UNIVERSIDADE FEDERAL DE MATO GROSSO DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM SAÚDE E DESENVOLVIMENTO NA REGIÃO CENTRO-OESTE

Avaliação toxicogenética e toxicoreprodutiva de larvicidas comercias utilizados no combate ao *Aedes aegypti*

Doutoranda: Juliana Miron Vani

Orientador: Prof. Dr. Rodrigo Juliano Oliveira

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UNIVERSIDADE FEDERAL DE MATO GROSSO DO SUL

Programa de Pós-graduação em Saúde e Desenvolvimento na Região Centro - Oeste

Juliana Miron Vani

Tese apresentada ao Programa de Pós-graduação em Saúde e Desenvolvimento na Região Centro-Oeste da Universidade Federal de Mato Grosso do Sul como requisito parcial para a obtenção do título de Doutora.

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COMISSÃO EXAMINADORA

Prof. Dr. Rodrigo Juliano Oliveira
Universidade Federal de Mato Grosso do Sul - UFMS

Profª. Dra. Elisvania Freitas dos Santos
Universidade Federal de Mato Grosso do Sul - UFMS

Profª. Dra. Ana Tereza Gomes Guerreiro
Fundação Oswaldo Cruz - FIOCRUZ

Profª. Dra. Carla Cardozo Pinto de Arruda
Universidade Federal de Mato Grosso do Sul - UFMS

Profª. Dra. Renata Trentin Perdomo
Universidade Federal de Mato Grosso do Sul - UFMS

Junho/2020

Dedicatória

Aos meus pais, Valter e Irene e à minha irmã, Jéssica,
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"...dono de toda ciÊncia, sabedoria e poder...ninguém explica Deus!"

Sumário

Lista de Tabelas

Piriproxifen

Diflubenzuron

VANI, Juliana Miron. Avaliação toxicogenética e toxicoreprodutiva de larvicidas comercias utilizados no combate ao *Aedes aegypti.* 2020. Tese de Doutorado. Universidade Federal de Mato Grosso do Sul.

Resumo

Dengue, Febre Chikungunya, Vírus Zika e Febre Amarela são arboviroses que causam epidemias em países tropicais e subtropicais. O *Aedes aegypti* e o *Aedes albopictus* são os mosquitos responsáveis pela transmissão destas doenças e estão disseminados no Brasil. Assim, a melhor maneira de prevenir as doenças é com a eliminação dos focos dos mosquitos transmissores. Atualmente, a utilização de compostos sintéticos é uma das alternativas para esse combate. Porém, sabe-se que esses compostos podem apresentar toxicidade para o meio ambiente (organismos não alvo) e para homem. Assim, uma das maiores preocupações passam a ser as mulheres gestantes, pois estas ficam mais susceptíveis a efeitos de xenobióticos. Frente ao exposto, o presente trabalho teve por objetivo avaliar os efeitos de larvicidas comerciais no desempenho reprodutivo, desenvolvimento embriofetal e na intergridade do DNA em modelo pré-clínico. Os resultados dos larvicidas de uso comercial demonstraram que Temefós e Piriproxifen não causaram toxicidade materna, não alteraram o desempenho reprodutivo e desenvolvimento embriofetal, bem como não alteraram a integridade do DNA nas doses e protocolos testados. O Diflubenzuron, por sua vez, não foi tóxico para a fêmea. Mas, causou malformações na prole. As malformações observadas de forma estatisticamente significativa e de maior preocupação foram as encontradas no coração. Na avaliação da integridade do DNA, o produto não causou nenhuma alteração. Diante do exposto, inferimos que Temefós e Piriproxifen não causam alterações do desempenho reprodutivo, desenvolvimento embriofetal e na intergridade do DNA. Mas, o Diflubenzuron é teratogênico e, por isso, sugere-se que o seu uso seja descontinuado.

Palavras-chave: Arboviroses; mosquitos transmissores; larvicidas; toxicidade; teratogenicidade.

1. Introdução

As arboviroses são doenças transmitidas por artrópodes e são responsáveis por causar surtos por todo o planeta (MUSSO; GLUBLER, 2016; KAURA et al., 2019). Dentre as principais arborviroses destacam-se a Dengue, a Febre Chikungunya, o Vírus Zika e a Febre Amarela (ALONSO-PALMARES et al., 2018).

A Dengue é uma doença endêmica e muitas vezes epidêmica, que afeta milhares de pessoas todos os anos, e o Brasil é um dos países que apresenta muitos casos (NUNES et al., 2019). Em 2014, um novo vírus chegou no país, provocando uma nova doença: a Febre Chikungunya, seguida pelo Vírus Zika no ano de 2015 (CUNHA; TRINTA, 2017; BONICA et al, 2019). Uma doença que já havia sido erradicada voltou a circular, a Febre Amarela (BACHA; JOHANSON, 2017).

Essas doenças apresentam sintomas semelhantes que até passam despercebidos. Porém, a Dengue e a Febre Amarela podem causar agravamento do quadro e evoluir ao óbito. A Febre Chikungunya, em sua forma mais grave, pode levar o paciente a desenvolver dores nas articulações, impossibilitado-o de desenvolver suas atividades por meses ou até anos (LASERNA et al., 2018; LITVOC; NOVAIS; LOPES, 2018; CUNHA; TRINTA, 2017).

Já o Vírus Zika, que afeta as gestantes, pode causar malformações congênitas em fetos e/ou recém-nascidos, como é o caso da microcefalia. Também ganha destaque a Síndrome de Guillan-Barré, uma doença autoimune desencadeada também pela presença do vírus (PLOURD; BLOCH, 2016).

Todas as doenças mencionadas anteriormente são transmitidas por mosquitos vetores do gênero *Aedes*, em especial o *Aedes aegypti* e o *Aedes albopictus*. Estes mosquitos são bem adaptados em regiões tropicais o que facilita sua reprodução (MONTEIRO et al., 2019). Uma outra preocupação é com a Febre do Mayaro, uma arbovirose que apresenta o ciclo silvestre no Brasil, e que possivelmente seguirá os caminhos da Febre Amarela, e na transmissão urbana o *Aedes aegypti* será o principal vetor (ESPOSITO; FONSECA, 2017).

Com excessão da Febre Amarela, não existe, até o momento, vacinas que sejam eficazes para prevenir essas arboviroses. Assim, a melhor maneira de combatê-las é por meio do manejo adequado dos focos dos mosquitos transmissores (PLOURDE; BLOCH, 2016; CUNHA; TRINTA, 2017; DOUAM; PLOSS, 2018; SALLES et al., 2018).

O uso de larvicidas sintéticos é uma das alternativas do Ministério da Saúde para fazer o controle destes focos. Porém, os mosquitos apresentam resistência à estes produtos, e assim não terá mais o efeito desejado, por isso, deve ser substituído de tempos em tempos (DE ARAÚJO et al., 2019). Esses produtos utilizados no manejo, em geral, são sintéticos e podem apresentar toxicidade para o meio ambiente e também para o ser humano (BARROS et al., 2013; SANTOS et al., 2017).

O Ministério da Saúde faz uso do Piriproxifen. Ele é um análogo de hormônio juvenil que não mata as larvas do mosquito. Mas, causa malformações e impede o desenvolvimento larval e se tornaram adultos sem capacidade reprodutiva. (WHO, 2002; BRASIL, 2014).

O fato do Piriproxifen® causar malformações em larvas sugere preocupação com mulheres grávidas já que essas podem consumir água que foi tratada com esse larvicida ou com outros.

Quando uma gestante e/ou uma fêmea prenhe está exposta a xenobióticos, pode acontecer alteração no desenvolvimento embriofetal, que determina o aparecimento de malformações congênitas (MOORE; PERSAUD, 1995; ROSENFELDI, 2015).

Frente ao exposto, o presente estudo tem por objetivo avaliar o desempenho reprodutivo, o desenvolvimento embriofetal e a integridade do DNA de camundogos *Swiss* prenhes expostas aos larvicidas comercias Temefós®, Diflubenzuron® e Piriproxifen® que são utilizados no combate ao mosquito *Aedes aegypti*.

2. Revisão de Literatura

2.1 Arboviroses

O termo arbovirose refere-se às doenças causadas por infecções virais e transmitidas por algum tipo de vetor, seja ele artrópode e que seja encontrado na natureza. Esses vetores são hematófagos e são, em especial, representados pelos mosquitos e carrapatos (MUSSO; GLUBER, 2016; ALONSO-PALOMARES et al., 2018; BRAACK et al., 2018).

Doenças transmitidas por alguma espécie de mosquito vetor são responsáveis por mais de 700 milhões de casos no mundo o que determina os surtos. Estima-se que pelo menos 1 pessoa, em cada 17 infectadas, morra devido a algum tipo de arbovirose (KAURA et al., 2019).

As arboviroses que acometem seres humanos estão distribuídas em três famílias principais: a Família Flaviviridade (Gênero *Flavivirus*) que responde pelo maior número de casos; a Família Togaviridae (Gênero *Alphavírus*) e a Família Bunyaviridade (Gênero *Ortobunyavírus*) (BRAACK et al., 2018).

Os *Flavivírus* possuem maior destaque pois compreendem o vírus da Dengue, do Vírus Zika e da Febre Amarela, as três transmitidas pelos mosquitos do gênero *Aedes*. O gênero *Alphavírus* também possuem destaque, pois são os responsáveis pela transmissão da Febre Chikungunya, doença esta que já se tornou um problema de saúde pública (BRAACK et al., 2018; CUNHA; TRINTA, 2017).

Regiões tropicais e subtropicais do mundo são as principais responsáveis pelo surgimento das arboviroses. Os vetores são bem adaptados a este tipo de clima. O tráfego internacional de pessoas, entre os mais diversos países, facilita a entrada de vírus que são transmitidos por mosquitos. Além disso, alterações ambientais também representam um fator importante para essa disseminação (BRAACK et al., 2018; DE MAJO et al., 2019).

2.2 Dengue

Dentre os principais problemas de saúde pública atuais, em países subdesenvolvidos, destaca-se a Dengue, uma doença que apresenta um padrão endo-epidêmico que afeta milhões de pessoas em todo o planeta (MAHMUD et al., 2019).

Esta doença é transmitida por mosquitos vetores do gênero *Aedes*, em especial o *Aedes aegypti* e o *Aedes albopictus*, que são bem adaptados em ambientes tropicais e subtropicais o que favorece sua proliferação.

As fêmeas desses mosquitos se alimentam de sangue, e ao picar um indivíduo infectado com a doença contrai o vírus e na próxima alimentação de sangue o vírus será transmitido para o hospedeiro humano (ALONSO-PALOMARES et al., 2018; BOCK; JAYATHUNGA, 2019).

Com 4 sorotipos diferentes, DENV 1, DENV 2, DENV 3 e DENV 4, a doença pode ser autolimitada, onde uma pequena parte progride para a forma mais grave e, apresenta uma sazonalidade entre 3 a 5 anos, o que causa surtos e rotatividade dos diferentes sorotipos (SALLES et al., 2018; NUNES et al., 2019).

O vírus da Dengue é um genoma de RNA de cadeia simples com sentido positivo. Pertence ao gênero *Flavivírus* da Família Flaviviridade composta por mais de 53 espécies

de vírus que são transmitidos por mosquitos, carrapatos ou vírus que não apresentam vetores conhecidos (MUSSO; GLUBER, 2016; HOYOS–LOPES; ATENCIA-PINEDA; GALLEGO-GOMEZ, 2019).

A Ásia e a África, são os continentes onde encontram-se os primeiros relatos de casos da doença, que foi em Manila, nas Filipinas nos anos de 1953 e 1954. Nos últimos anos ela se expandiu de forma generalizada para as Américas, e o Brasil por ser o 2º país mais populoso, apresenta uma alta taxa de contribuição dos casos relatados, que acontecem em maior quantidade no Nordeste do país (SALLES et al., 2018; NUNES et al., 2019; OLIVEIRA; ITRIA; LIMA, 2019).

No Brasil, os primeiros relatos de Dengue ocorreram no ano de 1846. Mas, somente em 1982 aconteceu o primeiro surto da doença na cidade de Boa Vista (RR) com incidência dos sorotipos DENV 1 e DENV 4. Devido à expansão e à proporção que a doença tomou, desde o ano de 1999 ela foi considerada uma doença tropical negligenciada (SALLES et al., 2018).

De acordo com a Organização Mundial de Saúde, estima-se que a Dengue pode afetar de 3,2 até 3,9 bilhões de pessoas todos os anos em todo o mundo (LIN; LEE; LEO, 2017; OLIVEIRA; ITRIA; LIMA, 2019). Sabe-se que mais de 20.000 mortes acontecem todos os anos por conta da doença e, também uma média de mais de 500.000 pessoas são hospitalizadas com sintomas que podem ou não ser confirmados (LIN; LEE; LEO, 2017; LASERNA et al., 2018). Esses fatos comprometem os sistemas de saúde pública, visto que os gastos para tratamento dos pacientes infectados gira em torno de U$9 a U$39 bilhões de dólares anualmente (LASERNA et al., 2018).

Em 2014, o Brasil adotou a nova classificação de Dengue, que deixou de ser classificada em Dengue Clássica ou Dengue Hemorrágica, que passou à Dengue com Complicações (DCC), Febre Hemorrágica da Dengue (FHD), Síndrome de Choque da Dengue (SSD) e Dengue severa (DS) (KATZELNICK; COLOMA; HARRIS, 2018; NUNES et al., 2019). As principais manifestações clínicas da doença são ínicio abrupto de febre alta acompanhado de calafrios, astenia, dor retro-ocular, cefaléia, dor lombar, milagia, artralgia, naúseas e vômitos. Esses sintomas são classificados como Dengue sem sinais de alerta, pois eles desaparecem de 2 a 7 dias após o ínicio das primeiras manifestações (LIN; LEE; LEO, 2017; LASERNA et al., 2018).

Nos casos mais severos e que são chamados de Dengue com sinais de alerta, dos quais incluem a nova classificação, os sintomas são problemas gastrointestinais,

hemorragia neurológica, leucopenia progressiva e trombocitopenia (LIN; LEE; LEO, 2017; LASERNA et al., 2018). Já para a classificação de Dengue grave, que pode levar o paciente à óbito, observa-se sangramento grave, extravazamento do plasma, envolvimento e comprometimento dos órgãos e desconforto respiratório (GLUBER, 1998; LIN; LEE; LEO, 2017; LASERNA et al., 2018). Um fato importante, é que após a infecção primária o paciente fica mais susceptível a desenvolver a forma mais grave da doença, esta que é potencialmente fatal, conhecida como síndrome do choque da Dengue (KUDLACEK ; METZ, 2019).

A empresa Sanofi Pasteur no ano de 2015 criou a Dengvaxia® uma vacina quimérica pentavalente que foi derivada dos 4 sorotipos de Dengue (DENV 1, 2, 3 e 4), (BOCK; JAYATHUNGA, 2019; OLIVEIRA; ITRIA; LIMA, 2019). Essa vacina tornou-se de acesso restrito porque ela protegia apenas as pessoas com imunidade adquirida ao DENV e as pessoas que não possuem imunidade adquirida tornavam-se suceptíveis à Dengue após receber vacina (KUDLACEK; METZ, 2019). O custo para a produção dessa vacina é alto. Esse fato impossibilita a sua disponibilização nos sistemas públicos de saúde. (SALLES et al., 2018; BOCK; JAYATHUNGA, 2019).

Diante dos fatos apresentados, sabe-se que a melhor maneira de prevenir a doença é por meio de manejo adequado da eliminação dos focos do mosquito transmissor. No entanto, a conscientização da população em eliminar os criadouros em suas residências, onde é encontrado a maior parte dos focos, ainda está longe de ser resolvida (SALLES et al., 2018).

2.3 Febre Chikungunya

Recentemente enfrentamos um surto de uma nova doença que é a Febre Chikungunya (MONTEIRO et al., 2019).

O vírus da Febre Chikungunya foi descrito pela primeira vez no ano de 1952, quando ocorreu um surto febril em Makonde, que é uma província do sul da Tanzânia. Este vírus, pertencente ao gênero *Alphavírus* da Família Togaviridae, apresenta uma cadeia simples de RNA que entra em células-alvo por meio de endocitose (CUNHA; TRINTA, 2017; MONTEIRO et al., 2019).

O termo Chikungunya, refere-se aquilo que se curva devido à maneira que os pacientes ficam após serem infectados pelo vírus. É como se os pacientes andassem em

uma marcha rígida (VAN AALST et al., 2017; SILVA; DERMODY, 2017). Este vírus também é transmitido por um mosquito vetor, no qual tem como seu principal representante o *Aedes aegypti* (MONTEIRO et al., 2019). Os primeiros relatos de surtos da Febre Chikungunya ocorreram na África, Oceano Índico e sul da Ásia e somente no ano de 2004 uma epidemia ocorreu no continente africano (LWAND et al., 2015; MONTEIRO et al., 2019).

A doença foi então se espalhando e chegou até as Américas. Depois disso se espalhou pelos outros países deste continente e chegou ao Brasil no ano de 2014. O primeiro caso autóctone descrito foi no estado do Amapá. Logo na sequência houve um surto da doença na cidade de Feira de Santana na Bahia (VAN AALST et al., 2017; MONTEIRO et al., 2019).

Esta doença pode ser assintomática e evoluir para casos mais severos que podem levar o paciente a sofrer por anos. Os principais sinais clínicos são como os da Dengue e por isso o diagnóstico diferencial é difícil (MADARIAGA; TICONA; RESURRECION, 2015; VAN AALST et al., 2017).

Cunha e Trinta (2017) destacaram que a Febre Chikungunya deve ser classificada em 3 fases: aguda, pós-aguda e crônica. A fase aguda caracteriza-se pelos sintomas mais simples e pode evoluir para manifestações cutâneas mais severas. Na fase pós-aguda, os sintomas voltam a aparecer após o 21º dia de infecção quando, o paciente já acreditava em possível cura. Nesse fase há o desencadeamento de tenossinovite, bursite, periostite e tendinite, por exemplo, que podem levar meses para regredir. A fase crônica é caracterizada por manifestações músculoesqueléticas que determina período de dores maiores que 3 meses podendo se prolongar por um longo período de tempo.

Não há uma vacina para o vírus da Febre Chikungunya e sua prevenção deve ser feita por meio da eliminação dos focos dos mosquitos transmissores (CUNHA; TRINTA, 2017).

2.4 Vírus Zika

Destaca-se o Vírus Zika encontrado no Brasil recentemente, mas que já provocou surtos e preocupa bastante as autoridades de Vigilância em Saúde pelo fato de ser pouco conhecido (RICO-MENDOZA et al., 2019).

O Vírus Zika é um *Flavivírus* pertencente a Família Flaviridade que foi isolado pela primeira vez em um macaco *Rhesus sentinela* na floresta Zika na Uganda. Depois de um ano este vírus foi isolado nesta mesma floresta em um mosquito *Aedes africanus*. Ele apresenta uma cadeia simples de RNA (PLOURDE; BLOCH, 2016; BONICA et al., 2019).

Somente no ano de 1966, na África, o Vírus Zika foi isolado pela 1ª vez em um mosquito *Aedes aegypti* e a partir dai começaram a surgir evidências de que este vírus poderia ser transmitido para seres humanos. Os relatos dos primeiros casos foram registrados na Nigéria e depois disto o vírus começou a circular por outros continentes (PLOURDE; BLOCH, 2016; BONICA et al., 2019).

Os primeiros surtos ocorreram primeiramente nas ilhas de Yap na Micronésia. Depois foram acontecendo em outras localidades como Polinésia Francesa, Ilhas Cook, Nova Caledônia e, recentemente, o vírus se instalou nas Américas. No Brasil, ele foi isolado pela primeira vez no ano de 2015, e, provavelmente, foi trazido por viajantes virêmicos que entraram no país durante o grande evento esportivo em 2014, a Copa do Mundo de futebol (TAPPE et al., 2016; PLOURDE; BLOCH, 2016; BONICA et al., 2019). Outras possibilidades é que o vírus possa ter entrado no país por participantes da Jornada Mundial da Juventude, em 2013, ou no campeonato de canoagem, em 2014 (KATZELNICK et al., 2018).

A transmissão deste vírus é semelhante à transmissão da Dengue e Febre Chikungunya, ou seja, ele é transmitido pelos mosquitos vetores, já infectados, e o *Aedes aegypti* é o principal (KATZELNICK et al., 2018).

Os sintomas do Vírus Zika são muitos parecidos com os das outras arboviroses. Por isso o diagnóstico diferencial é difícil e precisa-se de cuidado para não ser confundido (RICO–MENDOZA et al., 2019).

Os principais sintomas da infecção pelo Vírus Zika podem aparecer entre o terceiro e o décimo segundo dia após a picada do mosquito. No entanto, essa doença pode ser assintomática. Os sintomas dessa infecção são erupção cutânea, febre, artralgia, mialgia, fadiga, dores de cabeça e conjuntivite. Esses podem desaparecer em até, no máximo, duas semanas (MARANO et al., 2016; PLOURDE; BLOCH, 2016).

Esta doença não apresenta a forma hemorrágica. Porém a sua forma mais grave pode causar malformações congênitas como a microcefalia em fetos e/ou recém nascidos de mães infectadas pelo vírus no primeiro trimestre da gestação (BONICA et al., 2019).

Esse vírus pode também levar ao desenvolvimento da Síndrome de Guillan-Barré que é uma doença autoimune que ataca o sistema nervoso. Os primeiros sintomas dessa síndrome são fraqueza que se agrava ao decorrer dos dias, neuropatia (formigação de pés e pernas), paralisia e perda da bainha de mielina (DALUGAMA et al., 2018; KONUSKAN et al., 2018).

