	242.8 ± 11.81	224.4 ± 9.17
Brain (g / 100g BW)	3.00 ± 0.15	3.86 ± 0.11***	0.78 ± 0.03	0.80 ± 0.03
Uterus (mg)	26.11 ± 1.26	23.69 ± 3.06	448.7 ± 17.41	361.0 ± 14.45**
Ovaries (mg)	26.59 ± 2.07	24.89 ± 1.79	90.95 ± 4.69	89.28 ± 5.84
Pituitary (mg / 100g BW)	4.82 ± 0.56	7.40 ± 0.72*	5.00 ± 0.28	5.18 ± 0.46
Thyroids (mg / 100g BW)	10.54 ± 0.93	13.62 ± 2.12	6.46 ± 0.56	6.59 ± 0.38
Adrenals (mg / 100g BW)	31.91 ± 2.63	34.77 ± 3.76	39.51 ± 2.67	39.30 ± 3.91

Values expressed as mean ± SEM. Student's t-test. *p ≤ 0.05. **p ≤ 0.01. **** p≤ 0.0001. BW: Body weight.

3.1.5. Histological evaluation

Regarding the morphometry of the uterus, the percentage of contribution to the total height was altered in the endometrial and myometrial layers on EDS group. The endometrial contribution was decreased whereas the myometrial was increased (Table 5).

Table 5. Height (µm) and contribution to total height (%) of different uterine regions of F1 females on PND 90.

Parameters	Control (n=6)	EDS (n=4)
[1]*Height (µm)*		
Total	886.70 ± 69.68	867.90 ± 78.32
Luminal epithelium	23.88 ± 0.81	27.28 ± 2.10
Endometrium	537.10 ± 50.09	463.80 ± 53.70
Myometrium	313.20 ± 19.89	365.00 ± 34.14
Perimetrium	12.57 ± 1.20	11.85 ± 1.36
[2]*Contribution to total height (%)*		
Luminal epithelium	3.00 (2.00 - 3.25)	3.50 (3.00 - 4.00)
Endometrium	59.50 (58.75 - 62.50)	55.00 (48.25 - 56.50)**
Myometrium	36.00 (34.50 - 37.00)	40.50 (39.25 - 47.00)**
Perimetrium	2.00 (1.00 - 2.00)	1.50 (1.00 - 2.00)

[1]Values expressed as mean ± SEM. Student's t-test. [2]Values expressed as median and interquartile intervals (Mann-Whitney test). **$p \leq 0.01$.

On ovary sections, the percentage of structures counted was similar between control and EDS groups (Table 6).

Table 6. Percentage of structures counted on ovarian sections of F1 females on PND 90.

Ovarian structures (%)	Control (n=6)	EDS (n=4)
Primordial and primary follicles	23.50 (22.25 - 33.75)	27.00 (22.50 - 40.50)
Secondary follicles	22.00 (19.75 - 27.25)	12.50 (6.00 - 22.75)
Tertiary follicles	7.50 (4.50 - 15.75)	9.00 (4.75 - 14.75)
Corpora lutea	28.00 (20.75 - 36.00)	34.50 (30.00 - 36.00)
Atretic follicles	10.50 (8.00 - 17.00)	15.50 (6.50 - 17.75)

Values expressed as median and interquartile intervals (Mann-Whitney test). $p > 0.05$.

3.1.6. Sexual behavior and fertility test

The body weight of females from the sexual behavior and fertility test was record every six days since natural insemination (GD 0). The body weight of EDS females was reduced on every day recorded and so was the total weight gain (Figure 6).