A síndrome de Guillan-Barré tem outras cauas que não são a infecção do Vírus Zika. Mas, esse vírus como causador foi relatado pela primeira vez em 2013 na Polinésia Francesa (MARCONDES; XIMENES, 2016; KATZELNICK et al., 2018).

Esta é mais uma arbovirose que atinge milhares de pessoas e que não há uma vacina disponível no mercado. Logo, o melhor meio de combater e prevenir a infecção por Vírus Zika é eliminando os focos dos mosquitos transmissores. Entretanto, as complicações que o Vírus Zika podem trazer são de grande preocupação e assim ela foi declarada como uma emergência de Saúde Pública (MARANO et al., 2016; PLOURDE; BLOCH, 2016).

2.5 Febre Amarela

Das muitas arboviroses já conhecidas, uma que ganha destaque é a Febre Amarela, doença que já causou epidemias e mortes em países tropicais e subtropicais. Essa doença é originária da África e se disseminou e expandiu para as Américas por navios que transportavam escravos. A primeira epidemia nas Américas ocorreu em 1648 na península de Yucatan. Já no Brasil, as epidemias datam século XVII (BACHA; JOHANSON, 2017; LITVOC; NOVAES; LOPES, 2018).

A Febre Amarela é causada por um vírus do genêro *Flavivirus* da Família Flaviviridae e transmitida por mosquitos vetores (BACHA; JOHANSON, 2017). Este vírus é envelopado e apresenta uma cadeia simples de RNA de sentido positivo, vírus primo do DENV. Foi isolado pela primeira vez no ano de 1927 em um paciente de Gana. Essa doença apresenta duas formas de transmissão sendo uma de ciclo silvestre e outra de ciclo urbano as quais diferem pelas espécies de mosquitos vetores e hospedeiros (VASCONCELOS, 2003; DYER, 2017; LITVOC; NOVAES; LOPES, 2018).

O ciclo silvestre que acontece em florestas é bem complexo e envolve muitas espécies de mosquitos, mas os principais são o *Haemagogus janthinomys, Haemagogus*

leucocelaenus e o *Sabethes albiprivus*. Esses mosquitos picam, especialmente, hospedeiros primatas, que estão em seu nicho ecológico, tais como macacos bugios, saguis e pregos. Os homens servem de hospedeiros somente quando estes adentram a mata e comumente são mais infectados os lenhadores, os seringueiros, os vaqueiros, os garimpeiros, os caçadores e os turistas (VASCONCELOS, 2003; LITVOC; NOVAES; LOPES, 2018).

Já no ciclo urbano quem transmite é o *Aedes aegypti*, o mesmo que transmite outros arbovírus tais como o DENV, CHIKV e ZIKV. Esse mosquito pica diretamente o ser humano que começa a apresentar sintomas parecidos com uma gripe, após três dias da picada, e que depois podem evoluir para a forma mais grave da doença (DOUAM; PLOSS, 2018).

Outros sintomas que os indivíduos desenvolvem são febre alta, mialgia, cefaleia, falta de apetite e naúseas. Os sintomas da Febre Amarela são semelhantes aos da Dengue, Febre Chikungunya e Vírus Zika.

A forma grave da Febre Amarela leva a maioria de seus pacientes a óbito. Inicialmente, depois de uns 14 dias após infecção o paciente apresenta cura. Mas, os sintomas reaparecem e se agravam causando falência órgãos e sistemas. É nesse período que o paciente desenvolve a descoloração ictérica da pele, sangramento, disfunção renal e cardíaca (LITVOC; NOVAES; LOPES, 2018).

No período critico, que é entre 10 e 14 dias, a maioria dos pacientes não sobrevive e quando consegue sobreviver permanece com sequelas. O paciente uma fez infectado desenvolve imunidade, pois a infecção ocorre somente uma única vez (VASCONCELOS, 2003, LITVOC; NOVAES; LOPES, 2018).

Nos séculos XVIII e XIX, a Febre Amarela ficou mundialmente conhecida como a doença infecciosa mais perigosa, e no século XX ela passou a ser considerada uma doença tropical negligenciada. No ano de 1907, a Febre Amarela foi considerada controlada após ter causado muitos surtos. Em 1937 uma vacina foi desenvovida com vírus atenuado. Essa vacina confere imunidade vitalícia e por isso passou a ser usada em larga escala (VASCONCELOS, 2003).

Desde o ano de 1942, quando ocorreu uma epidemia no Brasil, na cidade de Sena Madureira (AC), não houve mais notificações da doença. No entanto, em 2016, notificações ocorreram nos estado da Bahia, de São Paulo e do Rio de Janeiro e, portanto, essa doença voltou a ser um problema de saúde pública. Mesmo com a distribuição da

vacina, o controle dos mosquitos vetores é de suma importância visto que eles tem aumentado a cada ano que passa e assim aumenta-se também os riscos de pessoas se infectarem (VASCONCELOS, 2013; DOUAM; PLOSS, 2018).

2.6 Febre do Mayaro

Os arbovírus estão em constante expansão no Hemisfério Ocidental e, sempre próximos às Américas onde desenvolvem surtos subsequentes (HOTEZ; MURRAY, 2017). Recentemente, um arbovírus tornou-se uma nova preocupação para as autoridades de saúde pública, pois ele está cada vez mais próximo de se tornar uma ameaça a população, que é a Febre do Mayaro (ESPOSITO; FONSECA, 2017).

O vírus causador da Febre do Mayaro, pertence ao gênero *Alphavírus* da Família Togaviridade, a mesma da Febre Chikungunya, ele apresenta uma fita simples de RNA (CAVALHEIRO et al., 2016). Foi isolado pela primeira vez, por Charles Anderson e colaboradores, em pacientes febris, no ano de 1954, em Trinidad e Tobago. A partir de então começou a se espalhar, principalmente, em regiões com florestas tropicais (MAVIAN et al., 2017; MUNOZ; NAVARRO, 2017).

No Brasil, o primeiro caso ocorreu no rio Guamá no estado do Pará e a primeira epidemia em uma aldeia próxima a este local. Algum tempo depois, vírus também foi encontrado em Conceição do Araguaia, no Pará, e em Peixe, no estado de Tocantins, no estado de Goiás, 343 casos foram notificados (ESPOSITO; FONSECA, 2017).

Os sintomas da Febre do Mayaro são febre moderada, dores de cabeça, dor retro-orbital, erupção cutânea e vômitos, podendo persistir por até 7 dias. Esse sintomas são semelhantes aos da Febre Chikungunya. Em casos mais graves essa doença pode se tornar uma doença incapacitante por um período prolongado de tempo, bem como causar complicações neurológicas e miocardia e em alguns casos pode levar o paciente a óbito (MUNOZ; NAVARRO, 2017; CAVALHEIRO et al., 2016; ACOSTA-AMPUDIA et al., 2018).

A transmissão do vírus Mayaro se dá pelas espécies de mosquitos *Haemagogus sp.*, principalmente, em florestas tropicais. Assim como acontece com a Febre Amarela, a Febre do Mayaro possui ciclo silvestre. Porém, há preocupação de que este vírus também ocorra em centros urbanos. No ano de 2016, o vírus Mayaro foi isolado de um paciente febril com 8 anos de idade e este mesmo paciente apresentou uma co-infecção de Dengue.

Esse fato levantou a hipótese de que o vetor principal da transmissão seja o *Aedes aegypti* (HOTES; MURRAY, 2017).

Acosta-Ampudia et al. (2018) relatam que é feita pelo gênero *Aedes* porque: (I) há homologia com o vírus da Febre Chikungunya, pois pertencem a mesma família; (II) a ocorrência do Mayaro é muito frequente nas regiões onde o *Aedes* é endêmico; (III) estudos com os mosquitos *Aedes* demonstram que eles tem competência para a transmissão do Mayaro, e (IV) o Mayaro se dissemina por meio de viajantes virêmicos e aves migratórias. Assim, há muita chance do vírus Mayaro começar a ser transmitido pelo *Aedes aegypti* e *Aedes albopictus* tornando-se uma ameaça à saúde pública. Logo, as medidas de prevenção devem ser inicadas já que não há vacina contra este vírus. A prevenção se dá da mesma maneira que para os demais arbovírus, ou seja, é necessária a eliminação dos focos dos mosquitos transmissores e de um manejo adequado (MOURÃO et al., 2012; HOTEZ; MURRAY, 2017; MAVIAN et al., 2017).

3. Mosquitos transmissores: *Aedes* ssp.

Os mosquitos do gênero *Aedes* de maior importância são as espécies *Aedes aegypti* (Linnaeus, 1762), originário das florestas africanas, e o *Aedes albopictus* (Skuse,1894), originário da Ásia, o Tigre Asiático. Ambos são de origem tropical e subtropical e estão espalhados por todas as partes do mundo. Estes mosquitos são predominantemente urbanos e peridomésticos por apresentarem uma relação com o homem (MONTEIRO et al., 2019).

O *Aedes aegypti* é a espécie encontrada, frequentemente, em ambientes urbanos em especial dentro das residências e o *Aedes albopictus* é mais frequente em regiões rurais. Mas, existem relatos de que o mesmo também é encontrado nas proximidades urbanas. Ambos são antropofílicos e se alimentam de sangue (no caso das fêmeas). Assim, o fato de estarem muito próximos à civilização, o homem se torna a maior fonte de alimentação e nesse caso a facilidade da propagação do vírus é intensa (ALONSO-PALOMARES et al., 2018; BRAACK et al., 2018; MONTEIRO et al., 2019).

Os mosquitos do gênero *Aedes* se diferenciam de outros pelo fato de apresentarem listras brancas, o *Aedes aegypti* apresenta um padrão dorsal brilhante, prateado e pernas brancas com faixas. Já o *Aedes albopictus* apresenta uma única listra dorsal prateada longitudinal e pernas brancas com faixa (MONTEIRO et al., 2019). A transmissão é descrita como vertical e horizontal. Na vertical os ovos são infectados e a horizontal, que

é a mais comum, ocorre após se alimentarem de sangue em um hospedeiro infectado com um vírus (BRAACK et al., 2018).

Essas espécies apresentam metamorfose completa: ovo, 4 estádios larvais, pupa e mosquito alado (adulto). Somente na fase adulta os mosquitos transmitem as doenças. As fêmeas podem ovipor até 250 ovos de cada vez quando seus oócitos estão cheios, e estes ovos podem permanecer no ambiente seco até 450 dias (SIQUEIRA et al., 2012).

Para que o vírus possa ser transmitido aos hospedeiros humanos, ele precisa passar por uma incubação extrínseca, ou seja, o vírus entra no intestino do mosquito e passa para a hemolinfa. A partir daí, o vírus infecta células de tecido adiposo, traquéia, hemócitos e glândulas salivares. Para que ocorra a transmição a um hospedeiro é necessário que o mosquito, neste caso fêmea, faça o repasto sanguíneo. Durante a picada o vírus passa então a ser secretado pela células epiteliais de seu hospedeiro (ALONSO-PALOMARES et al., 2018).

No Brasil, em meados do século XX, iniciou-se um projeto de erradicação do mosquito *Aedes aegypti* depois que ficou comprovado que ele era o transmissor urbano de Febre Amarela. Reformas sanitárias, eliminação de água parada, educação pública, associada ao uso de inseticidas, foram as estratégias das autoridades sanitárias para erradicar a espécie. Em 1942, o país estava livre deste mosquito. Porém, o fracasso em manter de pé os meios estratégicos de manejo, principalmente, pela falta de financiamento público fizeram com que o mosquito voltasse a circular (EPELBOIN et al., 2017).

A incidência do *Aedes aegypti* aumenta anualmente o que preocupa as autoridades em elaborar uma estratégia para a total erradicação deste mosquito. Mudanças demográficas, aumento da população em aglomerados, falta de saneamento bem como a deterioração dos sistemas de água, esgoto e lixo, criam condições cada vez mais favoráveis para o aumento da transmissão de doenças pelos mosquitos (GUBLER, 1998; TAPIA-LOPEZ et al., 2019).

4. Compostos sintéticos: Larvicidas, Inibidores de crescimento e Análogos de hormônio juvenil

As epidemias de Dengue, Febre Chikungunya, Vírus Zika e agora com a volta da Febre Amarela, são um desafio para as autoridades sanitárias e a população já que não existem vacinas efetivas, com exceção da Febre Amarela. Sendo assim, até o momento a melhor maneira de prevenção destas doenças é com a diminuição das populações dos

mosquitos transmissores por meio do uso de larvicidas sintéticos comerciais (OHBA et al., 2013; VIEIRA-SANTOS et al., 2017).

A principal estratégia do Programa Nacional de Combate à Dengue (PNCD) é utilizar inseticidas sintéticos que sejam eficazes na redução dos vetores. A primeira opção para o combate foi a utilização de um organofosforado, o Temefós®, produto larvicida contra as larvas do mosquito/vetor *Aedes aegypti* (DE ARAÚJO et al., 2019).

Desde o ano de 1967, o uso do Temefós® durante as epidemias de Dengue era preconizado no Brasil. Esse produto só deixou de ser indicado porque as larvas, em muitos municípios, tornaram-se resistentes (CORTE et al., 2018). Um mosquito se torna resistente a partir do momento em que ele consegue sobreviver a concentrações de inseticidas que são letais para outros da mesma espécie. Então, nesse momento, faz-se necessária a troca do produto por outro (DOS SANTOS DIAS et al., 2017).

Como os mosquitos vetores adquiriram resistência ao larvicida Temefós®, uma nova alternativa de combate começou a ser utilizada: os Inibidores de crescimento (IGRs). Dentre os principais destaca-se o Diflubenzuron® (DFB), pertencente à classe das benzoluréias que são utilizados para combater artrópodes. Esse foi o primeiro inibidor de síntese de quitina utilizado, comercialmente, para o controle de pragas e moscas de importância veterinária (BELLINATO; VALLE, 2015; BITENCOURT et al., 2019).

O DFB é um inibidor que interrompe a síntese e a deposição de quitina em mosquitos durante os estadios larvais além de dificultar a alimentação, causar malformações em pupas e nos adultos e inibição completa ou parcial de muda. A eficiência dsse produto foi reconhecida pela Organização Mundial da Saúde que passou a recomendar sua utilização em recipientes que contivessem focos dos vetores, inclusive, em água potável. Essa indicação e a substituição do larvicida Temefós® ocorreu no ano de 2003 (BELLINATO; VALLE, 2015; MARCOMBE et al., 2018; BITENCOURT et al., 2019).

Contudo, seguindo os passos do larvicida Temefós®, os mosquitos vetores também desenvolveram resistência ao uso do DFB e, uma outra alternativa teve de ser adotada para o combate ao *Aedes*, um novo composto então passou a ser utilizado: o Piriproxifen® (BELLINATO; VALLE, 2015; DE ARAÚJO et al., 2019).

Entre os anos 2013 e 2014, o DFB foi então substituído pelo Piriproxifen®, que permance até os dias atuais. Este composto é um análogo de hormônio juvenil, que suprime a embriogenêse e que altera as fases larvais e a metamorfose dificultando a

emergência de insetos adultos (MARCOMBE et al., 2018; OO et al., 2018; DE ARAÚJO et al., 2019).

Esse composto é formulado com um pequeno disco de resina, e por isso ocorre liberação lenta do composto. Essa tecnologia promove efeito residual por mais tempo do que facilita a reposição do mesmo pelos profissionais da saúde que pode fazer visitas em períodos mais longos (OO et al., 2018). Não existem relatos ainda de resistência ao Piriproxifen® pelos vetores. Por isso, não há previsão para que haja troca deste por um novo produto e, ele continuará sendo a escolha do Ministério da Saúde para o manejo dos focos e criadouros do mosquito *Aedes aegypti* (WHO, 2002; BRASIL, 2014).

4.1 Toxicidade dos compostos sintéticos

A utilização de compostos sintéticos, para o manejo adequado da redução das populações de mosquitos vetores que transmitem doenças como a Dengue, Febre Chikungunya, Zika Vírus e Febre Amarela são a melhor alternativa para prevenir a disseminação destas doenças. Porém, estes compostos podem ser tóxicos para a população e também para o meio ambiente (BARROS et al., 2013; SANTOS et al., 2017).

Aiub et al. (2002) demonstraram em seus estudos com sistemas-teste AMES e SOS (testes com microorganismos) que o larvicida Temefós® é genotóxico. Porém, esses dados não são corroborados por ensaios pré-clínicos com camundongos *Swiss* fêmeas prenhes. Vani et al. (2018) demonstraram que o Temefos® não apresentou toxicidade materna, nem causou alterações no desempenho reprodutivo e no desenvolvimento embriofetal, e também não alterou a integridade do DNA. Esses resultados sugerem que não há contra-indicações no uso deste produto em recipientes que contenham água potável.

O inibidor de crescimento DFB utilizado após a retirada do Temefós® do manejo, apresenta maiores preocupações pois ele tem descrições de toxicidade. Sapone et al. (2005) e Barros et al. (2013) demonstraram que o DFB possui potencial genotóxico para o ser humano e também possivelmente para o meio ambiente. Outros dados da literatura mostrados por Barros et al. (2014) relatam danos reprodutivos para machos após exposição ao composto.

Com relação ao Piriproxifen®, não existem muitos relatos na literatura que indiquem sua possível toxicidade para mamíferos. Maharajan et al. (2018), em seus

achados, no modelo de estudo zebrafish, observaram que o Piriproxifen® pode causar deformidades cardíacas e alteração na freqnuencia cardíaca. Além disso esse estudo ainda comprovou indução de danos genotóxicos e apoptose. Además, Viera-Santos et al. (2017) demonstram que o produto pode ser altamente tóxico para o meio ambiente em seus estudos com *Daphinia magna* e *Artemia Salina*.

O Piriproxifen® foi patê de polêmica em relação ao uso e a ocorrência de microcefalia já que o início do seu uso foi concomitante ao surgimento dos primeiros casos de microcefalia no Brasil. Um grupo de médicos da Argentina sugeriram microcefalia, inicial atribuídas à infecção pelo Vírus Zika, poderiam na verdade seram causadas pela intoxicação por Piriproxifen®. Por isso, alguns estados brasileiros interromperam o uso do produto (DZIECIOLOWSKA et al., 2017; PARENS et al., 2017).

Essas informações geraram grande interesse entre os pesquisadores em descobrir se o uso do larvicida tinha relação com o aparecimento de microcefalia. Albuquerque et al. (2016), em um estudo epidemiológico, e Dzieciolowska et al. (2017), em um modelo com zebrafish, demonstraram que a hipótese não era válida. No entanto, a contradição permanecia na literatura da área.

A partir desses relatos, iniciou-se buscas por novos compostos, já que os mosquitos vetores podem apresentar resistência, bem como o uso contínuo destes larvicidas pode ser tóxico ao meio ambiente e ao ser humano (VANI et al., 2018b).

5. Ensaios de Teratogênese

Sabe-se que fatores genéticos, sejam eles hereditários ou determinados por alguma alteração fisíca, química ou biológica, podem são responsáveis pela ocorrência de malformações congênitas. Logo, alterações no DNA, podem ser um fator que leva ao desenvolvimento destas. As alterações de DNA alteram os processos de mitose, meiose e de apoptose. O funcionamento inadequado interfere na embriogênese, organogênese e fetogênese (MOORE; PERSAUAD, 1995; OLIVEIRA ET AL., 2009; ROSENFELDI, 2015).

Mulheres e/ou fêmeas gestantes, são uma grande preocupação já que são mais susceptíveis a efeitos de xenobióticos que são capazes de atravessar a barreira placentária (ROSENFELDI, 2015). Logo, é evidente a necessidade de estudos de teratogênese

experimental, pois esse é o melhor meio para identificar possíveis agentes teratógenos e alertar as gestantes sobre os riscos que estão expostas (HOLMES, 2011).

Nesse sentido, mulheres grávidas, precisam ser orientadas sobre os atuais produtos larvicidas que são utilizados em caixas d'água e/ou depósitos contendo água utilizados para as atividade antropogênicas. Essa conscientização é importante para que as gestantes estejam alertas aos riscos e assim podem se proteger e ao seu embrião/feto (MOORE; PERSUAD, 1995; HOLMES, 2011; SINCLAIR ET AL., 2016).

6. REFERÊNCIAS

ACOSTA-AMPUDIA, Y.; MONSALVE, D.M.; RODRÍGUEZ, Y.; PACHECO, Y.; ANAYA, J.M.; RAMÍREZ-SANTANA, C. Mayaro: an emerging viral threat? **Emerging Microbes Infectious**. v.26, n.7, p.1:163, 2018. doi: 10.1038/s41426-018-0163-5.

AIUB, C.A.; COELHO, E.C.; SODRÉ, E.; PINTO, L.F.; FELZENSZWALB, I. Genotoxic evaluation of the organophosphorous pesticide temephos. **Genetics and Molecular Research**. v.30, n.1(2), p.159-66, 2002.

ALBUQUERQUE, M.F.P.M.; SOUZA, W.V.; MENDES, A.D.; LYRA, T.M.; XIMENES, R.A.; ARAÚJO, T.V.; BRAGA, C.; MIRANDA-FILHO, D.B.; MARTELLI, C.M.; RODRIGUES, L.C. Pyriproxyfen and the microcephaly epidemic in Brazil - an ecological approach to explore the hypothesis of their association. **Memórias do Instituto Oswaldo Cruz.** Dec, v.111, n. 12, p. 774-776, 2016. doi: 10.1590 / 0074-02760160291.

ALONSO-PALOMARES, L.A.; MORENO-GARCIA, M.; LANZ-MENDOZA, H.; SALAZAR, M.I. Molecular Basis for Arbovirus Transmission by *Aedes aegypti* Mosquitoes. **Intervirologia.** v.61, n.6, p. 255-264, 2018. doi: 10.1159 / 000499128.

BACHA, H.A.; JOHANSON, G.H. Yellow fever. **Revista da Associação Medica Brasileira (1992)**. v.63, n.4, p.291-292, 2017. doi: 10.1590/1806-9282.63.04.291.

BARROS, A.L.; DE SOUZA, V.V.; NAVARRO, S.D.; OESTERREICH, S.A.; OLIVEIRA, R.J.; KASSUYA, C.A.; ARENA, A.C .Genotoxic and Mutagenic Effects of Diflubenzuron, an Insect Growth Regulator, on Mice. **Journal of Toxicology Environmental Health A.** v.76, p.1003-1006, 2013. doi: 10.1080/15287394.2013.830585.

BARROS,A.L.; CAVALHEIRO, G.F.; SOUZA, A.V.M.; TRAESEL,G.K.; FRANCI, J.A.A.; KASSUYA, C.A.L.; ARENA, A.C. Subacute Toxicity Assessment of Difluobenzuron, an Insect Growth Regulator, in Adult Male Rats. **Environmental Toxicology.** v.31, n.4, p. 407-14, 2014. doi: 10.1002/tox.22054.

BELINATO, T.A.; VALLE, D. The Impact of Selection with Diflubenzuron, a Chitin Synthesis Inhibitor, on the Fitness of Two Brazilian *Aedes aegypti* Field Populations. **PLoS One.** v.24, n.10, p.6:e0130719, 2015. doi: 10.1371/journal.pone.0130719.

BITTENCOURT, V.C.E.; MOREIRA, A.M.D.S.; DA SILVA, J.G.; GOMIDES, A.F.F.; VELLOSO-RODRIGUES, C. KELMANN, R.G.; MENDONÇA, L.M.; LULA, I.S.; DENADAI, Â.M.L**.** Hydrophobic nanoprecipitates formed by benzoylphenylureas and β-cyclodextrin inclusion compounds: synthesis, characterization and toxicity against *aedes aegypti* larvae. **Heliyon.** v.5, n.5, p.7:e02013, 2019. doi: 10.1016/j.heliyon.2019.e02013.

BOCK, W.; JAYATHUNGA, Y. Optimal control of a multi-patch Dengue model under the influence of Wolbachia bacterium. **Mathematical Biosciences.** v.21, p.315:108219, 2019. doi: 10.1016/j.mbs.2019.108219.

BONICA, M.B.; GOENAGA, S.; MARTIN, M.L.; FEROCI, M.; LUPPO, V.; MUTTIS, E.; FABBRI, C.; MORALES, M.A.; ENRIA, D.; MICIELI, M.V.; LEVIS, S. Vector competence of *Aedes aegypti* for different strains of Zika virus in Argentina. **PLoS Negligencted Tropical Diseases.** v.12, p.13(6):e0007433, 2019. doi: 10.1371/journal.pntd.0007433.

BRAACK, L.; GOUVEIA DE ALMEIDA , A.P.; CORNEL, A.J.; SWANEPOEL, R.; DE JAGER, C. Mosquito-borne arboviruses of African origin: review of key viruses and vectors. **Parasites & Vectors**. v.9, p.11(1):29, 2018. doi: 10.1186/s13071-017-2559-9.

BRASIL - **Orientações técnicas para utilização do larvicida pyriproxyfen (0,5G) no controle de *Aedes aegypti* (2014).** Disponível em: < http://portalarquivos.saude.gov.br/images/pdf/2014/julho/15/Instruções-para-uso-de-pyriproxifen-maio-2014.pdf> Acesso em 26/04/2017.

CAVALHEIRO, M.G.; COSTA, L.S.; CAMPOS, H.S.; ALVES, L.S.; ASSUNÇÃO-MIRANDA, I.; POIAN, A.T.
Macrophages as target cells for Mayaro virus infection: involvement of reactive oxygen speciesin the inflammatory response during virus replication**. Anais da Academia**

Brasileira de Ciencias. v.88, n.3, p.1485-99, 2016. doi: 10.1590/0001-3765201620150685.

CORTE, R.; MELO, V.A.D.; DOLABELLA, S.S.; MARTEIS, L.S. Variation in temephos resistance in field populations of *Aedes aegypti* (Diptera: Culicidae) in the State of Sergipe, Northeast Brazil. **Revista da Sociedade Brasileira de Medicina Tropical.** v.51, n.3, p.284-290, 2018. doi: 10.1590/0037-8682-0449-2017.

CUNHA, R.V.D.; TRINTA, K.S. Chikungunya virus: clinical aspects and treatment - A Review. **Memórias do Instituto Oswaldo Cruz.** v.112, n.8, p. 523-531, 2017. doi: 10.1590 / 0074-02760170044.

DALUGAMA, C.; SHELTON, J.; EKANAYAKE, M.; GAWARAMMANA, I.B. Dengue fever complicated with Guillain-Barré syndrome: a case report and review of the literature. **Journal of Medicine Case Reports.** v.15, p.12, n. 1:137, 2018. doi: 10.1186/s13256-018-1626-y.

DE ARAÚJO, A.P.; PAIVA, M.H.S.; CABRAL, A.M.; CAVALCANTI, A.E.H.D.; PESSOA, L.F.F.; DINIZ, D.F.A.; HELVECIO, E.; DA SILVA, E.V.G.; DA SILVA, N.M.; ANASTÁCIO, D.B.; PONTES, C.; NUNES, V.; DE SOUZA, M.F.M.; MAGALHÃES, F.J.R.; DE MELO SANTOS, M.A.V.; AYRES, C.F.J. Screening *Aedes aegypti* (Diptera: Culicidae) Populations From Pernambuco, Brazil for Resistance to Temephos, Diflubenzuron, and Cypermethrin and Characterization of Potential Resistance Mechanisms. **Journal of Insect Science.** v.1, n.19, p.3 pii: 16, 2019. doi: 10.1093/jisesa/iez054.

DE MAJO, MS; ZANOTTI, G.; CAMPOS, RE; FISCHER, S. Efeitos de baixas temperaturas constantes e flutuantes no desenvolvimento de *Aedes aegypti* (Diptera: Culicidae) de uma região temperada**. Journal of Medical Entomology.** [Epub ahead of print], 2019. doi: 10.1093 / jme / tjz087.

DOUAM, F.; PLOSS, A. Yellow Fever Virus: Knowledge Gaps Impeding the Fight Against an Old Foe. **Trends in Microbiology.** v.26, n.11, p.913-928, 2018. doi: 10.1016/j.tim.2018.05.012.

DOS SANTOS DIAS, L.; MACORIS, M.L.; ANDRIGHETTI, M.T.; OTRERA, V.C.; DIAS, A.D.; BAUZER, L.G.; RODOVALHO, C.M.; MARTINS, A.J.; LIMA, J.B. Toxicity of spinosad to temephos-resistant *Aedes aegypti* populations in Brazil. **PLoS One.** v.16, n.12, p.3:e0173689, 2017. doi: 10.1371/journal.pone.0173689.

DYER, O. Yellow fever stalks Brazil in Zika's wake. **BMJ.** v. 8, p.356:j707, 2017. doi: 10.1136/bmj.j707.

DZIECIOLOWSKA, S.; LARROQUE, A.L. KRANJEC, E.A.; DRAPEAU, P.; SAMARUT, E. The larvicide pyriproxyfen blamed during the Zika virus outbreak does not cause microcephaly in zebrafish embryos**. Scientific Reports.** v.7, p. 40067, 2017. doi: 10.1038/srep40067.

EPELBOIN, Y.; TALAGA, S.; EPELBOIN, L.; DUSFOUR, I. Zika virus: An updated review of competent or naturally infected mosquitoes. **PLoS Negligencited Tropical Diseases.** v.16, n.11, p.11:e0005933, 2017. doi: 10.1371/journal.pntd.0005933.

ESPOSITO, D.L.A.; FONSECA, B.A.L.D. Will Mayaro virus be responsible for the next outbreak of an arthropod-borne virus in Brazil? **Brazilian Journal Infectious Diseases.** v.21, n.5, p.540-544, 2017. doi: 10.1016/j.bjid.2017.06.002.

GUBLER, D.J. Dengue and dengue hemorrhagic fever. **Clinical Microbiology Reviews.** v.11, n.3, p.480-96, 1998.

HOLMES, L.B. Human teratogens: update 2010. **Birth Defects Research Part A. Clinical and Molecular Teratology.** v. 91, n. 1, p. 1-7, 2011. doi: 10.1002/bdra.20748.

HOTEZ, P.J.; MURRAY, K.O. Dengue, West Nile virus, chikungunya, Zika-and now Mayaro? **PLoS Negligencted Tropical Diseases.** v.31, n.11, p.8:e0005462, 2017. doi: 10.1371/journal.pntd.0005462.

HOYOS-LÓPEZ, R.; ATENCIA-PINEDA, M.C.; GALLEGO-GÓMEZ, J.C. Phylogenetic analysis of Dengue-2 serotypes circulating in mangroves in Northern

Cordoba, Colombia. **Revista da Sociedade Brasileira de Medicina Tropical.** v.6, p. 52:e20190060, 2019. doi: 10.1590/0037-8682-0060-2019.

KAURA, T.; MEWARA, A.; ZAMAN, K.; SHARMA, A.; AGRAWAL, S.K.; THAKUR, V.; GARG, A.; SEHGAL, R.
Utilizing larvicidal and pupicidal efficacy of *Eucalyptus* and neem oil against *Aedes* mosquito: An approach for mosquito control. **Tropical Parasitology.** v. 9, n.1, p.12-17, 2019. doi: 10.4103/tp.TP_35_18.

KATZELNICK, L.C.; COLOMA, J.; HARRIS, E.
Dengue: knowledge gaps, unmet needs, and research priorities. **The Lancet Infectious Diseases.** v.17, n.3, p.:e88-e100, 2018. doi: 10.1016/S1473-3099(16)30473-X.

KONUŞKAN, B.; OKUYAZ, Ç; TAŞDELEN, B.; KURUL, S.H.; ANLAR, B.; Turkish Childhood Guillan-Barre Syndrome Study Group. Electrophysiological Subtypes and Prognostic Factors of Childhood Guillain-Barré Syndrome.**NoroPsikiyatr Arsivi.** v. 5, n. 55, p. 3:199-204, 2018. doi: 10.5152/npa.2017.16996.

KUDLACEK, S.T.; METZ, S.W. Focused dengue vaccine development: outwitting nature's design. **Pathogens and Diseases.** v.1, p.77(1), 2019. doi: 10.1093/femspd/ftz003.

LASERNA, A.; BARAHONA-CORREA, J.; BAQUERO, L.; CASTAÑEDA-CARDONA, C.; ROSSELLI, D. Economic impact of dengue fever in Latin America and the Caribbean: a systematic review**. Revista Panamericana de Salud Publica.** v.7, p.42:e111, 2018. doi: 10.26633/RPSP.2018.111.

LIN, R.J.; LEE, T.H.; LEO, Y.S. Dengue in the elderly: a review. **Expert Review Anti Infective Therapy.** v.15, n.8, p.729-735, 2017. doi: 10.1080/14787210.2017.1358610.

LITVOC, M.N.; NOVAES, C.T.G.; LOPES, M.I.B.F. Yellow fever. **Revista da Associação Medica Brasileira (1992).** v.64, n.2, p.106-113, 2018. doi: 10.1590/1806-9282.64.02.106.

LWANDE, O.W.; OBANDA, V.; GÖRAN BUCHT, G.; MOSOMTAI, G.; VIOLA OTIENO, V.; CLAS AHLM, C. EVANDER, M.· Global emergence of *Alphaviruses* that

cause arthritis in humans. **Infection Ecology & Epidemiology.** v. 5, p. 10.3402, 2015. doi: 10.3402/iee.v5.29853.

MADARIAGA, M., TICONA, E., RESURRECION, C. Chikungunya: bending over the Americas and the rest of the world. **The Brazilian Journal Infectious Diseases**. v.20, n.1, p.91-8, 2015. doi: 10.1016/j.bjid.2015.10.004.

MAHARAJAN, K.; MUTHULAKSHMI, S.; NATARAJ, B.; RAMESH, M.; KADIRVELU, K. Toxicity assessment of pyriproxyfen in vertebrate model zebrafish embryos (Danio rerio): A multi biomarker study. **Aquatic Toxicology.** v.196, p.132-145, 2018. doi: 10.1016/j.aquatox.2018.01.010.

MAHMUD, M.A.F.; ABDUL MUTALIP, M.H.; LODZ, N.A.; MUHAMMAD, E.N.; YOEP, N.; HASHIM, M.H.; PAIWAI, F.; RAJARETHINAM, J.; AIK, J.; MUHAMMAD, N.A. Environmental management for dengue control: a systematic review protocol. **BMJ Open.** v.15, n.9, p.5:e026101, 2019. doi: 10.1136/bmjopen-2018-026101.

MARANO, G., PUPELLA, S., VAGLIO, S., LIUMBRUNO, G.M., GRAZZINI, G. Zika virus and the never-ending story of emerging pathogens and transfusion medicine**.** **Blood Transfusion.** v.14, n.2, p.95-100, 2016. doi: 10.2450/2015.0066-15.

MARCOMBE, S.; CHONEPHETSARATH, S.; THAMMAVONG, P.; BREY, P.T. Alternative insecticides for larval control of the dengue vector *Aedes aegypti* in Lao PDR: insecticide resistance and semi-field trial study. **Parasits &Vectors.** v.3, n.11, p.1:616, 2018. doi: 10.1186/s13071-018-3187-8.

MARCONDES, C.B., XIMENES, M.F. Zika virus in Brazil and the danger of infestation by *Aedes* (Stegomyia) mosquitoes. **Revista da Sociedade Brasileira de Medicima Tropical.** v.49, n.1, p.4-10, 2016. doi: 10.1590/0037-8682-0220-2015.

MAVIAN, C.; RIFE, B.D.; DOLLAR, J.J.; CELLA, E.; CICCOZZI, M.; PROSPERI, M.C.F.; LEDNICKY, J.; MORRIS, J.G.; CAPUA, I.; SALEMI, M. Emergence of recombinant Mayaro virus strains from the Amazon basin. **Scientific Reports.** v.18, n.7, p.1:8718, 2017. doi: 10.1038/s41598-017-07152-5.

MONTEIRO, V.V.S.; NAVEGANTES-LIMA, K.C.; DE LEMOS, A.B.; DA SILVA, G.L.; DE SOUZA GOMES, R.; REIS, J.F.; RODRIGUES JUNIOR, L.C.; DA SILVA, O.S.; ROMÃO, P.R.T.; MONTEIRO, M.C. *Aedes*-Chikungunya Virus Interaction: Key Role of Vector Midguts Microbiota and Its Saliva in the Host Infection. **Frontiers in Microbiology.** v.9, p.10:492, 2019. doi: 10.3389/fmicb.2019.00492.

MOORE, B.A.; PERSUAD, T.V.N. **Embriologia Básica**. 4ª ed. Rio de Janeiro: Guanabara Koogan, 1995.

MOURÃO, M.P.; BASTOS, M.D.E.S; DE FIGUEIREDO, R.P.; GIMAQUE, J.B.; GALUSSO EDOS, S.; KRAMER, V.M.; DE OLIVEIRA, C.M.; NAVECA, F.G.; FIGUEIREDO, L.T. Mayaro fever in the city of Manaus, Brazil, 2007-2008. **Vector Borne Zoonotic Diseases.** v.12, n.1, p.42-6, 2012. doi: 10.1089/vbz.2011.0669.

MUÑOZ, M.; NAVARRO, J.C. Mayaro: a re-emerging Arbovirus in Venezuela and Latin America. **Biomedica.** v.32, n.2 p.:286-302, 2012. doi: 10.1590/S0120-41572012000300017.

MUSSO, D.; GUBLER, D.J. Vírus Zika. **Clinical Microbiology Reviews.** v. 29, n.3, p. 487-524, 2016. doi: 10.1128 / CMR.00072-15.

NUNES, P.C.G.; DAUMAS, R.P.; SÁNCHEZ-ARCILA, J.C.; NOGUEIRA, R.M.R.; HORTA, M.A.P.; DOS SANTOS, F.B. 30 years of fatal dengue cases in Brazil: a review. **BMC Public Health.** v.21, n.19, p.1:329, 2019. doi: 10.1186/s12889-019-6641-4.

OHBA, S.Y.; OHASHI, K.; PUJIYATI, E.; HIGA, Y.; KAWADA, H.; MITO, N.; TAKAGI, M. The effect of pyriproxyfen as a "population growth regulator" against *Aedes albopictus* under semi-field conditions**. PLoS One.** v.2, n.8, p.7:e67045, 2013. doi: 10.1371/journal.pone.0067045.

OLIVEIRA, R. J.; KANNO, T. Y. N ; SALLES, M. J. S ; LOURENÃ O, A. C. S. ;RIBEIRO, L. R. ; FREIRIA, G. A. ; MATIAZI, .H.JO ; MANTOVANI,M.S.; SILVA,A.F. Effects of the polysaccharide ß-glucan on clastogenicity and teratogenicity caused by acute exposure to cyclophosphamide in mice. **Regulatory Toxicology and Pharmacology.** v. 53, p.3, n. 164-73, 2009.

OLIVEIRA, L.N.D.S.; ITRIA, A.; LIMA, E.C. Cost of illness and program of dengue: A systematic review. **PLoS One.** v.20, n.14, p.2:e0211401, 2019. doi: 10.1371/journal.pone.0211401. eCollection 2019.

OO, S.Z.M.; THAUNG, S.; MAUNG, Y.N.M.; AYE, K.M.; AUNG, Z.Z.; THU, H.M.; THANT, K.Z.; MINAKAWA, N. Effectiveness of a novel long-lasting pyriproxyfen larvicide (SumiLarv®2MR) against Aedes mosquitoes in schools in Yangon, Myanmar. **Parasites & Vectors.** v.6, n.11, p.1:16, 2018. doi: 10.1186/s13071-017-2603-9.

PARENS, R.; NIJHOUT, H.F.; MORALES, A.; XAVIER COSTA, F.; BAR-YAM, Y. A Possible Link Between Pyriproxyfen and Microcephaly. **PLoS Currents Outbreaks.** v.27, p.9, 2017. doi: 10.1371/currents.outbreaks.5afb0bfb8cf31d9a4baba7b19b4edbac.

PLOURDE, A.R.; BLOCH, E.M. A Literature Review of Zika Virus. **Emerging Infectious Diseases.** v.22, n.7, p.1185-92, 2016. doi: 10.3201/eid2207.151990.

RICO-MENDOZA, A.; ALEXANDRA, P.R.; CHANG, A.; ENCINALES, L.; LYNCH, R. Co-circulation of dengue, chikungunya, and Zika viruses in Colombia from 2008 to 2018. **Revista Panamericana Salud Publica.** v. 7, p.43:e49, 2019. doi: 10.26633/RPSP.2019.49.

ROSENFELD, C.S. Sex-Specific Placental Responses in Fetal Development. **Endocrinology.** v.156, n.10, p.3422-34, 2015. doi: 10.1210/en.2015-1227.

SALLES, T.S.; DA ENCARNAÇÃO SÁ-GUIMARÃES, T.; DE ALVARENGA, E.S.L.; GUIMARÃES-RIBEIRO, V.; DE MENESES, M.D.F.; DE CASTRO-SALLES, P.F.; DOS SANTOS, C.R.; DO AMARAL MELO, A.C.; SOARES, M.R.; FERREIRA, D.F.; MOREIRA, M.F. History, epidemiology and diagnostics of dengue in the American and Brazilian contexts: a review. **Parasites & Vectors.** v.24, n.11, p.1:264, 2018. doi: 10.1186/s13071-018-2830-8.

SANTOS, V.S.; CAIXETA, E.S.; OLEGÁRIO, D.E.; CAMPOS JÚNIOR, E.; PEREIRA, B.B. Ecotoxicological effects of larvicide used in the control of *Aedes aegypti* on nontarget organisms: Redefining the use of pyriproxyfen. **Journal of**

Toxicology Environmental Health A. v. 80, n.3, p.155-160, 2017. doi: 10.1080/15287394.2016.1266721.

SAPONE, A.; POZZETTI, L.; CANISTRO, D.; BROCCOLI, M.; BRONZETTI, G.; POTENZA, G.; AFFATATO, A.; BIAGI, G.L.; CANTELLI-FORTI, G.; PAOLINI, M. CYP superfamily perturbation by diflubenzuron or acephate in different tissues of CD1 mice. **Food Chemical Toxicology.** v.43, n.1, p.173-83, 2005.

SINCLAIR, S.M.; MILLER R.K.; CHAMBERS, C.; COOPER, E.M. Medication Safety During Pregnancy: Improving Evidence-Based Practice. **Journal of Midwifery & Womens Health.** v. 61, n. 1, p.52-67, 2016. doi: 10.1111/jmwh.12358.

SILVA, L.A.; DERMODY, T.S. Chikungunya virus: epidemiology, replication, disease mechanisms, and prospective intervention strategies. **The Journal of Clinical Investigation.** v.1, n.127 (3), p.737-749, 2017. doi: 10.1172/JCI84417**.**

SIQUEIRA, S. C. F ; PIGNATTI, M. G. ; ATANAKA, M. ; ESPINOSA, M. M. ; SANTOS, R. S. . Vulnerabilidade espacial da dengue no Estado de Mato Grosso no período de 2007-2009. **Questões ambientais em Saúde Coletiva.** p. 73-94, 2012.

TAPPE, D.; PÉREZ-GIRÓN, J.V.; ZAMMARCHI, L.; RISSLAND, J.; FERREIRA, D.F.; JAENISCH, T.; GÓMEZ-MEDINA, S.; GÜNTHER, S.; BARTOLONI, A.; MUÑOZ-FONTELA, C.; SCHMIDT-CHANASIT, J. Cytokine kinetics of Zika virus-infected patients from acute to reconvalescent phase. **Medical Microbiology Immunology.** v.205, n.3, p.269-73, 2016. doi: 10.1007/s00430-015-0445-7.