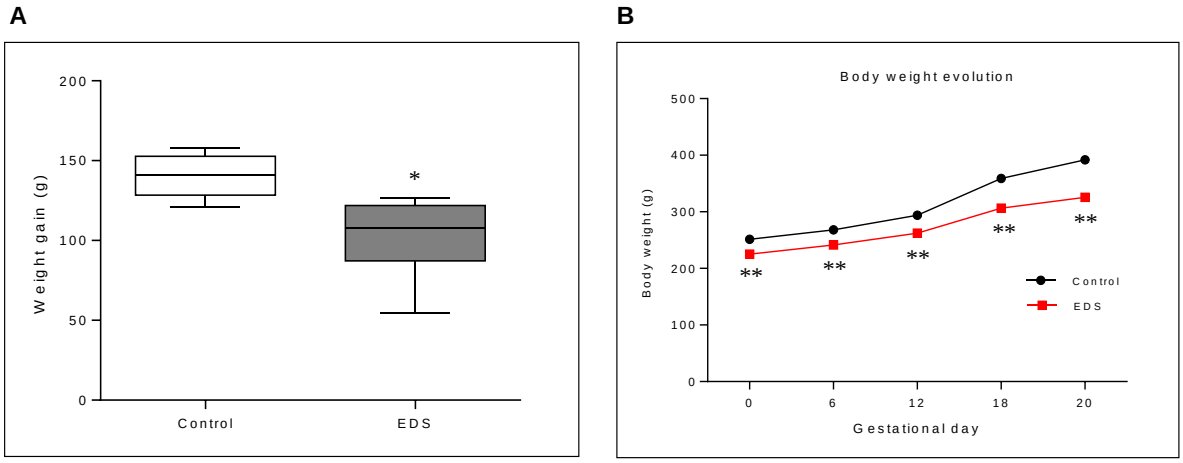

Figure 6. Weight gain (A) and body weight evolution (B) of pregnant F1 females from control (n=6) and EDS (n=7) groups from the fertility test. Values expressed as mean ± SEM. Mann-Whitney's test. *p ≤ 0.05. **p ≤ 0.01.

Sexual behavior (represented by lordosis coefficient) was not altered. The body weight of the dams, the weight of the uterus with fetuses, the number of corpora lutea and the implantation number were reduced on EDS group. Post-implantation loss and fetus weight, however, were increased on EDS group compared to control (Table 7).

Table 7. Lordosis coefficient and fertility test of F1 females.

Parameters	Control (n=6)	EDS (n=7)
[1]Lordosis coefficient	100.00 (70.00 - 100.00)	100.00 (62.50 - 100.00)
[2]Pregnancy rate (%)	83.33	100.00
[1]Fertility potential (%)	93.75 (81.49 - 97.22)	92.86 (84.62 - 100.00)
[1]Pre-implantation loss (%)	6.25 (2.78 - 18.51)	7.14 (0.00 - 15.38)
[1]Post-implantation loss (%)	0.00 (0.00 - 6.67)	9.09 (8.33 - 23.08)*
[3]Body weight of the dams (g)	392.10 ± 12.73	325.50 ± 8.93**
[3]Uterus weight with fetuses (g)	89.47 ± 4.94	58.77 ± 8.54*
[3]Corpora lutea number	17.00 ± 1.41	12.43 ± 0.37**
[3]Implantation number	15.20 ± 0.92	10.43 ± 1.43*
[3]Reabsorption number	0.40 ± 0.24	1.29 ± 0.36
[3]Fetus weight (g)	4.15 ± 0.07	4.46 ± 0.06*
[3]Placental weight (g)	0.60 ± 0.02	0.61 ± 0.02

[1]Values expressed as median and interquartile intervals (Mann-Whitney test). [2]Values expressed as percentage (Chi-Square test). [3]Values expressed as mean ± SEM (Student's t-test). *p≤0.05. **p≤0.01.

3.2. Study 2 - Hormonal assays

Testosterone levels were decreased on F1 adult females of EDS group. The other hormones evaluated were unaltered (Figure 7). Also, no signs of general toxicity were observed.