TAPIA-LÓPEZ, E.; BARDACH, A.; CIAPPONI, A.; ALCARAZ, A.; GARCÍA-PERDOMO, H.A.; RUVINSKY, S.; BELIZÁN, M. Experiences, barriers and facilitators to the implementation of interventions for controlling *Aedes aegypti* in Latin America and the Caribbean: a qualitative study. **Cadernos de Saúde Pública.** v. 20, n.35, p.5:e00092618, 2019. doi: 10.1590/0102-311X00092618.

VAN AALST, M.; NELEN, C.M.; GOORHUIS, A.; STIJNIS, C.; GROBUSCH, M.P. Long-term sequelae of chikungunya virus disease: A systematic review. **Travel**

Medicine and Infectious Diseases. v.15, p.8-22,2017. doi: 10.1016/j.tmaid.2017.01.004.

VANI, J.M.; DE CARVALHO SCHWEICH, L.; DE OLIVEIRA, K.R.W.; AUHAREK, S.A.; CUNHA-LAURA, A.L. ANTONIOLLI-SILVA, A.C.M.B.; NAZARIO, C.E.D. OLIVEIRA, R.J. Evaluation of the effects of the larvicides temephos on reproductive performance, embryofetal development and DNA integrity of Swiss mice. **Pesticide Biochemistry and Physiology.** v.148, p.22-27, 2018a. doi: 10.1016/j.pestbp.2018.03.010.

VANI, J.M.; MONREAL, M.T.F.D.; AUHAREK, S.A.; CUNHA-LAURA, A.L.; DE ARRUDA, E.J.; LIMA, A.R.; DA SILVA, C.M.; ANTONIOLLI-SILVA, A.C.M.B.; DE LIMA, D.P.; BEATRIZ, A.; OLIVEIRA, R.J. The mixture of cashew nut shell liquid and castor oil results in an efficient larvicide against *Aedes aegypti* that does not alter embryo-fetal development, reproductive performance or DNA integrity. **PLoS One.** v.19, n.13, p.3:e0193509, 2018b. doi: 10.1371/journal.pone.0193509.

VASCONCELOS, P.F. Yellow Fever. **Revista da Sociedade Brasileira de Medicina Tropical.** v.36, n.2, p.275-93, 2003. Epub 2003 Jun 10.

VIEIRA SANTOS, V.S.; CAIXETA, E.S.; CAMPOS JÚNIOR, E.O.; PEREIRA, B.B. Ecotoxicological effects of larvicide used in the control of *Aedes aegypti* on nontarget organisms: Redefining the use of pyriproxyfen. **Journal of Toxicology Environmental Health A.** v.80, n.3, p.155-160, 2017. doi: 10.1080/15287394.2016.1266721.

WHO - **Recommended Classification of Pesticides by Hazard. Guidelines to classification 2000-2002.** Disponível em: <http://www.who.int/ipcs/publications/en/pesticides_hazard.pdf> Acesso em 26/04/2017.

7. Objetivos

7.1 Objetivo Geral

Avaliar o desempenho reprodutivo, desenvolvimento embriofetal e integridade do DNA de camundongos *Swiss* prenhes expostas aos larvicidas comerciais Temefós, Diflubenzuron e Piriproxifen que são utilizados no combate ao *Aedes aegypti*.

7.2 Objetivos específicos

Avaliar o efeito dos larvicidas Temefós, Diflubenzuron e Piriproxifen nos parâmetros biométricos de camundongos *Swiss* fêmeas prenhes;

Avaliar o efeito dos larvicidas Temefós, Diflubenzuron e Piriproxifen nos parâmetros reprodutivos e no desenvolvimento embriofetal por meio dos ensaios de desempenho reprodutivo e teratogênese em camundongos *Swiss* fêmeas prenhes;

Avaliar o efeito genotóxico dos larvicidas Temefós, Diflubenzuron e Piriproxifen por meio do ensaio de micronúcleo em sangue periférico;

Artigo Nº 1: Larvicida comercial Temefós publicado na revista: Pesticide Biochemistry and Physiology. Qualis Capes Interdisciplinar A2. Anexo I.

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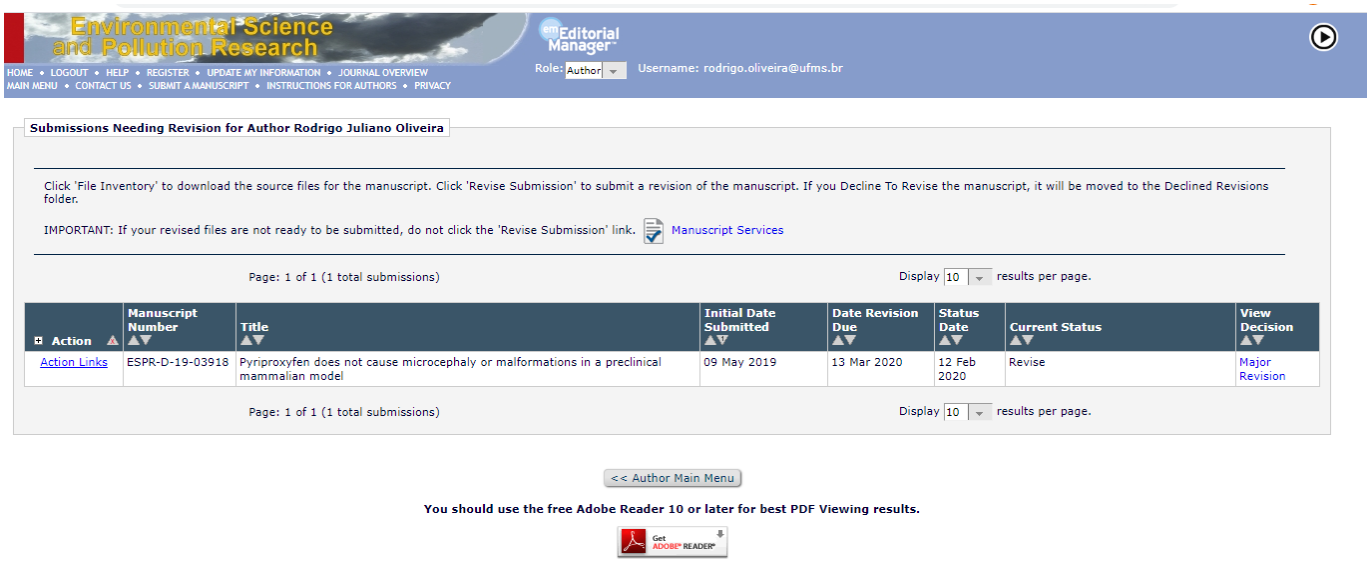
Evaluation of the effects of the larvicides temephos on reproductive performance, embryofetal development and DNA integrity of Swiss mice

Juliana Miron Vani[a,b], Laynna de Carvalho Schweich[a,b], Karla Regina Warszawski de Oliveira[c,d], Sarah Alves Auharek[e], Andréa Luiza Cunha-Laura[f], Andréia Conceição Millan Brochado Antoniolli-Silva[a,b], Carlos Eduardo Domingues Nazario[c,d], Rodrigo Juliano Oliveira[a,b,g,*]

[a] Centro de Estudos em Células Tronco, Terapia Celular e Genética Toxicológica - CeTroGen, Hospital Universitário Maria Aparecida Pedrossian – HUMAP, Universidade Federal de Mato Grosso do Sul - UFMS, Campo Grande, MS, Brazil
[b] Programa de Pós-graduação em Saúde e Desenvolvimento na Região Centro-Oeste, Faculdade de Medicina "Dr. Hélio Mandetta" – FAMED, Universidade Federal de Mato Grosso do Sul – UFMS, Campo Grande, MS, Brazil
[c] Programa de Pós-graduação em Química - INQUI, Universidade Federal de Mato Grosso do Sul- UFMS, Campo Grande, MS, Brazil
[d] Laboratório de Combustíveis - LABCOM, Instituto de Química - INQUI, Universidade Federal de Mato Grosso do Sul- UFMS, Campo Grande, MS, Brazil
[e] Faculdade de Medicina do Mucuri, Universidade Federal dos Vales do Jequitinhonha e do Mucuri – UFVJM, Teófilo Otoni, MG, Brazil
[f] Programa de Mestrado em Farmácia, Instituto de Biociências - INBIO, Universidade Federal de Mato Grosso do Sul – UFMS, Campo Grande, MS, Brazil
[g] Programa de Pós-graduação em Genética e Biologia Molecular, Centro de Ciências Biológicas – CCB, Universidade Estadual de Londrina, Londrina, PR, Brazil

Manuscrito 1: Piriproxifen. Submetido a revista: Environmental Science and Pollution Research. Status: Aguardando revisão. Qualis Capes Interdisciplinar: A2.



Pyriproxyfen does not cause microcephaly or malformations in a preclinical mammalian model

Rodrigo Juliano Oliveira[a,b,c,d*], Juliana Miron Vani[a,b], Sarah Alves Auharek[e] Laynna de Carvalho Schweich[a,b], Andréia Conceição Millan Brochado Antoniolli-Silva[a,b],

[a]Centro de Estudos em Células Tronco, Terapia Celular e Genética Toxicológica - CeTroGen, Hospital Universitário Maria Aparecida Pedrossian – HUMAP, Universidade Federal de Mato Grosso do Sul – UFMS, Campo Grande, MS, Brasil.

[b]Programa de Pós-graduação em Saúde e Desenvolvimento na Região Centro-Oeste, Faculdade de Medicina Dr. Hélio Mandetta – FAMED, Universidade Federal de Mato Grosso do Sul – UFMS, Campo Grande, MS, Brasil.

[c]Programa de Pós-graduação em Genética e Biologia Molecular, Centro de Ciências Biológicas – CCB, Universidade Estadual de Londrina, Londrina, PR, Brasil.

[d]Programa de Pós-graduação em Ciências Farmacêuticas, Faculdade de Ciências Farmacêuticas, Alimento e Nutrição – FACFAN, Universidade Federal de Mato Grosso do Sul – UFMS, Campo Grande, MS, Brasil.

[e]Faculdade de Medicina do Mucuri, Universidade Federal dos Vales do Jequitinhonha e do Mucuri – UFVJM, Teófilo Otoni, MG, Brasil.

Corresponding author:
Rodrigo Juliano Oliveira
Medicine College, Federal University of Mato Grosso do Sul. Cidade Universitária, S/N
Campo Grande, MS, 79070-900, Brasil.
Telephone: +55 (67) 3345-3068
Fax: +55 (67) 3345-7190
E-mail: rodrigo.oliveira@ufms.br

Abstract

Pyriproxyfen is used in Brazil to combat epidemics of Dengue Fever, Chikungunya Fever and Zika virus. This study assessed the effects of pyriproxyfen on reproductive performance, embryo-fetal development, head measurements and DNA integrity in a preclinical model. Thirty pregnant mice were divided into three groups (n=10): control (drinking water-0.1 ml/10 g (body weight-b.w., gavage) and treated with pyriproxyfen 0.0002 mg/kg and 0.0021 mg/kg (b.w., gavage) during the gestational period. Analysis of biometric, reproductive performance and embryo-fetal development parameters related to control presented no significant differences, suggesting no maternal or embryo-fetal toxicity. Head measurements showed no differences except an increase in anterior/posterior measurement and glabella/external occipital protuberance. Analysis of DNA integrity showed an increase in micronucleus only at 72 h for the lowest dose group. Thus, we infer that pyriproxyfen is not related to the occurrence of microcephaly, nor does it alter reproductive performance, embryo-fetal development or DNA integrity.

Keywords: Larvicide; reproductive performance; embryo-fetal development; teratogenesis; genotoxicity.

1. Introduction

Since 2014, the larvicide pyriproxyfen has been used to combat epidemics of Dengue Fever, Chikungunya Fever and Zika Virus in Brazil by indication of the Ministry of Health (WHO, 2002; Brasil, 2014). This compound has a pyridine base, which is classified as a juvenile hormone analog that regulates a wide range of processes in mosquito development, including for *Aedes aegypti* (Albuquerque et al., 2016; Dzieciolowska et al., 2017).

In Brazil the increase in microcephaly cases was concomitant with the Zika Virus epidemic and the use of pyriproxyfen. Because of these simultaneous occurrences, a group of Argentine researchers raised the hypothesis that pyriproxyfen was correlated with the development of microcephaly (REDUAS, 2016). This finding corroborates the study by Parens et al. (2017) that reports the correlation between the use of piriprofixen and the development of microcephaly and/or neurological changes. However, studies from Saegusa (1988) and Sumitomo (s.d.), initially indicated the absence of a correlation between the use of pyriproxifen and the occurrence of microcephaly in a preclinical model. Then, our investigation aimed to address these questions.

Dzieciolowska et al. (2017) and Albuquerque et al. (2016) demonstrated the absence of a correlation between a point of exposure and pyriproxyfen in the development of microcephaly in a zebrafish model and an epidemiological study. Thus, these studies corroborate the initial hypothesis of Saegusa (1988) and Sumitomo (s.d.).

Considering this, there is no consensus in the literature on the correlation between the use of pyriproxifen and the development of microcephaly. The recent literature has no information about an experimental mammalian model yet, in addition to the data produced by the larvicide manufacturer itself (Parens et al., 2017). These facts reinforce

the need for new independent studies that can contribute to this discussion. This observation highlights the uniqueness of the present study.

Thus, the present study assessed the effects of pyriproxifen on reproductive performance, embryo-fetal development, including head measurements, and DNA integrity in a preclinical model with *Swiss* pregnant mice.

2. Materials and methods

2.1. Chemical agents

Sumilarv® 0.5 G (pyriproxyfen) (Sumitomo Chemical do Brasil Representações Ltda, Lot: 6574F4), an insecticide product indicated for the control of *A. aegypti* larvae, is composed of 4-phenoxyphenyl (RS)-2-(2pyridyloxil) propyl ether pyriproxyfen 0.5 w/w, surfactant, diluent excipient. The product was diluted in distilled water.

According to Word Health Organization (WHO, 2014), the recommended dose of pyriproxyfen is 0.5 ppm. The Ministry of Health also recommends the use of this concentration (Brasil, 2014).

To calculate the lower dose, we used the commercial dose (0.0002 mg/mL of pyriproxyfen when ingested by the mouse via gavage, corresponding to the amount of pyriproxyfen that would be present in 3 L of water containing pyriproxyfen at 0.5 ppm) that corresponds to when pyriproxyfen is deposited in a water tank and 3 L of water are ingested daily by a 70 kg person; this dose was determined (Vitolo, 2008) using the following formulation (pyriproxyfen in 0.5 ppm x 30 g)/70.000 g). The dose was subsequently adjusted to the mice's weight.

The 0.0002 mg / kg (b.w., a.o.) dose is based on the larvicide dose for commercial use, and the higher dose of 0.0021 mg / kg (b.w., a.o.) is 10x higher than the recommended dose (OECD, 2001; OECD, 2009).

2.2. Experimental animals

Swiss mice (*Mus musculus*) (30 females and 15 males) of reproductive age (8-10 weeks) with an average weight of 30 g were obtained from the State Bureau of Animal and Plant Health Protection (Agência Estadual de Defesa Sanitária Animal e Vegetal - IAGRO). All procedures and protocols followed approved guidelines for the ethical treatment of animals according to the Ethics Committee for Animal Experimentation of the Federal University of Mato Grosso do Sul (No. 808/2016).

The animals were maintained in propylene boxes, with the males in isolation and the females in pairs. They went through an adaptation period of seven days. Light and temperature were controlled, with a photoperiod of 12 hours (12 hours of light: 12 hours of dark), and temperature maintained at 22 ± 2ºC, in an ALESCO® ventilated cabinet. The mice were provided with commercial feed (Nuvital®) and filtered water *ad libitum*.

Overnight mating was performed at a ratio of 1 male : 2 females, and detection of pregnancy was performed based on the observation of vaginal plug formation (considered day zero of gestation) (Oliveira et al., 2009; Gonçalves et al., 2013; David et al., 2014; Gonçalves et al., 2014; Oliveira et at., 2015; Vani et al., 2018a; Vani et al., 2018b).

2.3.Experimental design

The pregnant females were divided into three experimental groups (n=10). Control group: animals received drinking water at the proportion of 0.1 mL/10 g body weight (b.w.) administered by gavage during all days of gestation (1st to 18th). In the other groups, animals received pyriproxyfen by gavage at a dose of either 0.0002 mg/kg (Pyri.

D1; commercial dose) or 0.0021 mg/kg (Pyri. D2; security dose – 10x commercial dose) (OECD, 2009) daily throughout gestation.

3. Biological assays

3.1 Reproductive performance and embryonic and fetal development (teratogenicity)

On the 18th day of gestation, the animals were euthanized, followed by laparotomy. The spleen, heart, liver, lungs and kidneys were collected and weighed. The fetuses and placentas were also weighed. An external systematic analysis of the fetuses was performed to detect possible external malformations. Subsequently, the sex of each fetus was identified. The number of implantation was recorded; when there was doubt, the Salewski (1964) technique was used. To check the implantation sites, the dissected uteri were immersed in an ammonium sulfide solution that allows visualizing the hemorrhagic areas that correspond to the implantation sites (Salewski, 1964; Harris et al., 2020). The number of resorptions and live and dead fetuses were recorded. Based on these data, fetal viability (number of live fetuses/number of implantations × 100), the postimplantation loss rate (number of implantations – number of live fetuses × 100/number of implantations), the resorption rate (number of resorptions × 100/number of implantations), the placental index (placental weight/fetal weight) and the sex ratio (number of male fetuses/number of female fetuses) were obtained (Oliveira et al., 2009; Gonçalves et al., 2013; David et al., 2014; Gonçalves et al., 2014; Oliveira et at., 2015; Vani et al., 2018a; Vani et al., 2018b). Then, the suitability of the observed fetal weight for the gestational age was determined according to Oliveira et al. (2009), with the fetuses being classified as follows: fetuses with an appropriate weight for their gestational age (AWGA), showing a body weight within the mean weight of the control group fetuses

plus or minus the standard deviation; fetuses with a low weight for their gestational age (LWGA), showing a body weight lower than the mean weight of the control group fetuses minus the standard deviation of the same group; or fetuses overweight for their gestational age (OVGA), showing a body weight higher than the mean weight of control group fetuses plus the standard deviation of the same group.

Subsequently, the fetuses were randomly divided into two subgroups. The first group was for visceral analysis, which the fetuses were fixed in Bodian's solution (distilled water (142 mL), acetic acid (50 mL), formaldehyde (50 mL) and 95% ethanol (758 mL)) for at least seven days. Visceral analysis was performed via microdissection with strategic cuts to examine the chest and abdomen, according to Barrow and Taylor (1969), and to examine the head, according to Wilson (1965), as modified by Oliveira et al. (2009). Visceral changes were described based on the studies by Taylor (1986), Manson and Kang (1994), Damasceno et al. (2008) and Oliveira et al. (2009). The second subgroup of fetuses was for skeletal analysis using the alizarin red technique proposed by Staples and Schnell (1964), as modified by Oliveira et al. (2009). The fetuses were fixed in acetone for at least seven days. For the diaphanization process, the fetuses were eviscerated and placed in a solution of KOH (0.8%). Then, four drops of alizarin were added. This solution was replaced every 24 hours over four days. After this period, the KOH solution was discarded, and the fetuses were placed in a bleaching solution (1 L glycerin: 1 L of ethyl alcohol: 0.5 L of benzyl alcohol), which was replaced every 24 hours for seven days. Skeletal changes were classified according to Taylor (1986), Manson et al. (1982), Damasceno et al. (2008) and Oliveira et al. (2009).

All analyses were performed under a stereomicroscope (NIKON SMZ745T).

3.2. Analysis of head measurement parameters

For head measurement parameters, the child neurology protocol (Montenegro; Guerreiro, s.d.) was used as a reference. The first measurement was anterior/posterior (glabella and external occipital protuberance passing over the sagittal suture and bregma-measure 1), the second measurement was made in the glabella and external occipital protuberance (measure 2), and the third measurement was binaural (reference point to the superior insertion of the ears, with the tape measure over the coronal suture and passing through the bregma-measure 3), all carried out with aid of a digital caliper (ZAAS Precision®).

Posteriorly, the fourth measurement was made using a tape measure, so the head circumference (measure 4) was obtained, and afterward, the area of the head circumference (measure 5) was calculated by the formula $A = \pi r^2$.

3.3. Micronucleus assay

The technique used for the micronucleus assay was based on Hayashi et al. (1990), as modified by Oliveira et al. (2009). A total of 20 μL of peripheral blood was collected via tail vein puncture, deposited on a slide that was previously stained with acridine orange (1 mg/mL) and then covered with a coverslip. Samples were collected on the 16th, 17th and 18th gestational day (i.e., at the end of the experiment) to assess whether pyriproxyfen had the ability to cause cumulative damage. The slides were stored in a freezer at -20ºC for at least 15 days. A total of 2,000 cells/animal were analyzed under an epifluorescence microscope (Motic®; Model BA 410) at a magnification of 400×.

3.4. Statistical analysis

The data are presented as the mean ± standard error of the mean (SEM) and were evaluated according to the nature of their distribution (parametric: ANOVA/Tukey test;

nonparametric: Kruskal-Wallis/Dunn test). For comparisons of the frequencies (percent) between the control and experimental groups, the chi-square test was used. For qualitative data and frequencies, the litter was utilized as the unit basis, as recommended in the literature (Hanseman; Hogan, 1995). However, for quantification of the visceral and skeletal malformations, the fetus was used as the basic unit according to Moreira et al. (2005) and Oliveira et al. (2009). The level of significance was set at $p<0.05$.