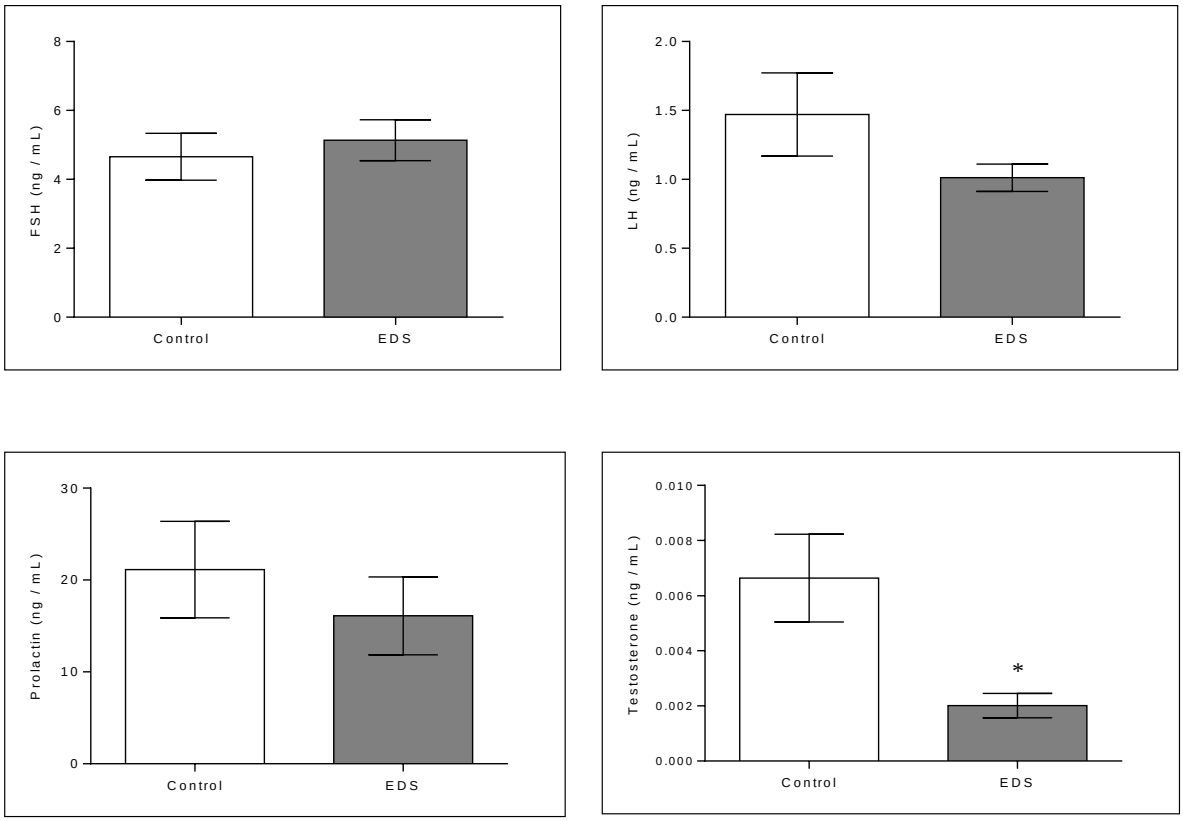

Figure 7. Hormone levels of female rats at PND 90. Values expressed as mean ± SEM. * $p \leq 0.05$.

4. DISCUSSION

Evident signs of general toxicity and stress derived from the treatment were not observed on both studies, neither on F0, nor on F1 females. Even so, F1 females from EDS group had impairment of sexual development and fertility, marked by puberty onset delay, alteration of estrous cyclicity, reduction of uterine weight and the contribution of its functional layer (endometrium) to the total epithelium height, decreased weight gain during pregnancy, reduction of pregnant uterus weight and the number of implantations, and increase in post-implantation losses. Additionally, testosterone levels of EDS adult females were reduced.

In the present study, we observed a reduction of food intake by F0 pregnant and lactating EDS females. Although the food was offered *ad libitum*, these animals ingested an average of 72% of what was ingested by the control group during the experimental period. Food intake may have been one of the factors for the reduction of body weight, associated with the EDS action itself, possibly due to mechanisms unrelated to the palatability of the formulated chow, since adult animals exposed to EDS through the intraperitoneal route present weight loss (Sahinturk et al., 2007). Busulfan, an alkylating agent similar to EDS, causes reduced body

weight (Abdollahifar et al., 2020) and decreased appetite in patients receiving this treatment (Clemmons et al., 2015).

On the fertility test of F0 females, we observed a reduction on the body weight of the dams, weight of the fetuses and the placentas of the EDS-treated group. This was probably caused by both a reduction in food consumption, and by an effect of EDS itself. Tarka-Leeds (2003b) exposed mice intraperitoneally to 160 mg / kg during gestation and observed a reduction on the weight of pups on PND 1, without a reduction on the weight of the dams. The placenta is responsible for the distribution of nutritional resources for the fetuses (Burton and Fowden, 2012), and the reduction of the weight of this organ implies that EDS-exposed fetuses received fewer nutritional resources than control fetuses. Also, EDS exposure did not lead to an interruption of pregnancy and did not promote fetal malformation.