4. Results

4.1. Evaluation of biometric parameters and reproductive performance

4.1.1. Evaluation of biometric parameters

The initial weight, final weight, uterine weight and net weight gain showed no significant differences between groups ($p>0.05$). The weight gain decreased in the Pyri.D1 group ($p<0.05$) in relation to the control group.

In relation to the absolute weight of the organs, we observed a decrease in spleen and kidney weight ($p<0.05$) for the groups Pyri.D2 and Pyri.D1, respectively. Heart, lung and liver weight showed no significant differences between groups ($p>0.05$). The relative weight of the organs showed no significant differences between the groups ($p>0.05$) (Table 1).

4.1.2. Evaluation of reproductive performance

For the parameters of reproductive performance, number of implants, number of live fetuses, fetal viability, postimplantation loss rate, number of resorptions, resorption rate and sexual ratio, there were no significant differences present between

groups ($p>0.05$). However, it was also observed that a decrease in placental weight in Pyri.D2 and in placental index in Pyri.D1 and Pyri.D2 ($p<0.05$) (Table 2).

4.2. Evaluation of embryo-fetal development

4.2.1. Adequacy of weight to gestational age

The fetuses of the Pyri.D1 group showed an increase in weight in relation to the control group and therefore were considered overweight for their gestational age. The Pyri.D2 group presented fetuses with appropriate weight for their gestational age (Table 2).

4.2.2. Analysis of head measurement parameters

In the head measurement parameters, increases in the anterior/posterior measurements and glabella and external occipital protrusion ($p<0.05$) were observed in the Pyri.D1 group. In the measurements of biauricular and occipitofrontal circumference and circumferential area, no significant differences were observed between the groups ($p>0.05$) (Table 3).

4.2.3. External malformations, visceral and skeletal

External malformations (unilateral posterior retroversion, tail coma, hydrops and gastroschisis) were found to be similar in all groups ($p>0.05$) (Table 4).

We found visceral malformations in brain (hydrocephalus) and urogenital tract (hydronephrosis). The urogenital malformations showed no significant differences between groups ($p>0.05$), and the malformations found in the brain were decreased in Pyri.D1 ($p<0.05$) (Table 5).

The skeletal malformations (agenesis, ossification irregular or reduced) occurred similarly in all groups ($p>0.05$), and the most frequent were in the limbs

(phalanges, metacarpals and metatarsals) and in the head (palate, presphenoid and occipital). In relation to the sternum (alterations of sternum centers, xiphoid process and manubrium), there was an increase in the frequency in Pyri.D2 ($p<0.05$) (Table 6).

4.2.3. Toxicogenic assess: Micronucleus test

The micronucleus frequency increased after 72 h exposure to pyriproxyfen in the animals of Pyri.D1 ($p<0.05$). At the other times (24 and 48 h), no significant differences were present between the groups ($p>0.05$) (Table 7).

5. Discussion

Pyriproxyfen, an analog of the juvenile hormone, was the product chosen by the Ministry of Health to combat the focus of *A. aegypti*, which is the transmitter of Dengue Fever, Chikungunya Fever and Zika Virus (WHO, 2002; Brasil, 2014). In 2015, an outbreak of Zika Virus was observed in Brazil, so there was an overlap between the period of use of pyriproxyfen, the identification of the Zika Virus and the increased occurrence of microcephaly. This fact has raised speculations of whether microcephaly is caused by viral infection and/or environmental exposure to larvicide, in particular due to the report REDUAS (2016). Still, in 2016, a group of Brazilian researchers, through an epidemiological study, demonstrated an absence of correlation between the use of pyriproxifen and the prevalence of microcephaly (Albuquerque et al., 2016). The following year, a study with zebrafish showed that piriproxifen isolated, does not cause changes in neurological development (Dzieciolowska et al., 2017). Our results corroborate these studies since in a Swiss mouse model we also did not observe a correlation between the use of priproxifen and the development of microcephaly and/or other changes in embryo-fetal development.

According to Parens et al. (2017), who reanalyzed the data from Saegusa (1988) and Sumitomo (s.d.), piriprofixen is a candidate for causal effect and, therefore, correlates with the development of microcephaly. The authors affirm that, after reviewing the primary evidence, including studies by the manufacturer itself, it is possible to conclude that piriproxifen is a candidate for a causal effect. This finding demonstrates the need for independent studies that could understand the relationship between piriproxifen and the development of microcephaly and/or other changes that may be of importance to the individual or their offspring. There is a need to compile the knowledge already produced and in light of it to reinterpret new findings. For this purpose, our work brings a relevant contribution to the literature in the area since we systematically analyze the effects of piriproxifen on the reproductive performance of females; in embryofetal development (systemic, external, visceral and skeletal malformations - teratogenesis); and the integrity of DNA. These tests were chosen for their ability to predict harmful effects of piriprofixen for the individual and for future generations. In addition, these tests are recommended by OECD guidelines for assessing reproductive toxicity, embryo-fetal development (OECD, 2015) and genotoxicity (OECD, 2014).

In this study, we used a commercial dose of pyriproxyfen and a 10× higher dose, as recommended by international area guidelines (OECD, 2001; OECD, 2009). Thus, we simulated the average ingestion of 3 liters of water per day by pregnant women (Vitolo, 2008), who have been exposed to water from sources treated with pyriproxyfen throughout the gestational period. This fact is common in Brazil, especially in places with lower socioeconomic power, considering that water tanks that do not have coverage often receive treatment with larvicides, and the water is subsequently used for human consumption.

The animals were treated with pyriproxyfen no presented clinical signs of toxicity (maternal deaths, alteration in locomotor activity, occurrence of piloerection, diarrhea, vaginal blood loss and indication of discomfort or stress). A reduction in the weight of the spleen and kidneys was observed for the groups Pyri.D2 and Pyri.D1, respectively. However, these differences were not maintained in the relative weight. This fact suggests that the variations are due to differences in the size of the animals and not an effect of the treatment. This type of distortion is effectively corrected whenever the organ weight is divided by the animal's final weight, that is, when the relative weight is calculated (Carvalho, 2013).

In a general context, reproductive parameters do not show toxicity neither altered reproductive performance and embryo-fetal development. However, in PyriD1, there was a reduction in the placental index and an increase in fetal weight and the offspring presented an overweight for gestational age. In Pyri.D2, there was a reduction in the weight of the placenta and the placental index. However, the fetal weight did not differ from the control and the offspring presented an appropriate weight for gestational age. Our results shows that piriproxifen does not compromise the nutritional supply to the fetuses since, even with the reduced placental index in Pyri.D2, the fetuses weight was normal. In the case of Pyri.D1, we suggest that the increase in fetal weight and classification as OVGA is not a worrying fact. In the general context, we infer variance from normality. However, the isolated biological data, not interpreted in the global context, suggests macrosomia and these should receive attention as they can cause metabolic disorders during adulthood (Usta et al., 2017). However, our study demonstrates that offspring, despite being macrosomic fetuses, had no present associated malformations or maternal complications.

The congenital malformations observed (external, visceral and skeletal) are considered variants of normality since they occurred at similar frequencies between the control and treated groups, but there was an increase in the frequency of malformations in the sternum (agenesis, reduction or irregular ossification). According to Taylor (1986), this can occur when the fetuses are withdrawn prematurely, and these malformations can regress until the end of the gestation or even after the birth.

When the head measurement parameters were analyzed, it was observed that the fetuses of mothers treated with pyriproxyfen showed an increase in glabella / external occipital protuberance measurement. But, there was no change in the other parameters. This increase occurred in the group classified as macrosomal fetuses (Pyri.D1). In view of the above, we verified that the use of piriprofixen is not correlated to the reduction in head measurements, which allows us to be more aware that it is not responsible for microcephaly according to this experimental design. This fact is reinforced by the study by Tang (2016) that reports that the measurements of the occipitofrontal circumference and circumference area, when reduced, are truly important to verify changes in the head of the newborn with microcephaly.

In summary, our data indicate that piriproxifen did not alter the reproductive performance of females and embryonic development. These data are not corroborated by Parens et al. (2017) and even these authors propose that piriproxifen can cause malformations by a mechanism similar to that of retinoic acid. Since these two compounds are fat-soluble terpenoids, they have molecular similarities and are capable of some degree of cross-reactivity such as binding to specific receptors for retinoic acid (Parens et al. (2017). It is possible to suggest that pyriproxifen could trigger cellular signaling, regulation of gene expression and, consequently, modulation of insect reproduction and metamorphosis (Parens et al., 2017) that could be similar in embryonic

development of mammals. However, our study provide strong data on the absence of embryo-fetal changes in animals exposed to pyriproxifen.

The exposure to pyriproxyfen did not increase chromosomal instability. Although there was an increase in the frequency of micronucleus, especially in the Pyri.D1 group, it was only at the assessment at 72 h. This suggests a need for more attention. However, this isolated fact is not enough to indicate a genotoxic effect for pyriproxyfen. These results, despite the difference in the model, corroborate the study by Maharajn et al. (2018) who reported the absence of genotoxicity in zebrafish embryos exposed to pyriproxifen. However, Vieira-Santos et al. (2018) reports that piriproxifexen carries an increased environmental risk even at the doses recommended for the control of outbreaks of *A. aegypti* and, therefore, can have adverse effects on the aquatic ecosystem in non-target organisms such as *Daphnia magna* and *Artemia salina*.

In view of the results obtained, we suggest that pyriproxyfen does not cause maternal toxicity; it does not alter reproductive performance or embryo-fetal development, so it is not teratogenic; and it does not cause changes in DNA. In addition, we confirmed in a pioneering way, in a preclinical model, that pyriproxyfen is not responsible for cases of microcephaly.

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The authors declare that there are no conflicts of interest.

6. References

Albuquerque MFPM, Souza WV, Mendes AD, Lyra TM, Ximenes RA, Araújo TV, Braga C, Miranda-Filho DB, Martelli CM, Rodrigues LC (2016). Pyriproxyfen and the microcephaly epidemic in Brazil - an ecological approach to explore the hypothesis of their association. Mem Inst Oswaldo Cruz. 111, (12) 774-776. doi: 10.1590 / 0074-02760160291.

Barros AL, Souza VV, Navarro SD, Oesterreich AS, Oliveira RJ, Kassuya CAL, Arena AC (2013). Genotoxic and Mutagenic Effects of Diflubenzuron, an Insect Growth Regulator, on Mice. J Toxicol Environ Health A. 76, (17) 1003-1006. doi: 10.1080/15287394.2013.830585.

Barrow MV, Taylor WJ (1969). A rapid method for detecting malformation in rat fetuse. J Morphol. 127, (3) 291-305.

Brasil - Orientações técnicas para utilização do larvicida pyriproxyfen (0,5G) no controle de *Aedes aegypti* (2014). Disponível em: < http://portalarquivos.saude.gov.br/images/pdf/2014/julho/15/Instru----es-para-uso-de-pyriproxifen-maio-2014.pdf >Acess 10/01/2019.

Damasceno DC, **Cristina** P, **Francisco JR, Volpato GT, Consonni M, Rudge MVC, Kempinas WG (2008).** Anomalias congênitas: estudos experimentais, Editora Média, Belo Horizonte.

David N, Mauro MO, Gonçalves CA, Pesarini JR, Strapasson RL, Kassuya CA, Stefanello ME, Cunha-Laura AL, Monreal AC, Oliveira RJ (2014). *Gochnatia polymorpha* ssp. floccosa: Bioprospecting of an anti-inflammatory phytotherapy for use during pregnancy. J Ethnopharmacol. 154, (2) 370-9. doi: 10.1016/j.jep.2014.04.005.

Dzieciolowska S, Larroque AL, Kranjec EA, Drapeau P, Samarut E (2017). The larvicide pyriproxyfen blamed during the Zika virus outbreak does not cause microcephaly in zebrafish embryos. Sci Rep. 7, 40067. doi: 10.1038/srep40067.

Gonçalves CA, Siqueira JM, Carollo CA, Mauro MO, de Davi N, Cunha-Laura AL, Monreal AC, Castro AH, Fernandes L, Chagas RR, Auharek SA, Oliveira RJ (2013). Gestational exposure to *Byrsonima verbascifolia*: Teratogenicity, mutagenicity and immunomodulation evaluation in female Swiss mice. J Ethnopharmacol. 150, (3) 843-850. doi: 10.1016/j.jep.2013.09.012.

Gonçalves CA, Silva NL, Mauro MO, David N, Cunha-Laura AL, Auharek SA, Monreal AC, Vieira MC, Silva DB, Santos FJ, Siqueira JM, Oliveira RJ (2014). Evaluation of mutagenic, teratogenic, and immunomodulatory effects of *Annona nutans* hydromethanolic fraction on pregnant mice. Genet Mol Res. 13, (2) 4392-4405. doi: 10.4238/2014.

Hanseman JK, Hogan MD (1995). Selection of the experimental unit in teratology studies. Teratology. 12, (2) 165-72.

Harris SB, Hardisty JF, Hayes AW, Weber K. (2020). Developmental and reproductive toxicity studies of BIA 10-2474 Regul Toxicol Pharmacol. Mar;111:104543. doi: 10.1016/j.yrtph.2019.104543.

Hayashi M, Morita T, Kodama Y, Sofundi T, Ishidate Junior M (1990). The micronucleus assay with mouse peripheral blood reticulocytes using acridine Orange-coated slides. Mutat Res. 245, (4) 245-249.

Manson JM, Kang YJ (1994). Test methods for assessing female reproductive and developmental toxicology. In: Hayes, A.W. (Ed). Principles and methods of Toxicology, Raven Press, New York.

Maharajan K, Muthulakshmi S, Nataraj B, Ramesh M, Kadirvelu K. Toxicity assessment of pyriproxyfen in vertebrate model zebrafish embryos (Danio rerio): A multi biomarker study (2018). Aquat Toxicol. Mar;196:132-145. doi: 10.1016/j.aquatox.2018.01.010.

Montenegro MA, Guerreiro MM (s.d.). Protocolos em neurologia infantil. Universidade Estadual de Campinas – UNICAMP.

Moreira CQ, Faria MJSS, Baroneza JE, Oliveira RJ, Moreira EG (2005). Developmental exposure to fenproporex: reproductive and morphological evaluation. Hum Exp Toxicol. 24, (8) 397-402.

OECD (2001) - Guidelines for Testing of Chemicals Section 4: Health Effects. Test No. 414: Prenatal Development Toxicity Study.

OECD (2009) - Guidelines for Testing of Chemicals. Draft Proposal for an Extended One-Generation Reproductive Toxicity Study. No.28.

OECD – Guideline for testing of chemicals. Mammalian erythrocyte Test. Teste nº 474 de 26/09/2014.

OECD – Guideline for testing of chemicals. Reproduction/Developmental Toxicity Screening Test. Teste nº 421 de 28/07/2015.

Oliveira RJ, Salles MJ, da Silva AF, Kanno TY, Lourenço AC, Freiria GA, Matiazi HJ, Ribeiro LR, Mantovani MS (2009). Effects of the polysaccharide ß-glucan on clastogenicity and teratogenicity caused by acute exposure to cyclophosphamide in mice. Regul Toxicol Pharmacol. 53, (3)164-73. doi: 10.1016/j.yrtph.2008.12.007.

Oliveira RJ, Mantovani MS, Pesarini JR, Mauro MO, da Silva AF, Souza TR, Ribeiro LR (2015). 6-Dimethylaminopurine and cyclohexamide are mutagenic and alter reproductive performance and intrauterine development *in vivo*. Genet Mol Res. 14, (1) 834-849. doi: 10.4238/2015.

OMS – Organização Mundial da Saúde. Larvicidas recomendados pela Organização Mundial da Saúde para utilização em água potável.

http://portalarquivos2.saude.gov.br/images/pdf/2014/abril/28/larvicida.pdf. Acess 27/02/2018.

Parens R, Nijhout HF, Morales A, Xavier Costa F, Bar-Yam Y. A Possible Link Between Pyriproxyfen and Microcephaly. PLoS Curr. (2017) Nov 27;9. doi: 10.1371/currents.outbreaks.5afb0bfb8cf31d9a4baba7b19b4edbac.

REDUAS - Universidade Vermelha de Ambiente e Saúde. *RELATÓRIO dos médicos da cidade pulverizada por culturas sobre dengue-zika, microcefalia e pulverização maciça com venenos químicos, 2016*. http://reduas.com.ar/report-from-physicians-in-the-crop-sprayed-town-regarding -diga-zika-microcefalia e pulverização maciça com venenos químicos. Acess 14/02/2020).

Saegusa T. SUMILARV - Study of S-31183 by oral administration during the period of fetal organogenesis in rats (1988).

Salewisk E (1964). Färbemethoden zum makroskopischen nachweis von implantationsstellen am uterus der ratte. Naunym-Schmiedeberg Archiv Exp Pathol. Pharmkol. 247, 367.

Santos VS, Caixeta ES, Olegário de Campos Júnior E, Pereira BB (2017). Ecotoxicological effects of larvicide used in the control of *Aedes aegypti* on nontarget organisms: Redefining the use of pyriproxyfen. J Toxicol Environ Health A. 17, (3) 1-6. doi: 10.1080/15287394.2016.1266721.

Staples RE Schenell VL, (1964). Refinements in rapid clearing technic in the KOH-alizarin red method for fetal bone. Stain Technol. 39, 61-63.

Sumitomo (not publicly released; as public record obtained free by request from California Department of Pesticide Regulation and from Sumitomo).

Tang BL (2016). Zika virus as a causative agent for primary microencephaly: the evidence so far. Arch Microbiol. 198, (7) 595-601. doi: 10.1007/s00203-016-1268-7.

Taylor P, (1986). Pratical Teratology, Academic Press, New York.

Usta A, Usta CS, Yildiz A, Ozcaglayan R, Dalkiran ES, Savkli A, Taskiran M (2017). Frequency of fetal macrosomia and the associated risk factors in pregnancies without gestational diabetes mellitus. Pan Afr Med J. 2, 26-62. doi: 10.11604/pamj.2017.26.62.11440.

Vani JM, Monreal MTFD, Auharek SA, Cunha-Laura AL, de Arruda EJ, Lima AR, da Silva CM, Antoniolli-Silva ACMB, de Lima DP, Beatriz A, Oliveira RJ (2018). The mixture of cashew nut shell liquid and castor oil results in an efficient larvicide against *Aedes aegypti* that does not alter embryo-fetal development, reproductive performance or DNA integrity. PLoS One. 13, (3) e0193509. doi: 10.1371/journal.pone.0193509a.

Vani JM, Schweich LC, Oliveira KRW, Auharek SA, Cunha-Laura AL, Antoniolli-Silva ACMB, Nazario CED, Oliveira RJ (2018). Evaluation of the effects of the larvicides temephos on reproductive performance, embryofetal development and DNA integrity of *Swiss* mice. Pestic Biochem Physiol. 148, 22-27. doi.org/10.1016/j.pestbp.2018.03.010b.

Vieira Santos VS, Caixeta ES, Campos Júnior EO, Pereira BB. Ecotoxicological effects of larvicide used in the control of Aedes aegypti on nontarget organisms: Redefining the use of pyriproxyfen (2017). J Toxicol Environ Health A. 2017; 80 (3): 155-160. doi: 10.1080 /15287394.2016.1266721.

Vitolo MR (2008). Especificidades dos Nutrientes. (Ed) Nutrição da gestação ao envelhecimento. Rio de Janeiro.

WHO - Recommended Classification of Pesticides by Hazard. Guidelines to classification 2000-2002. Disponível em: <http://www.who.int/ipcs/publications/en/pesticides_hazard.pdf> Acess 10/01/2019.

Wilson JG (1965). Methods for administering agentes and detecting malformations in experimental animals. In: WILSON, J.G.; WAEKANY, J. (Eds.). Teratology: Principles snd Thecniques, The University of Chicago Press, Chicago.

Table 1: Parameters related to the growth development and organ weight of the female treated with Pyriproxyfen.

Experimental Groups	Biometrics Parameters				
	Initial Weight	**Final Weight**	**Weight Gain**	**Weight Utero**	**Liquid Weight Gain**
Control	29.90±0.50[a]	55.77±1.25[a]	25.87±1.29[b]	19.45±1.47[a]	6.42±0.72[a]
Pyri. D1	30.50±0.69[a]	51.26±1.47[a]	20.76±1.14[a]	16.84±1.52[a]	3.92±0.61[a]
Pyri. D2	32.44±1.64[a]	54.28±2.05[a]	21.84±1.01[a.b]	16.72±1.54[a]	5.07±0.80[a]
	Absolut Weight Organs (g)				
	Heart	**Lung**	**Spleen**	**Liver**	**Kidneys**
Control	0.21±0.01[a]	0.26±0.02[a]	0.16±0.008[b]	0.39±0.01[a]	2.84±0.09[b]
Pyri. D1	0.19±0.01[a]	0.25±0.02[a]	0.14±0.006[a.b]	0.37±0.01[a]	2.39±0.06[a]
Pyri. D2	0.17±0.01[a]	0.27±0.02[a]	0.13±0.010[a]	0.40±0.02[a]	2.58±0.07[a.b]
	Relative Weight of Organs				
	Heart	**Lung**	**Spleen**	**Liver**	**Kidneys**
Control	0.004±0.0003[a]	0.005±0.0004[a]	0.003±0.0001[a]	0.007±0.0002[a]	0.05±0.002[a]
Pyri. D1	0.004±0.0002[a]	0.005±0.0004[a]	0.003±0.0002[a]	0.007±0.0002[a]	0.05±0.001[a]
Pyri. D2	0.003±8.96[a]	0.005±0.0004[a]	0.002±0.0002[a]	0.007±0.0002[a]	0.05±0.001[a]

Legend: Different letters (a and b) indicate statistically significant differences (Test: [1] ANOVA/Tukey; $p<0.05$).

Table 2: Reproductive parameters for female treated with Pyriproxyfen.

Reproductive Parameters	Experimental Groups		
	Control	**Pyri. D1**	**Pyri. D2**
Implants[1]	14.20±0.65[a]	12.60±0.67[a]	11.78±1.09[a]
Live Fetuses[1]	12.70±1.03[a]	10.60±1.13[a]	10.89±1.17[a]
Fetal Viability[1]	88.83±4.79[a]	83.11±6.66[a]	91.27±2.94[a]
Post-implantational Losses[1]	74.63±4.63[a]	70.51±6.47[a]	79.49±2.52[a]
Resorption[1]	1.50±0.69[a]	2.00±0.75[a]	0.89±0.31[a]
Resorption Rate[1]	11.17±4.80[a]	16.89±6.66[a]	8.73±2.94[a]
Placental Weight (g)[2]	0.10±0.002[b]	0.10±0.002[a.b]	0.09±0.002[a]
Placental Index[1]	0.08±0.002[a]	0.07±0.001[b]	0.07±0.001[b]
Fetuses Weight (g)[1]	1.21±0.009[a]	1.26±0.01[b]	1.22±0.01[a]
SWGA	-	OVGA	AWGA
SR[1]	1.84±0.46[a]	1.90±0.66[a]	0.96±0.30[a]

Legend: SWGA: suitability of weight for gestational age; AWGA: appropriate weight for their gestational age; LWGA: low weight for their gestational age; OVGA: overweight for their gestational age. Different letters (a and b) statistically significant differences (Test: [1]ANOVA/Tukey; [2] Kruskall-Wallis/Dunn; $p<0.05$).

Table 3: Head measurements of offspring of females treated with Pyriproxyfen.

	Experimental Groups		
Parameters (mean in mm)	**Controle**	**Pyri. D1**	**Pyri. D2**
Measure 1 - Anterior/Posterior	$9.73\pm0.20^{a.b}$	10.50 ± 0.15^{b}	10.27 ± 0.16^{a}
Measure 2 - Glabella and external occipital protuberance	7.59 ± 0.20^{a}	8.37 ± 0.08^{b}	$8.19\pm0.08^{a.b}$
Measure 3 - Binaural	5.19 ± 0.20^{a}	6.02 ± 0.06^{a}	5.94 ± 0.05^{a}
Measure 4 – Occiptofrontal circunference	3.05 ± 0.03^{a}	3.40 ± 0.31^{a}	3.16 ± 0.15^{a}
Measure 5 - Area of head circunference	0.74 ± 0.01^{a}	0.76 ± 0.01^{a}	0.73 ± 0.01^{a}

Legend: Measure 1 - anterior/posterior- glabella and external occipital protuberance passing over the sagittal suture and bregma; 2 glabella and external occipital protuberance; 3 binaural-reference point to the superior insertion of the ears; 4 head circumference; 5 area of the head circumference. Different letters (a and b) means statistically significant differences (Test: Kruskall-Wallis/Dunn; $p<0.05$).

Table 4: Relationship and frequency of external malformations in offspring of females treated with Pyriproxyfen.

	Experimental Groups		
Parameters	**Control**	**Pyri. D1**	**Pyri. D2**
		Members	
Analyzed Fetuses	127	124	95
Normal Fetuses	122	117	89
Retr. Post. Unilateral	5	7	5
Hematoma	0	0	1
Malf. Freq.	5	7	6
%M.F.	3.94	5.64	5.26
		Tail	
Normal Fetuses	126	120	89
Rolled up tail	1	4	6
Malf. Freq.	1	4	6
%M.F.	0.79	3.22	6.31
		Sistemic Malformation and Abdomen	
Normal Fetuses	125	124	94
Hydrops	2	0	0
Gastroschisis	0	0	1
Freq.Malf.	2	0	1
%M.F.	1.57	0.00	1.05

Legend: Malf.Freq. – Frequency of malformation; %M.F. – average value percentage of malformations; Retr. – Retroversion; Post. – Posterior. Statistically compared witch the control. (Chi – Square test. $p>0.05$).

Table 5: Relationship and frequency of visceral malformations in the offspring of females treated with Pyriproxyfen.

	Experimental Groups		
Parameters	**Control**	**Pyri. D1**	**Pyri. D2**
	Brain- Hydrocephalus		
Analyzed Fetuses	64	54	50
Normal Fetuses	7	15	7
Mild Hydroc.	46	29	34
Severe Hydroc.	11	10	9
Malf. Freq.	57	39	43
%M.F.	89.06	72.22*	86.00
	Region Urogenital- Hydronephrosis		
Normal Fetuses	56	51	47
Mild Hydron.	8	3	3
Severe Hydron.	0	0	0
Malf. Freq.	8	3	3
%M.F.	12.5	5.56	6.00

Legend: Malf.Freq. – Frequency malformation; %M.F. – average value percentage of malformation; Hydroc. – Hydrocephalus; Hydron. – Hydronephrosis. *Statistically witch the control. (Chi-Square test. $p>0.05$).

Table 6: Relationship and frequency of skeletal malformations in the offspring of females treated with Pyriproxyfen.

Parameters		Experimental Groups		
		Control	**Pyri. D1**	**Pyri. D2**
		Members		
Analyzed Fetuses		63	62	47
Normal Fetuses		0	0	0
Phalanges	Absence	55	53	41
	R.O.	3	1	0
Metac.Metat.	Absence	5	8	6
Freq.Malf.		63	62	47
%M.F.		100	100	100
		Sternum		
Normal Fetuses		56	58	32
Sternum Centers	Absence	1	0	1
	R.O.	0	4	4
	Irregular	4	0	3
Xiphoid Process	Absence	1	0	0
	Irregular	1	0	6
Manubrium	Absence	0	0	1
	Irregular	0	0	0
Malf. Freq.		7	4	22
%M.F.		11.11	6.45	46.81*
		Head and Jaw		
Normal Fetuses		40	45	29
Pal.Pres.	Absence	0	0	0
Occiptal	R.O.	0	0	1
Malf. Freq.		23	17	18
%M.F.		36.51	27.42	38.30

Legend: Malf.Freq. – Frequency malformation; %M.F. – average value percentage of malformation; Metac. – Metacarpus; Metat. – Metatarsus; Pal. – Palate; Presf. – Presphenoide; R.O. – Reduced ossification. *Different statistically in relation to control. (Chi-Square test. $p<0.05$).

Table 7: Micronucleus assay

Experimental Groups	**Micronucleus**		
	24h	**48h**	**72h**
Control	5.70 ± 1.32^{a}	5.20 ± 1.06^{a}	$8.90\pm1.35^{a.b}$
Pyri. D1	7.11 ± 3.29^{a}	13.44 ± 4.27^{a}	21.40 ± 6.10^{b}
Pyri. D2	9.67 ± 1.86^{a}	5.22 ± 1.21^{a}	7.67 ± 1.22^{a}

Legend: Different letters (a and b) indicate statistically significant differences. (Test: ANOVA/Tukey $p<0.05$).

Manuscrito 2: Larvicida comercial Diflubenzuron: Recusado na revista: Drug & Chemical Toxicology

The teratogenical potential of Diflubenzuron in a preclinical model

Rodrigo Juliano Oliveira[1,2,3,4], Sarah Alves Auharek[5], Juliana Miron Vani[1,2,3], Gabriela Lamberti Sabine Escobar[1], Antonio Carlos Duenhas-Monreal[1], Andréia Conceição Millan Brochado Antoniolli-Silva[1,2], Andréa Luiza Cunha-Laura[3*]

[1]Centro de Estudos em Células Tronco, Terapia Celular e Genética Toxicológica - CeTroGen, Hospital Universitário Maria Aparecida Pedrossian – HUMAP, Universidade Federal de Mato Grosso do Sul – UFMS, Campo Grande, MS, Brasil.

[2]Programa de Pós-graduação em Saúde e Desenvolvimento na Região Centro-Oeste, Faculdade de Medicina Dr. Hélio Mandetta – FAMED, Universidade Federal de Mato Grosso do Sul – UFMS, Campo Grande, MS, Brasil.

[3]Programa de Mestrado em Farmácia, Faculdade de Ciências Farmacêuticas, Alimentos e Nutrição - FACFAN, Universidade Federal de Mato Grosso do Sul – UFMS, Campo Grande, MS, Brasil.

[4]Programa de Pós-graduação em Genética e Biologia Molecular, Centro de Ciências Biológicas – CCB, Universidade Estadual de Londrina - UEL, Londrina, PR, Brasil.

[5]Faculdade de Medicina do Mucuri, Universidade Federal dos Vales do Jequitinhonha e do Mucuri – UFVJM, Teófilo Otoni, MG, Brasil.

Autor correspondente:

Andrea Luiza Cunha-Laura, e-mail: andrea.laura@ufms.br

Abstract

Diflubenzuron (DFB) is an insecticide and acaricide growth regulator used in Brazil to combat Aedes aegypti. Male mice studies showed that DFB present hazardous effects on male reproduction. In view of the above, the present study aims to evaluate the toxic effects, reproductive performance, embryofetal development and DNA integrity in *Swiss* pregnant mice. The females were treated with different dosages of DFB (0.3 mg/kg, 3 mg/kg and 30 mg/kg (w/v) in the following windows: the pre-implantation period (1st to the 4th day of gestation); during organogenesis (5th to 15th day of gestation) and thorough gestation (1st to 18th). The results showed that biometric and reproductive parameters were similar between controls and DFB exposed animals, which suggests absence of maternal toxicity. Moreover, the analysis of the embryofetal development presented significant differences in relation to the control, showing concern, since this compound has a high teratogenic potential. Regarding DNA integrity analysis, there was an increase in pre-implantation micronucleus frequency, but an increase in splenic phagocytosis was also observed, suggesting that the blood micronuclei were sequestered by the spleen. We can thus infer that Diflubenzuron is not maternal-toxic, it does not alter the reproductive performance neither the integrity of the DNA, but it has a high teratogenic potential.

Keywords: Diflubenzuron, pregnancy, teratogenicity

1. Introduction

Compounds that exhibit insect regulators (IGR) have been an alternative for the control of insects that are resistant to traditional insecticides (Belinato and Valle, 2015). These compounds have also been promising in the fight against *Aedes aegypti*, the mosquito transmitter of Dengue and Chikungunya fever and Zika virus. However, studies have already shown that they may be toxic to non-target populations (Fischer and Hall, 1992; Barros et al., 2013; Barros et al., 2014).

Diflubenzuron (DFB) is chitin growth inhibitor belonging to the benzoylphenyl urea family, used initially to combat flies and pests present in agriculture and for veterinary use. The DFB shows great effectiveness in controlling insects, as it acts during ecdysis, rendering the development of the larvae impracticable (Sapone et al., 2005; Abe et al., 2014; Darriet, 2015). DFB was used in the Brazilian Program to Combat Dengue to combat *Aedes aegypti* around 1990 when mosquitoes presented resistance to the larvicidal Temefós (Benze et al., 2014; Belianto and Valle, 2015).

Some studies have already been conducted using the product Diflubenzuron (Sapone et al., 2005; Barros et al., 2013) and demonstrated that it has genotoxic potential for the environment and humans. This fact leads to concern with pregnant women, since this condition makes the woman more susceptible to the xenobiotics effects (Sally et al., 2013; 90 Zhang et al., 2014), and there is still no data in literature about the possible DFB teratogenic effect. Based on the fact DFB could induce the curve U dose–response, since this insecticide, when administered by gavage, showed reproductive toxicity only at the lowest dose tested in male rats, our study extended this finding to investigating the effects of DFB on reproductive performance. Taken this into account, the present study aims to

evaluate the toxic effects, reproductive performance, embryofetal development and DNA integrity in *Swiss* pregnant mice exposed to DFB.

2. Material and methods

2.1 Experimental Animals

Swiss (*Mus musculus*) mice of both sexes were used (30 females and 15 males), aged 8-10 weeks, with an average weight of 30 g, from the State Agency of Animal and Plant Health Protection (IAGRO). All procedures and protocols followed approved guidelines for the ethical treatment of animals, according to the Ethics Committee in Animals Experimentation from the Federal University of Mato Grosso do Sul under protocol # 808/2016.

The animals were kept in polypropylene boxes isolated in the case of males and in double in the case of females. They went through an adaptation period of seven days. The temperature and luminosity were monitored and a 12-hour photo (12-hour clearing: 12-hour dark) was used with temperature maintained at 22 ± 2 ° C on an ALESCO® ventilated shelf and fed with commercial feed (Nuvital®) and filtered water *ad libitum*.

The mating was overnight, in the proportion of 1macho: 2 females and the detection of pregnancy was done by observing the vaginal plug, this day being considered zero day of gestation (Oliveira et al., 2009; Gonçalves et al., 2013; David et al., 2014; Gonçalves et a., 2014; Oliveira et al., 2015).

2.2 Experimental Design

The pregnant females were randomly divided into 10 experimental groups (n = 10): Control group - the animals received drinking water at the rate of 0.1ml/10g body weight (bw.) oral (v.o. - gavage) during all days of gestation (1st to 18th). Pre-implantation group – the animals received Diflubenzuron (v.o. - gavage) at doses of 0.3 (D1); 3 (D2) and 30 (D3) mg/kg respectively, from the 1st to the 4th day of gestation. Group Organogenesis - the animals received Diflubenzuron (v.o. - gavage) at doses 0.3 (D1); 3 (D2) and 30 (D3) mg/kg respectively from the 5th to 15th day of gestation. Gestational Group - the animals received Diflubenzuron (v.o. - gavage) at doses 0.3 (D1); 3 (D2) and 30 (D3) mg/kg mg/kg respectively during all days of gestation (1st to 18th).

The dose of 0.3 mg/kg (w/v) was based on the commercial use of the product. For its calculation was considered the commercial dose being deposited in a box of water and a person of 70 kg ingesting 3L of water daily as recommended (Vitolo, 2008). The 3mg/kg (w/v) is the safety dose, since it is 10x higher than the dose of use. The dose of 30 mg/kg (w/v) is 100x greater than the dose of use, as indicated by area guidelines (OECD, 2001; OECD, 2009).

2.3 Biological Assay

2.3.1. Reproductive performance and embryofoetal development (teratogenicity)

On the 18th day of pregnancy the animals were submitted to euthanasia followed by laparotomy, hysterectomy and omphalectomy. Spleen, heart, liver, lung and kidneys were collected and weighed. Fetuses and placentas were weighed. A systematized external analysis of fetuses was performed to detect possible external malformations. Subsequently, the fetuses were sexed and recorded the number of implantations, resorptions, live fetuses and dead fetuses. The fetal viability (number of live fetuses/number of implantations x 100), post-implantation loss rate (number of implantations - number of live fetuses x 100/number of implantations), resorption rate number of implantations), placental index (placental weight/fetal weight) and sex ratio (number of male fetuses/number of female fetuses) were estimated (Oliveira et al., 2009; Gonçalves et al., 2013; David et al., 2014; Gonçalves et al., 2014; Oliveira et al., 2015). Then, fetal weight was adjusted to the age of pregnancy according to Oliveira et al. (2009). The relative weight of the organs were calculated by organ weight/ final b.w.

Subsequently, the fetuses were randomly distributed into two subgroups. The first was intended for visceral analysis and the fetuses were fixed in a solution of Bodian's (distilled water (142 ml), acetic acid (50 ml), formaldehyde (50 ml) and alcohol 95% (758 ml)) for at least seven days. The visceral analysis was performed through microdissection with strategic sections for the study of the thorax and abdomen according to Barrow, Taylor (1969), and for head study according to Wilson (1965), with modifications of Oliveira et al. (2009). The visceral changes were based on the studies of Taylor (1986), Manson and Kang (1994), Damasceno et al. (2008), Oliveira et al. (2009).

The second subgroup of fetuses was assigned to the skeletal analysis by the red alizarin technique proposed by Staples; Schnell (1964), with modifications of Oliveira et

al. (2009). The fetuses were fixed in acetone for at least seven days. For the diaphanization process, the fetuses were eviscerated and placed in a solution of KOH (0.8%). Then, four drops of alizarin were added. This solution was changed every 24 hours over four days. After this period the KOH solution was discarded and the fetuses were placed in whitening solution (1 liter of glycerin: 1 liter of ethyl alcohol: 0.5 liters of benzyl alcohol) and exchanged for 24 hours for seven days. Skeletal changes were classified according to the studies of Taylor (1986), Manson et al. (1982), Damasceno et al. (2008) and Oliveira et al. (2009). All analyzes were performed in stereomicroscope (NIKON SMZ745T).

2.3.2 Micronucleus Assay

The technique used was based on Hayashi et al. (1990) with modifications of Oliveira et al. (2009). 20μL of peripheral blood was collected by puncturing the caudal vein and deposited on a slide previously stained with Acridine Orange (1mg / mL) and then covered by cover slip. The collections were performed on the 18th gestational day, that is, at the end of the experiment to verify if the TaLCC had the capacity to cause cumulative damages. The slides were stored in a freezer at -20 ° C for at least 15 days. 2,000 cells / animal was analyzed in an epifluorescence microscope (Motic® Model BA 410) in a 400-fold increase.

2.3.3 Splenic Phagocytosis Assay

At the end of the gestational period (18ºth) the animals were submitted to euthanasia. The spleen was collected and macerated in physiological solution, obtaining homogeneous suspension of cells by successive aspirations with a pipette. 100 μL of cell suspension were deposited on a slide previously stained with Acridine Orange (1mg / mL) and then covered by cover slip. The slides were stored in a freezer (-20ºC) for further analysis. This was done in a fluorescence microscope (Motic® Model BA 410) at a magnification of 400x, with a 420-490nm filter and a 520nm barrier filter. 200 cells / slide / animal were analyzed. The absence or presence of phagocytosis was based on the description of Carvalho et al. (2015).

2.4 Statistical analysis

We used the ANOVA / Tukey test for parametric data, the Kruskal-Wallis / Dunn test for non-parametric data and the Chi-square test for comparisons of frequencies between groups. Data were presented in Mean ± Standard Error of Mean and the established level of significance was $p < 0.05$. The analyzes were done in the GraphPad InStat 5 program.

3. Results

3.1 Evaluation of biometric parameters and reproductive performance

The initial and final weight, weight gain, uterine weight and net weight did not present significant differences ($p > 0.05$) between the groups that received diflubenzuron dose in comparison to the control group (Table 1). The same tendency was observed for the absolute and relative weight of the organs (heart, lung, spleen, kidneys and liver), that were similar ($p < 0.05$) between DFB exposed group and controls (Table 1).

Reproductive parameters such as number of implants, live fetuses, post-implantation loss rate, number of resorptions, rate of resorption and sex ratio were similar ($p > 0.05$) in all groups investigated (Table 2).

Pre-implantation D3 and Gestacional D2 groups presented an increased placenta weight ($p < 0.05$). The placental index was also augmented ($p < 0.05$) in Pre-implantation D3 and Gestacional D2 groups, in comparison to control. In all DFB exposed groups, the weight of the fetuses did not differ from the control ($p > 0.05$) (Table 2).

3.2. Evaluation of embryo-fetal development

The assessment of fetal weight did not show differences between the control and the treated groups ($p > 0.05$). However, the adequacy of fetal weight at the age of pregnancy demonstrated that the Organogenesis D1and Gestational D2 groups are adequate. The Pre-Implantation D3 and Gestational D1 groups present low gestational birth weight fetuses and the Pre-Implantation D1 and D2, Organogenesis D2 and D3 and Gestational D3 groups show fetuses with high weight for gestational age (Table 2).

3.3. External, visceral and skeletal malformations

In comparison to controls, external malformations found in the anterior and posterior limbs showed a significant increase in the Pre-implantation D1 and D3, Organogenesis (all doses) and Gestational D2 and D3 groups. In all dosages in the DFB Organogenesis exposed group, obstructed choana malformation were observed. Cardiac changes (ventriculomegaly and bifurcate heart) were observed in Gestational D2 and D3 groups. Hydronephrosis was present in Pre-Implantation D3, Organogenesis D1 and Gestational D1 and D2 groups. The other malformations investigated (tail and abdomens) did not diverge from control (Table 3 and 4).

Skeletal malformations were found in the anterior and posterior limbs, sternum, head and mandible, and in the spine. The limb malformations were increased ($p < 0.05$) in the Pre-implantation D1 and D2 and Organogenesis D1. In comparison to control sternum malformations were augmented ($p < 0.05$) in the Pre-implantation D2 and D3 and Organogenesis D1 and D2. Regarding to head and jaw malformations, all DFB exposed groups presented a significant increase in this parameter comparing to the control group ($p < 0.05$). However, the spinal malformation were similar ($p > 0.05$) in all investigated groups (Table 5).

The malformations previously described were found in fetuses of different litters and for none of the parameters analyzed, the malformations were restricted to fetuses of only one mother.

3.4 Toxicogenic evaluation: Micronucleus test

At Table 6 is possible to observe that micronucleus test showed significant increase in the Pre-Implantation group D1 and D3 only in the time of 48 hours. The remaining times (24h and 48h) were similar to the control ($p > 0.05$).

3.5 Evaluation of splenic phagocytosis

In comparison to the control group, the phagocytosis assay presented a significant increase in the Pre-Implantation D1 and D2, Organogenesis D2 and D3 and Gestacional (all doses) groups (Table 7).

4. Discussion

The growth inhibitor Diflubenzuron that is widely used in Brazilian agriculture has proven its action on mosquito mortality, and its effectiveness has been extended to combat *Aedes aegypti* since 1990 in the Brazil (Benze et al., 2014)

Many studies have already shown that this compound has genotoxic action and can cause damage to the non-target population (Sapone et al., 2005; Fischer and Hall, 1992). The present study investigated the action of the DFB on pregnant women, since no data of this product on the embryofoetal development were found in the literature. In this sense, an experimental condition was performed simulating in mice, the average consumption of 3 liters of water/day with DFB by pregnant women (Vitolo, 2008).