Reduced food intake may lead to reduced energy stock. In light of a reduced energy stock, the brain is capable of preferably allocating energy supply to itself, as explained by the selfish brain theory (Peters et al., 2004). The hypothalamic-pituitary-adrenal axis plays an important role in energy management (Fehm et al., 2006). It is suggested that for that reason, relative brain and pituitary weights were increased in F1 females from EDS group on PND 21, associated with body weight reduction at that same age. Also, maternal malnutrition can cause underdevelopment of the central nervous system of the offspring, which includes changes in memory, social and aggressive behavior, food preference and food intake (Lagisz et al., 2014). As for body weight, developmental reflexes milestones and physical development, our results were similar to Smart et al. (1990), except for the delay on eye opening on EDS group. This difference could be explained by the treatment period and exposure route. In this study, F0 rats were exposed from GD 12 to the end of lactation, whereas Smart et al. (1990) exposed pups intraperitoneally from PND 4 to 15.

Body weight is an important factor for the onset of puberty (Castellano et al., 2018; Ojeda and Skinner, 2006). In F1 females from EDS group, both parameters of puberty onset were delayed and the weight observed was similar between experimental groups, as also shown by Smart et al. (1990). Especially in females, it is necessary to have an energy reservoir at the time of installation of puberty so that the animal can continue with pregnancy and lactation, which justifies the similar weight and delay in observing the external parameters of puberty (Castellano and Tena-Sempere, 2016). In addition, the onset of puberty depends on the activation of the hypothalamic-pituitary-gonadal axis (HPG) (Ojeda and Skinner, 2006) and, although the action of EDS on female steroidogenesis is unknown, we observed a reduction in

serum testosterone concentration in females treated in the midgestational and lactational period. Since we were not able to measure estrogen serum levels, it is not possible to determine whether the decrease in testosterone concentration is associated with a decrease in estrogen levels, or if testosterone is reduced due to an increase in the conversion of this androgen to estrogen.

In females, androgens are produced by theca cells and adrenal glands. Theca cells originate from the interstitium of the developing gonad such as Leydig cells, and it is believed that these cell types have a common progenitor (Potter et al., 2016). It is possible, then, that EDS affects the fetal progenitors of the theca cells, which may have compromised androgenic production in F1 females of the EDS group. In addition, androgens produced by male fetuses participate in the fetal development of females (Martinez-Arguelles et al., 2013), which indicates that a potential reduction in male androgenic production caused by fetal Leydig cells depletion may have interfered with female offspring development. It is also noted that exposure to antiandrogens in the fetal period causes changes in the pituitary secretion pattern (Rossi et al., 1991).

The integrity of the HPG axis can be assessed by estrous cyclicity and be an indicator of female reproductive function (Goldman et al., 2007). F1 females from EDS group had an increased frequency of diestrus compared to the control group, which shows a disruption in estrous cyclicity. Sexual behavior, the number of sexually active females, pregnancy rate, and fertility potential were not altered, but pregnant F1 EDS-females gained less weight during pregnancy and a reduced weight of the uterus with the fetuses.

Steroid hormones promote proliferation and preparation of the uterine epithelium. Androgens and estrogens have complementary actions in the regulation of the trophic environment and uterine cytoarchitecture (Nantermet et al., 2005; Weihua et al., 2002). Uterine growth of the functional layer (endometrium) also depends on estrous cyclicity and steroid hormones stimuli. EDS adult females had decreased uterus weight and a smaller contribution of the endometrial layer to the total height of the uterine epithelium. An endometrium that is not adequately receptive is responsible for two-thirds of implantation failure (Taylor and Gomel, 2008).