The biometric parameters of the females exposed to DFB showed no maternal toxicity. The reproductive parameters reinforce the idea of the DFB absence of toxicity, since they were similar to the control group. However, the placenta weight also increased in pre-implantation and gestation DFB exposed groups. The placenta index had an increase in the same groups as the placenta weight. When there is an increase in the weight of the placenta, an attempt is made by the organism to nourish the fetus during gestation when it presents a lower weight. This fact occurs in order to the newborn to reach its ideal weight before birth, and the same occurs when the placental index also increases (Erikson et al., 1982; Erikson et al., 1989; Brett et al., 2014). It was not observed a lower weight for the DBF exposed fetuses and since they presented a similar form to the control, we can then infer that this increase in placental weight and placental index was a random event.

Regarding embryofoetal development, the data show that DFB is capable of increasing the frequency of external malformations in limbs, especially leg retroversion. This compound also increases the frequency of visceral malformations and among them the induction of hydrocephalus, choana obstruction, ventriculomegaly, bifurcated heart and hydronephrosis. In relation to skeletal malformations DFB increased the frequency of absence/reduction of ossification in the skull, sternum, vertebrae and anterior and posterior limbs. These malformations were distributed in three different doses and in three different protocols tested. Thus, these results suggest that DFB is not embryo lethal since even females treated only in the pre-implantation period presented gestational

development and fetuses. In addition, there were no differences in the number of implants between the control and treated groups in the pre-implantation period. Thus, if this compound was embryo lethal, it was expected that gestation would not have continued because the embryos would not implant when the females were treated at that specific period.

Although not embryo lethal, Diflubenzuron is capable of altering embryofetal development independently of the treatment window. Therefore, the results indicate that this compound is embryotoxic and teratogenic. It is suggested that the compound is embryotoxic because females that are treated only during the pre-implantation period had the offspring malformed. It can also be confirmed that DFB is teratogenic since the females that received the treatment only in the period of organogenesis also had their offspring malformed. However, we can not affirm that the effects of Diflubenzuron are cumulative since the highest percentages of malformations are not present in the treated groups throughout the gestational period.

Regarding the visceral analysis, the main malformations were hydrocephalus and hydronephrosis and since this type of malformation regress after birth, they could be considered variants of normality (Taylor (1986), Damasceno et al., 2008; Oliveira et al., 2009; Gonçalves et al., 2013). However, in this study it was observed that both malformations (hydrocephalus and hydronephrosis) showed a significant increase in the treated groups when compared to the control group, and this increase is very high and therefore can not be correlated as a variant of normality.

Reinforcing the idea of attention in the visceral analysis, heart malformations (ventriculomegaly and bifurcate heart) were also observed, which showed a significant increase in the DBF gestational groups. Heart malformations are of great concern as they are difficult to diagnose during pregnancy and are those with a high mortality rate in newborns (Rivera et al., 2007). Together these data are important because, in all the analyzes, an increase in malformations was observed in the groups treated with Diflubenzuron, a product that was already widely used in Brazil during periods of Dengue epidemic (Belinato and Valle, 2015). Thus, exposure by pregnant women during this application can be harm to the newborn, since the product presented a high teratogenic potential.

In the evaluation of DNA integrity, an increase in the frequency of micronuclei in the pre-implantation window in 48 hours was observed. Although this was an indication of genotoxicity, the other times (24 and 48h) did not diverge from the control, including at the highest dose that is 100x greater than the dose used commercially. Corroborating with the data from the micronucleus assay we can observe the splenic phagocytosis assay that was increased in the treated groups.

In general, the spleen is responsible for the removal of micronucleated cells that are circulating in the blood, and their increase may be related to the absence of micronuclei from the treated groups (Carvalho et al., 2015; Martello et al., 2016; Schneider et al., 2016). It is suggested that the compound Diflubenzuron causes damage in the DNA and leads to the presence of micronuclei, but the increase of phagocytosis is the indication that the spleen could removal them into the blood.

Barros et al. (2013), conducted a study with Diflubenzuron, in male mice. Their DNA integrity data diverged from our study, since in males the amount of micronuclei increased significantly in all treated groups, which shows the genotoxic action of this compound. Despite the divergence of data, it is worth mentioning that our study was carried out with females and it is known that males and females present different hormonal profiles (OECD, 2008) a fact that may explain this occurrence.

It is also noteworthy that genetic factors are the most frequent cause of malformations and these can result from changes in the structure of DNA or expression of genes (Oliveira et al., 2009). However, the literature consulted does not demonstrate a direct correlation between somatic mutations and changes in embryo-fetal development. Thus, the micronucleus assay, even though it is representative of genotoxic damage, cannot be used as a direct measure or related to birth defects. However, this is a trial of indirectly predicting embryotoxic effects as demonstrated by Oliveira et al. (2014). In the present study, no correlations were observed between the frequency of genotoxic damage and the teratogenic potential of DFB. However, our group previously reported the induction of genotoxicity by this pesticide (Barros et al., 2014). In the present study, the frequency of micronuclei may be underestimated due to the splenic activation that DFB caused in pregnant females, in addition to the hormonal differences already mentioned. Therefore, these results deserve special attention and cannot be ignored in a larger context, which means that it is not possible to affirm that the DFB did not cause DNA damage.

In view of the above, we can infer that the product Diflubenzuron does not present maternal toxicity, nor does it alter reproductive performance when used by pregnant females. However, DFB presents a high teratogenicity and therefore, in case of accidental poisoning in pregnant women, this product can alter the embryo-fetal development. This fact suggests the discontinuation of its use.

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References

Abe FR, Coleone AC, Machado AA, Gonçalves MNJ. 2014. Ecotoxicity and environmental risk assessment of larvicides used in the control of *Aedes aegypti* to *Daphnia magna* (Crustacea, Cladocera).. J Toxicol Environ Health A 7737-7745.

Barros AL, Cavalheiro GF, de Souza AV, Traesel GK, Anselmo-Franci JA, Kassuya CA, Arena AC. 2013. Subacute toxicity assessment of diflubenzuron, an insect growth regulator, in adult male rats. Environ Toxicol 31:407-414.

Barros AL, Cavalheiro GF, Souza AVM, Traesel GK, Franci JAA, Kassuya CAL, Arena AC. 2014. Subacute Toxicity Assessment of Difluobenzuron, an Insect Growth Regulator, in Adult Male Rats. Environ Toxicol 31(4):407-414.

Barrow MV, Taylor WJ. 1969. A rapid method for detecting malformation in rat fetuse. J Morphol 127:291-305.

Benze TP, Sakuragui MM, Zago LH, Fernandes MN. 2014. Subchronic exposure to diflubenzuron causes health disorders in neotropical freshwater fish, *Prochilodus lineatus*. Environ Toxicol 31:533-542.

Belinato TA, Valle D. 2015. The Impact of Selection with Diflubenzuron, a Chitin Synthesis Inhibitor, on the Fitness of Two Brazilian *Aedes aegypti* Field Populations. PLoS One. 24;10(6).

Brett KE, Ferraro ZM, Yockell-Lelievre J, Gruslin A, Adamo KB. 2014. Maternal–Fetal Nutrient Transport in Pregnancy Pathologies: The Role of the Placenta. Int J Mol Sci 15:16153-16185.

Carvalho PC, Santos EA, Schneider BUC, Matuo R, Pesarini JR, Cunha-Laura AL, Monreal ACD, Lima DP, Brochado-Antoniolli ACM, Oliveira RJ. 2015. Diaryl sulfide analogs of combretastatin A-4: Toxicogenetic, immunomodulatory and apoptotic evaluations and prospects for use as a new chemotherapeutic drug. Environ Toxicol Pharmacol 40:715-721.

Damasceno DC, Kempinas WG, Volpato GT, Consonni M, Rudge MVC. Paumgartten, FJR. 2008. Anomalias congênitas: estudos experimentais.Editora Média, Belo Horizonte.

Darriet, F. 2015. An anti-mosquito mixture for domestic use, combining a fertiliser and a chemical or biological larvicide. Pest Manag Sci. 72(7):1340-1345.

David N de, Mauro MO, Gonçalves CA, Strapasson, RLB, Kassuya CAL, Stefanello MÉA, Cunha-Laura AL, Monreal ACD, Oliveira RJ. 2014. *Gochnatia polymorpha* ssp. floccosa: Bioprospecting of an anti-inflammatory phytotherapy for use during pregnancy. J Ethnopharmacol 154: 370-379.

Eriksson UJ, Dahbstrom E, Larsson KS, Hellertron C. 1982. Increased incidence of congenital malformations in the offspring of diabetics rats and their prevention by maternal insulin teraphy. Diabetes 3:1-6.

Eriksson RSM, Thumberg L, Eriksson UJ. 1989. Effectts of interrupted insulin treatment on fetal outcome of pregnant diabetic rats. Diabetes 38:764-72.

Fischer SA, Hall LW Jr. 1992. Environmental concentrations and aquatic toxicity data on diflubenzuron (dimilin). Crit Rev Toxicol 22:45-79.

Gonçalves CA, Siqueira JM, Carollo CA, Mauro MO, David N, Cunha-Laura AL, Monreal ACD, Castro AH, Fernandes L, Chagas R, Auharek SA, Oliveira RJ. 2013. Gestational exposure to *Byrsonima verbascifolia*: Teratogenicity, mutagenicity and immunomodulation evaluation in female Swiss mice. J Ethnopharmacol 150:843-850.

Gonçalves CA, Silva NL, Mauro MO, David N, Cunha-Laura AL, Auharek SA, Monreal ACD, Vieira MC, Silva DB, Santos FJL, Siqueira JM, Oliveira RJ. 2014. Evaluation of mutagenic, teratogenic, and immunomodulatory effects of *Annona nutans* hydromethanolic fraction on pregnant mice. Genet Mol Res 13:4392-4405.

Hayashi M, Morita T, Kodama Y, Sofundi T, Ishidate Junior, M. 1990. The micronucleus assay with mouse peripheral blood reticulocytes using acridine Orange-coated slides. Mutat.Res. 245;245-249.

Manson JM, Zenich H, Costlowr D. 1982. Teratology test methods for laboratory animal. In: Hayes, A.W. (Ed). Principles and methods of toxicology. Raven Press, New York.

Manson JM, Kang YJ. 1994. Test methods for assessing female reproductive and developmental toxicology. In: Hayes, A.W. (Ed). Principles and methods of Toxicology. Raven Press, New York.

Martello MD, David N, Matuo R, Carvalho PC, Navarro SD, Monreal ACD, Cunha-Laura, AL, Cardoso CAL, Kassuya CAL, Oliveira RJ. 2016. *Campomanesia adamantium* extract induces DNA damage, apoptosis, and affects cyclophosphamide metabolism. Genet Mol Res 26:15(2).15027678.

OECD-2001. Guidelines for Testing of Chemicals Section 4: Health Effects. Test No. 414: Prenatal Development Toxicity Study.

OECD- 2009. Guidelines for Testing of Chemicals. Draft Proposal for an Extended One-Generation Reproductive Toxicity Study. No.28.

Oliveira RJ, Kanno TYN, Salles MJS, Lourenão ACS. Ribeiro LR, Freiria GA, Matiazi HJO, Mantovani MS, Silva AF. 2009. Effects of the polysaccharide ß-glucan on clastogenicity and teratogenicity caused by acute exposure to cyclophosphamide in mice. Regul Toxicol Pharmacol. 53(3):164-173.

Oliveira RJ, Mantovani MS, Pesarini, JR, Mauro MO, da Silva, AF, Souza TR, Ribeiro LR. 2015. 6-Dimethylaminopurine and cyclohexamide are mutagenic and alter reproductive performance and intrauterine development *in vivo*. Genet Mol Res 14:834-849.

Oliveira RJ, Pesarini JR, Salles MJS, Kanno TYN, Lourenço ACS, Leite VS, Silva AF, Matiazi HJ, Ribeiro LR, Mantovani MS. 2014. Effects of β-glucan polysaccharide revealed by the dominant lethal assay and micronucleus assays, and reproductive performance of male mice exposed to cyclophosphamide. Genet Mol Biol 37:111-119.

Rivera IR, da Silva MAM, Fernandes JMG, Thomaz JMG, Soriano CFR, de Souza MGB. 2007. Cardiopatia congênita no recém-nascido: da solicitação do pediatra à avaliação do cardiologista. Arq Bras Cardiol 89:6-10.

Sally EOF, Anjos LA, Wahrlich V. 2013. Metabolismo Basal durante a gestação: revisão sistemática. Ciên S C 18:413-430.

Sapone A, Pozzetti L, Canistro D, Broccoli M, Bronzetti G, Potenza G, Affatato A, Biagi GL, Cantelli-Forti G, Paolini M. 2005. CYP superfamily perturbation by diflubenzuron or acephate in different tissues of CD1 mice. Food Chem Toxicol 43:173-183.

Shneider BUC, Meza A, Beatriz A, Pesarini JR, Carvalho PC, Mauro MO, Karaziack CB, Cunha-Laura AL, Monreal ACD, Matuo R, Lima DP, Oliveira RJ. 2016. Cardanol: toxicogenetic assessment and its effects when combined with cyclophosphamide. Gen Mol Biol 39, 279-289.

Staples RE, Schenell VL. 1964. Refinements in rapid clearing technic in the KOH-alizarin red method for fetal bone. Stain Technology 39-61-63.

Taylor, P. 1986. Pratical Teratology. Academic Press, New York.

Vitolo MR. 2008.Especificidades dos Nutrientes. (Ed) Nutrição da gestação ao envelhecimento. Rio de Janeiro.

Wilson JG. 1965. Methods for administering agentes and detecting malformations in experimental animals. In: Wilson, J.G., Waekany, J. (Eds.). Teratology: Principles snd Thecniques. The University of Chicago Press, Chicago.

Zhang C, Xu D, Luo H, Lu J, Ping J, Wang H. 2014. Prenatal xenobiotic exposure and intrauterine hypothalamus-pituitary-adrenal axis programming alteration. Toxicol 325:74-8.

Table 1: Biometric Parameters

Experimental Groups	Biometric Parameters				
	Inicial weight	Final weight	Weight gain	Uterine weight	Liquid weight
Control	30±1.17[a]	51.9±2.44[a]	21.9±1.59[a]	18.57±1.84[a]	0.35±0.03[a]
Pre-Imp. D1	29.9±0.53[a]	55.4±2.43[a]	25.5±2.20[a]	19.55±1.73[a]	0.35±0.02[a]
Pre-Imp. D2	32.2±1.23[a]	57.7±1.55[a]	25.5±1.95[a]	20.09±1.33[a]	0.34±0.02[a]
Pre-Imp. D3	32.3±0.97[a]	52.9±3.15[a]	20.6±3.04[a]	15.64±2.55[a]	0.28±0.03[a]
Organo. D1	29.4±0.73[a]	49.7±1.77[a]	20.3±2.09[a]	16.07±1.88[a]	0.31±0.03[a]
Organo. D2	29.6±1.42[a]	53.8±2.19[a]	24.2±1.81[a]	17.90±2.31[a]	0.33±0.04[a]
Organo. D3	28.6±1.24[a]	53.6±2.49[a]	25±1.56[a]	18.29±1.31[a]	0.34±0.01[a]
Gest. D1	29.3±0.92[a]	49.7±2.91[a]	20.4±2.88[a]	15.96±2.86[a]	0.30±0.05[a]
Gest. D2	29.8±1.01[a]	53.8±1.52[a]	24±1.82[a]	18.31±1.86[a]	0.33±0.03[a]
Gest. D3	30.22±1.08[a]	53.5±1.51[a]	21±2.62[a]	17.54±1.59[a]	0.32±0.03[a]
	Absolute weight of organs (g)				
	Heart	Lung	Spleen	Kidney	Liver
Control	0.16±0.01[a]	0.20±0.01[a]	0.11±0.01[a]	0.36±0.01[a]	2.07±0.12[a]
Pre-Imp. D1	0.16±0.01[a]	0.19±0.01[a]	0.11±0.01[a]	0.37±0.01[a]	2.19±0.12[a]
Pre-Imp. D2	0.16±0.01[a]	0.18±0.01[a]	0.12±0.01[a]	0.35±0.01[a]	2.37±0.09[a]
Pre-Imp. D3	0.18±0.01[a]	0.22±0.03[a]	0.13±0.02[a]	0.38±0.01[a]	2.36±0.13[a]
Organo. D1	0.16±0.004[a]	0.19±0.01[a]	0.13±0.005[a]	0.32±0.02[a]	2.06±0.11[a]
Organo. D2	0.17±0.02[a]	0.19±0.01[a]	0.12±0.01[a]	0.35±0.01[a]	2.20±0.08[a]
Organo. D3	0.14±0.01[a]	0.19±0.02[a]	0.11±0.01[a]	0.35±0.01[a]	2.17±0.11[a]
Gest. D1	0.17±0.01[a]	0.22±0.02[a]	0.20±0.10[a]	0.35±0.02[a]	2.05±0.13[a]
Gest. D2	0.17±0.01[a]	0.19±0.01[a]	0.14±0.02[a]	0.33±0.01[a]	2.21±0.05[a]
Gest. D3	0.18±0.01[a]	0.19±0.01[a]	0.12±0.01[a]	0.35±0.02[a]	2.03±0.16[a]
	Relative weight of organs				
	Heart	Lung	Spleen	Kidney	Liver
Control	0.0030±0.0002[a]	0.0040±0.0003[a]	0.0022±0.0003[a]	0.0070±0.0002[a]	0.0402±0.0018[a]
Pre-Imp. D1	0.0030±0.0002[a]	0.0036±0.0003[a]	0.0019±0.0002[a]	0.0091±0.0002[a]	0.0397±0.0017[a]
Pre-Imp. D2	0.0028±±0.0002[a]	0.0030±0.0002[a]	0.0020±0.0002[a]	0.0062±0.0003[a]	0.0410±0.0011[a]
Pre-Imp. D3	0.0033±±0.0002[a]	0.0044±0.0006[a]	0.0026±0.0005[a]	0.0074±0.0004[a]	0.0451±0.0018[a]
Organo. D1	0.0032±±0.0001[a]	0.0040±0.0003[a]	0.0026±0.0002[a]	0.0066±0.0007[a]	0.0416±0.0015[a]
Organo. D2	0.0031±±0.0003[a]	0.0035±0.0002[a]	0.0023±0.0002[a]	0.0067±0.0004[a]	0.0413±0.0002[a]
Organo. D3	0.0027±±0.0002[a]	0.0035±0.0003[a]	0.0021±0.0001[a]	0.0067±0.0004[a]	0.0407±0.0017[a]
Gest. D1	0.0035±±0.0002[a]	0.0046±0.0003[a]	0.0040±0.0019[a]	0.0067±0.0003[a]	0.0413±0.0015[a]

Gest. D 2	0.0032±±0.0002[a]	0.0055±0.0018[a]	0.0026±0.0004[a]	0.0063±0.0003[a]	0..0411±0.0006[a]
Gest. D3	0.0033±±0.0002[a]	0.0036±0.0002[a]	0.0023±0.0002[a]	0.0065±0.0003[a]	0.0380±0.0003[a]

Test: [1] Analysis of Variance /Tukey; [2] Kruskall-Wallis/Dunn; $p<0.05$).

Table 2: Reproductive Parameters

Parameters	**Experimental groups**									
	Control	**Pre-Imp. D1**	**Pre-Imp. D2**	**Pre-Imp. D3**	**Organo. D1**	**Organo. D2**	**Organo. D3**	**Gest. D1**	**Gest. D2**	**Gest. D3**
Implants	12.6±4.09[a]	12.5±4.17[a]	14.5±4.33[a]	11.2±5.24[a]	12.1±2.77[a]	12±5.18[a]	12±3.13[a]	10.6±5.64[a]	13.2±2.97[a]	11.33±3.54[a]
Life fetuses	12.3±4.03[a]	12±3.97[a]	13.3±3.86[a]	9.6±6.08[a]	10.8±4.55[a]	11.4±5.04[a]	11.3±3.09[a]	10.1±5.68[a]	11.9±4.04[a]	10.9±3.41[a]
FV	97.76±3.67[a]	96.44±5.1[a]	92.67±8.58[a]	86.92±30.95[a]	82.73±30.7[a]	94.89±5.93[a]	94.4±7.56[a]	93.84±12.39[a]	87.19±21.43[a]	96.38±4.36[a]
PILR	2.24±3.67[a]	3.56±5.1[a]	7.31±8.58[a]	13.07±30.95[a]	17.27±30.7[a]	5.1±5.93[a]	5.6±7.56[a]	6.16±12.39[a]	12.81±21.43[a]	9.61±4.36[a]
Resorption	0.2±0.42[a]	0.5±0.71[a]	1.2±1.32[a]	0.3±0.67[a]	0.9±1.29[a]	0.4±0.52[a]	0.7±0.95[a]	0.3±0.48[a]	0.9±1.2[a]	0.44±0.53[a]
RR	1.33±2.81[a]	3.56±5.1[a]	7.31±8.58[a]	2.36±9.99[a]	7.27±10.2[a]	3.89±5.6[a]	5.6±7.56[a]	3.57±6.66[a]	8.66±13.64[a]	3.61±4.36[a]
PW (g)	0.09±0.004[a]	0.11±0.001[a.b.c.d]	0.09±0.005[a.b]	0.10±0.002[d]	0.09±0.002[a.b]	0.09±0.002[a.b]	0.09±0.007[a.b]	0.10±0.001[a.b.c]	0.10±0.002[d]	0.10±0.008[a.b.c.d]
FW (g)	1.23±0.01[a.b]	1.31±0.01[b]	1.30±0.01[b]	1.17±0.01[a]	1.22±0.01[a]	1.25±0.01[a.b]	1.31±0.01[b]	1.19±0.02[a]	1.23±0.01[a]	1.27±0.02[a.b]
PI	0.07±0.005[a.b.c]	0.08±0.009[a.b.c.d]	0.07±0.005[a.b]	0.08±0.005[e]	0.07±0.002[a.b.c]	0.07±0.002[a.b]	0.07±0.005[a]	0.09±0.011[a.b.c.d.e]	0.08±0.002[e]	0.07±0.007[a.b.c.d]
SR	1.89±0.91[a]	1.36±0.26[a]	1.04±0.20[a]	1.13±0.28[a]	1.12±0.25[a]	1.48±0.52[a]	1.02±0.15[a]	1.09±0.22[a]	2.06±1.03[a]	0.72±0.12[a]
AWGA		AWGA	AWGA	OVGA	AWGA	AWGA	AWGA	OVGA	AWGA	AWGA

Legend: Different letters indicate significant differences.VF: fetal viability; PILR: post-implantation loss rate; RR: reabsorption rate; W: placental weight; PF: fetal weight; PI: placental index; SR: sexual reason. AWGA: fetuses with an appropriate weight for their gestational age; OVGA: body weight higher than the mean weight of the control group fetuses. (Test: [1] Analysis of Variance /Tukey; [2] Kruskall-Wallis/Dunn; $p<0.05$).