As decidua and metrial gland are maternal components of the placenta that originate from the endometrium, abnormal decidualization could be responsible for an increase in post-implantation loss on EDS females (Das, 2009). Also, pregnancy maintenance is dependent on steroid hormones (Whittaker et al., 2018), and in rats they are mainly supplied by the corpora lutea (Gaytán et al., 1997). In androgen receptor knockout mice, fecundity reduction is mostly

due to a decreased number of ovulated oocytes and a thinner endometrium (Astapova et al., 2019; Hu et al., 2004). Both corpora lutea number and the number of implantations were reduced on EDS females, meaning a smaller litter size, and the placental weight was not altered. Since the number of fetuses was reduced on EDS females, they had more room and nutrients available to grow, which caused their weight to be increased compared to the control group.

All these results lead to a decrease in reproductive efficacy of the females treated with the dietary formulation with EDS. An ideal pest control method that reduces fertility should be effective on both males and females (Shuster et al., 2018). Another concern regarding pest control is the risk of primary and secondary poisoning of domestic and wild animals, and humans accidentally exposed (Bertero et al., 2020; Durão and Machado, 2016; Rodrigues Mendonça et al., 2016; Sánchez-Barbudo et al., 2012). EDS is a cytotoxic agent that specifically depletes Leydig cells and leads to male transitory infertility (Kerr et al., 1985), and in this study we show that EDS also reduces fertility parameters in females, potentially by the common embryonic origin of male and female steroidogenic cells (Potter et al., 2016). Yet, this is still to be confirmed. Even so, EDS has the potential to reduce fertility in both male and female rats, and this combined effect could lead to a decrease in pest populations without systemic toxic effects and the development of pest resistance.

5. CONFLICT OF INTEREST

The authors Lethícia Valencise, Patrícia Villela e Silva, Ana Flávia Quiarato Lozano, Jorge Willian Franco de Barros, Cibele dos Santos Borges, Gary Robert Klinefelter and Wilma De Grava Kempinas are developing a pest control method of EDS oral exposure (Patent Protocol 18CI097).

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Conclusões

Em relação ao desenvolvimento de um método alternativo para o controle de animais sinantrópicos, o EDS adicionado ao alimento sólido se mostra promissor. Os animais foram receptivos ao tratamento e este não aparentou provocar efeitos tóxicos sistêmicos sobre o organismo (F0, F1 e F2), além de ser eficaz em reduzir a fertilidade tanto em F1 (machos e fêmeas) quanto F2 (machos). O efeito intergeracional combinado pode levar a um controle populacional mais seguro e humanitário, reduzindo a proliferação desenfreada sem causar prejuízos à qualidade de vida dos roedores. Ademais, este trabalho pode incentivar a investigação desta metodologia alternativa em outras espécies.

Resumo dos resultados					
Geração	Peso corpóreo	Ingestão de alimento	Peso de órgãos genitais	Parâmetros de fertilidade	Testosterona
F0	↓	↓	Não se aplica	Não se aplica	Não se aplica
F1 masculina	↓	↓	↓	↓	↓ (intratesticular)
F1 feminina	↓ (jovem)	Não se aplica	↓ (útero no DPN 90)	↓	↓ (sérica)
F2	Não alterado	Não se aplica	↓ (testículo no DPN 21)	Não se aplica	Não se aplica

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Anexo I: Certificado de aprovação da pesquisa pela Comissão de Bioética

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

Instituto de Biociências de Botucatu - Unesp

Certificado

Certificamos que o projeto intitulado "Efeitos da exposição intrauterina e lactacional ao etano dimetanosulfonato (EDS) sobre o desenvolvimento sexual e a fertilidade de ratos Wistar", Protocolo nº **1090-CEUA**, sob a responsabilidade de **Wilma De Grava Kempinas**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 9 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela ***COMISSÃO DE ÉTICA NO USO DE ANIMAIS*** (CEUA), nesta data.

Finalidade:	( ) Ensino	(x) Pesquisa Científica
Vigência do Projeto:	Início: 1/1/2019	Término: 30/6/2020
Espécie/linhagem:	*Rato Wistar*	
Nº de animais:	*280*	
Peso:	*250-500g*	Idade: 90 dias
Sexo:	*Macho e fêmea*	
Origem	*Biotério Central da Unesp – Câmpus de Botucatu/SP.*	

Botucatu, 4 de maio de 2018.

Prof. Dr. Bruno Cesar Schimming
Presidente da CEUA

Comissão de Ética no Uso de Animais IBB-Unesp

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