Table 3: External abnormalities found on the progeny in the different experimental groups.

Parameters	Control	Pre-Impl.D1	Pre-Impl.D2	Pre-Impl.D3	Organo.D1	Organo.D2	Organo.D3	Gest.D1	Gest.D2	Gest.D3
Anterior and posterior limb										
Analyzed Fetuses	118	120	113	89	90	125	112	105	121	115
Normal Fetuses	99	58	86	50	45	64	74	76	86	76
Unilateral posterior retroversion	10	31	14	21	23	29	17	7	16	18
Unilateral anterior retroversion	5	20	9	10	13	17	14	8	10	8
Unilateral posterior introversion	0	0	0	1	1	1	4	2	0	1
Unilateral anterior introversion	0	0	0	9	1	3	1	4	2	0
Anterior Unilateral Stretch	0	0	0	0	0	0	0	0	0	0
Poterior Unilateral Stretch	0	0	1	3	2	0	0	0	0	2
Bilateral posterior retroversion		7	2	3	5	4	2	4	5	6
Bilateral anterior retroversion	0	2	0	0	0	2	0	0	2	3
Bilateral posterior introversion	0	0	1	0	0	0	0	1	0	0
Bilateral anetrior introversion	0	0	0	1	0	1	0	1	0	0
Inferior short limb	2	2	0	1	0	4	0	0	0	1
Malformation frequency.	**19**	**62**	**27**	**49**	**45**	**61**	**38**	**27**	**35**	**39**
%M.F.	**16.10**	**51.67***	**23.89**	**55.06***	**50***	**48.8***	**33.93***	**25.71**	**28.92***	**33.91***
Tail										
Normal Fetuses	110	116	110	86	86	118	109	103	117	109
Comma tail	8	4	3	3	4	7	3	2	4	6
Malformation frequency.	**8**	**4**	**3**	**3**	**4**	**7**	**3**	**2**	**4**	**6**
%M.F.	**6.78**	**3.33**	**2.65**	**3.37**	**4.44**	**5.6**	**2.68**	**1.94**	**3.3**	**5.22**
Column										
Normal Fetuses	118	117	111	89	88	121	111	100	116	113
Scoliosis	0	3	2	0	4	4	1	5	5	2
Malformation frequency.	**0**	**3**	**2**	**0**	**4**	**4**	**1**	**5**	**5**	**2**
%M.F.	**0**	**2.5**	**1.77**	**0**	**4.44**	**3.2**	**0.89**	**4.76**	**4.13**	**1.74**
Nose										
Normal Fetuses	63	57	113	87	76	115	94	55	50	58
Obstructed coana	0	4	0	2	14	10	18	0	0	1
Malformation frequency.	**0**	**4**	**0**	2	14	10	18	**0**	**0**	**1**
%M.F.	**0**	**6.56**	**0**	**2.25**	**15.55***	**8***	**16.07***	**0**	**0**	**1.72**
Abdomen										

Normal Fetuses	118	120	113	87	90	125	111	103	121	114
Gastroschisis	0	0	0	2	0	0	1	2	0	1
Malformation frequency	**0**	**0**	**0**	**2**	**0**	**0**	**1**	**2**	**0**	**1**
%M.F.	**0**	**0**	**0**	**2.25**	**0**	**0**	**0.89**	**1.90**	**0**	**0.87**

Legend: %M.F. Mean value of malformation percentage; Compared statistically with the control.* Significant difference (Test: Chi-square; $p<0.05$).

Table 4: Visceral abnormalities found in the encephalic and urogenital regions in the offspring of different experimental groups.

Parameters	**Control**	**Pre-Impl.D1**	**Pre-Impl.D2**	**Pre-Impl.D3**	**Organo.D1**	**Organo.D2**	**Organo.D3**	**Gest.D1**	**Gest.D2**	**Gest.D3**
Brain-Hydrocephalus										
Analyzed Fetuses	63	61	66	48	41	60	57	55	50	59
Normal Fetuses	42	49	39	19	18	34	26	24	19	35
Weghtless hyd..	9	1	9	10	10	9	10	10	10	5
Medium hyd.	4	8	12	11	12	17	19	14	8	10
Serious hyd.	8	3	6	8	1	0	2	7	13	9
Malformation frequency	**21**	**12**	**27**	**29**	**23**	**26**	**31**	**31**	**31**	**24**
%M.F.	**33.33**	**19.67**	**40.91**	**60.42***	**56.10***	**43.33**	**54.38***	**56.36***	**62***	**40.68**
Urogenital Region-Hydronephrosis										
Normal Fetuses	59	57	55	26	26	53	49	36	32	52
Weghtless hy.	2	2	5	7	11	5	3	5	3	5
Medium hy.	0	2	5	10	4	2	4	2	2	1
Serious hy.	2	0	1	5	0	0	1	12	13	1
Malformation frequency	**4**	**4**	**11**	**22**	**15**	**7**	**8**	**19**	**18**	**7**
%M.F.	**6.35**	**6.56**	**16.67**	**45.83***	**36.58***	**11.67**	**14.03**	**34.54***	**36***	**11.86**
Heart										
Normal Fetuses	118	120	113	88	86	124	108	54	46	53
Ventriculomegaly	0	0	0	0	4	0	0	0	0	1
Bifurcated Heart	0	0	0	1	0	1	4	1	4	5
Malformation frequency.	**0**	**0**	**0**	**1**	**4**	**1**	**4**	**1**	**4**	**6**
%M.F.	**0**	**0**	**0**	**1.12**	**4.44**	**0.8**	**0.35**	**1.82**	**8***	**10.17***

Legenda: %M.F. Mean value of malformation percentage. Hyd.-Hidrocephalus; Hy.- Hydronephrosis. Compared statistically with the control.* Significant difference (Test: Chi-square; $p<0.05$).

Table 5: Skeletal abnormalities found in anterior and posterior limbs. sternum. head and mandible and column of different experimental groups.

Parameters		Control	Pre-Impl.D1	Pre-Impl.D2	Pre-Impl.D3	Organo.D1	Organo.D2	Organo.D3	Gest.D1	Gest.D2	Gest.D3
Anterior and Posterior Limbs											
Analyzed Fetuses		55	59	47	41	49	65	55	50	71	56
Normal Fetuses		52	46	34	33	34	59	47	41	58	46
Phalanges	Absent	0	0	0	0	0	0	0	0	0	0
	R.O.	3	12	11	7	14	6	6	5	10	9
Metacarpus-metatarsus	Absent	0	0	0	0	0	0	0	0	0	0
	R.O	0	1	2	1	1	0	2	4	3	1
Radio	Irregular	0	0	0	0	0	0	0	0	0	0
Humerus	Irregular	0	0	0	0	0	0	0	0	0	0
Malformation frequency		**3**	**13**	**13**	**8**	**15**	**6**	**8**	**9**	**13**	**10**
%M.F.		**5.45**	**22.03***	**27.66***	**19.51**	**30.61***	**9.23**	**14.54**	**18**	**18.31**	**16.95**
Sternum											
Normal Fetuses		36	31	15	3	1	18	35	34	45	38
Sternum centers	Absent	3	0	3	0	0	0	1	2	2	0
	R.O	0	0	0	0	0	0	0	8	17	0
	Irregular	13	14	15	17	19	18	7	3	2	12
Manubrium	Absent	0	0	0	1	1	0	0	0	0	1
	R.O	0	0	0	0	0	0	0	0	0	0
	Irregular	3	5	4	6	5	2	0	0	0	2
Xiphoid Process	Absent	0	0	0	0	0	0	0	0	0	0
	R.O	0	0	0	0	0	0	0	0	0	0
	Irregular	0	9	13	14	23	27	12	4	5	3
Malformation frequency		**19**	**28**	**32**	**38**	**48**	**47**	**20**	**17**	**26**	**18**
%M.F.		**34.54**	**47.46**	**68.08***	**92.68***	**97.96***	**72.31***	**3.64**	**34**	**36.62**	**32.14**
Head and Jaw											
Normal Fetuses		112	74	71	47	57	83	71	83	94	90
Palate	Irregular	6	46	42	34	33	41	41	4	27	25
	R.O	0	0	0	0	0	0	0	0	0	0
Par.Fron.Occip.	Absent	0	0	0	8	0	1	0	0	0	0
	R.O	0	0	0	0	0	0	0	18	0	0

Malformation frequency		**6**	**46**	**42**	**42**	**33**	**42**	**41**	**22**	**27**	**25**
%M.F.		**5.08**	**38.33***	**37.17***	**47.19***	**36.67***	**33.6***	**36.61***	**20.95***	**22.31***	**21.74***
					Spine						
Normal Fetuses		53	55	46	41	49	65	55	42	70	56
Vertebrae	Absent	0	1	0	0	0	0	0	0	0	0
	Irregular	2	3	1	0	0	0	0	8	1	0
Malformation frequency		**2**	**4**	**1**	**0**	**0**	**0**	**0**	**8**	**1**	**0**
%M.F.		**3.64**	**6.78**	**2.17**	**0**	**0**	**0**	**0**	**16**	**1.41**	**0**

Legend: %M.F: Mean value of malformation percentage; Par.-Parietal; Fron.-Frontal; Occip.-Occipital; R.O- Reduced Ossification. Compared with the control.* Significant difference (Test: Chi-square; $p<0.05$).

Table 6: Micronucleus

Experimental groups	**Micronucleus**		
	24h	**48h**	**72h**
Control	2.5 ± 0.54^{a}	2.6 ± 0.48^{a}	$3.6\pm0.54^{a.b}$
Pre-Impl.D1	3.33 ± 0.67^{a}	6.25 ± 0.37^{b}	3.33 ± 0.96^{a}
Pre-Impl.D2	3.8 ± 0.97^{a}	$4.5\pm0.75^{a.b}$	$3.7\pm0.37^{a.b}$
Pre-Impl.D3	6.2 ± 0.66^{a}	5.9 ± 0.89^{b}	$5.2\pm0.7^{a.b}$
Organo.D1	4.4 ± 0.73^{a}	$3.7\pm0.67^{a.b}$	$4.1\pm0.43^{a.b}$
Organo.D2	5.67 ± 0.68^{a}	$5.89\pm0.84^{a.b}$	$5.22\pm0.59^{a.b}$
Organo.D3	4.2 ± 1.26^{a}	$4.2\pm0.66^{a.b}$	$5.88\pm0.90^{a.b}$
Gest.D1	3.8 ± 0.74^{a}	$4.22\pm0.49^{a.b}$	3 ± 0.63^{a}
Gest.D2	6.2 ± 0.73^{a}	$5\pm0.73^{a.b}$	$4\pm0.82^{a.b}$
Gest.D3	6 ± 1.46^{a}	$5.89\pm1.15^{a.b}$	6.7 ± 0.63^{b}

Legend: Different letters indicate significant differences. (Test: [1]ANOVA; [2] Tukey; $p<0.05$).

Table 7: Phagocytosis

Experimental groups	Phagocytosis With evidence
Control	21.2±2.53[a]
Pre-Impl.D1	31.1±1.32[b]
Pre-Impl.D2	29.7±1.81[b]
Pre-Impl.D3	28.2±1.26[a.b]
Organo.D1	27.8±0.61[a.b]
Organo.D2	28.8±1.07[b]
Organo.D3	32.2±1.81[b]
Gest.D1	33.1±2.50[b]
Gest.D2	29.5±1.80[b]
Gest.D3	30.7±1.04[b]

Legend: Different letters indicate significant differences. (Teste: [1]ANOVA; [2] Tukey; $p<0.05$).

Conclusões:

O Temefós não apresentou toxicidade maternal, não alterou o desempenho reprodutivo e desenvolvimento embriofetal, e também não causou alterações na integridade do DNA.

Piriproxifen não apresentou toxicidade maternal, não alterou o desempenho reprodutivo e desenvolvimento embriofetal, e também não causou alterações na integridade do DNA. E de forma pioneira em modelos pré-clínicos, mostrou que este produto não tem relação com os casos de microcefalia.

Diflubenzuron não apresentou toxicidade maternal, não alterou o desempenho reprodutivo e não causou alterações na integridade do DNA. Porém, este produto induziu malformações, em especial, em de coração. Esse efeito teratogênico sugere que o uso deve seja descontinuado.

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Evaluation of the effects of the larvicides temephos on reproductive performance, embryofetal development and DNA integrity of Swiss mice

Juliana Miron Vani[a,b], Laynna de Carvalho Schweich[a,b], Karla Regina Warszawski de Oliveira[c,d], Sarah Alves Auharek[e], Andréa Luiza Cunha-Laura[f], Andréia Conceição Millan Brochado Antoniolli-Silva[a,b], Carlos Eduardo Domingues Nazario[c,d], Rodrigo Juliano Oliveira[a,b,g,*]

[a] Centro de Estudos em Células Tronco, Terapia Celular e Genética Toxicológica - CeTroGen, Hospital Universitário Maria Aparecida Pedrossian – HUMAP, Universidade Federal de Mato Grosso do Sul - UFMS, Campo Grande, MS, Brazil
[b] Programa de Pós-graduação em Saúde e Desenvolvimento na Região Centro-Oeste, Faculdade de Medicina "Dr. Hélio Mandetta" – FAMED, Universidade Federal de Mato Grosso do Sul – UFMS, Campo Grande, MS, Brazil
[c] Programa de Pós-graduação em Química - INQUI, Universidade Federal de Mato Grosso do Sul- UFMS, Campo Grande, MS, Brazil
[d] Laboratório de Combustiveis - LABCOM, Instituto de Química - INQUI, Universidade Federal de Mato Grosso do Sul- UFMS, Campo Grande, MS, Brazil
[e] Faculdade de Medicina do Mucuri, Universidade Federal dos Vales do Jequitinhonha e do Mucuri – UFVJM, Teófilo Otoni, MG, Brazil
[f] Programa de Mestrado em Farmácia, Instituto de Biociências - INBIO, Universidade Federal de Mato Grosso do Sul – UFMS, Campo Grande, MS, Brazil
[g] Programa de Pós-graduação em Genética e Biologia Molecular, Centro de Ciências Biológicas – CCB, Universidade Estadual de Londrina, Londrina, PR, Brazil

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ABSTRACT

Temephos is considered the gold standard by the Ministry of Health for controlling the larvae of the mosquito *Aedes aegypti*. The present study evaluated the effects of Temephos larvicide on the reproductive performance, embryo-fetal development and DNA integrity of Swiss mice. This study used 30 pregnant female mice: 10 were controls treated with drinking water at a dosage of 0.1 mL/10 g (body weight – b.w., administered orally - a.o.), and 20 were treated with Temephos at doses of 0.0043 mg/kg and 0.043 mg/kg (b.w., a.o.) during the gestational period. Statistical analysis showed that Temephos did not alter the biometric or reproductive parameters. Comparing the weight of the fetus to the stage of pregnancy demonstrated that the 0.0043 mg/kg dosage increased the size of the fetuses. No external malformations were detected. However, the 0.043 mg/kg dosage induced changes in the sternum, with the main change being the center of the sternum, xiphoid processes and absence of the manubrium. The other skeletal and visceral alterations did not differ from the control group and are considered variants of normality. The analysis of head measurements showed an increase in the anterior/posterior measurements of the glabella, the external occipital protuberance and the biauricular plane. The circumference and area of the head did not present significant differences. The micronucleus test showed only a 0.043 mg/kg increase in 48 h. Thus, it is considered that Temephos has a low teratogenic and genotoxic risk.

1. Introduction

Brazil is one of the biggest consumers of insecticides in the world, and the insecticide is used for both pest control in agriculture as well as the elimination of mosquitoes that transmit diseases in urban areas [1]. However, the use of these chemical agents (larvicides, insecticides and/or growth inhibitors) may correlate with genetic/genomic damage that predisposes individuals to chronic diseases such as cancer [2–4].

Control programs for insect vectors in different countries use organophosphates and pyrethroids on a large scale [5]. Temephos larvicide, an organophosphate, is commonly used to combat mosquitoes and black flies. Additionally, it is often used to kill the larvae of the mosquito *Aedes aegypti* (*Ae. Aegypti*) and *Aedes albopictus* to combat epidemics of Dengue fever, and more recently Chikungunya fever and Zika Virus. The Ministry of Health in Brazil indicates that the continued use of this product for 12 years is considered safe for humans and suitable for handling programs. However, it was eventually replaced after *Ae. aegypti* mosquitoes became resistant [6–8]. Nevertheless, this compound is still available on the market.

This larvicide can be deposited in water tanks near mosquito outbreaks, as well as in other water containers. It can also be used for fumigation [9]. The greatest concern about this type of application is

* Corresponding author at: Medicine College, Federal University of Mato Grosso do Sul, Cidade Universitária, S/N, Campo Grande, MS 79070-900, Brazil.
E-mail address: rodrigo.oliveira@ufms.br (R.J. Oliveira).

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Table 1
Parameters related to growth development and organ weight of the females treated witch Temephos.

Biometrics parameters					
Experimental groups	Initial weight[1]	Final weight[1]	Weight gain[1]	Weight utero[1]	Liquid weight gain[1]
Control	29,90 ± 0,50[a]	55,77 ± 1,25[a]	25,87 ± 1,29[b]	19,45 ± 1,47[a]	6,42 ± 0,72[a]
Gest. D1	29,30 ± 0,54[a]	54,06 ± 1,42[a]	24,76 ± 1,28[a,b]	19,16 ± 1,04[a]	5,60 ± 0,60[a]
Gest. D2	30,30 ± 0,61[a]	51,12 ± 1,67[a]	20,82 ± 1,28[a]	15,31 ± 1,40[a]	5,51 ± 0,44[a]
Absolut weight organs (g)					
	Heart[1]	Lung[1]	Spleen[1]	Liver[1]	Kidneys[1]
Control	0,21 ± 0,015[a]	0,26 ± 0,02[a]	0,16 ± 0,008[a]	0,39 ± 0,01[a]	2,84 ± 0,09[a]
Gest. D1	0,18 ± 0,007[a]	0,24 ± 0,01[a]	0,14 ± 0,011[a]	0,38 ± 0,01[a]	2,60 ± 0,09[a]
Gest. D2	0,19 ± 0,010[a]	0,24 ± 0,03[a]	0,17 ± 0,015[a]	0,39 ± 0,01[a]	2,73 ± 0,12[a]
Relative weight of organs					
	Heart[1]	Lung[1]	Spleen[1]	Liver[1]	Kidneys[1]
Control	0,004 ± 0,0003[a]	0,005 ± 0,0004[a]	0,003 ± 0,0001[a]	0,007 ± 0,0002[a]	0,05 ± 0,002[a]
Gest. D1	0,003 ± 0,0001[a]	0,004 ± 0,0002[a]	0,003 ± 0,0001[a]	0,007 ± 0,0002[a]	0,05 ± 0,001[a]
Gest. D2	0,004 ± 0,0001[a]	0,005 ± 0,0005[a]	0,003 ± 0,0001[a]	0,008 ± 0,0002[b]	0,05 ± 0,001[a]

Legend: Different letters (a and b) indicate statistically significant differences. (Test: [1]ANOVA/Tukey; p<0,05).

Table 2
Reproductive parameters for females treated witch Temephos.

Experimental groups			
Reproductive parameters	Control	Gest. D1	Gest. D2
Implants[1]	14,20 ± 0,65[a]	13,40 ± 0,58[a]	12,70 ± 0,99[a]
Live fetuses[1]	12,70 ± 1,03[a]	12,40 ± 0,73[a]	9,50 ± 1,03[a]
Dead fetuses[2]	0,00 ± 0,00[a]	0,00 ± 0,00[a]	0,30 ± 0,21[a]
Number fetuses	127,00	124,00	95,00
Fetal viability[1]	88,83 ± 4,79[a,b]	93,00 ± 4,47[b]	76,50 ± 7,15[a]
Post-implantational losses[1]	74,63 ± 4,63[a]	79,60 ± 4,66[a]	63,80 ± 7,54[a]
Resorption[1]	1,50 ± 0,69[a]	1,00 ± 0,63[a]	2,90 ± 1,05[a]
Reabsorção rate[1]	11,17 ± 4,80[a]	7,00 ± 4,47[a]	20,65 ± 6,81[a]
Placental weight (g)[2]	0,10 ± 0,002[a]	0,10 ± 0,003[a]	0,10 ± 0,002[a]
Placental index[2]	0,08 ± 0,010[a]	0,08 ± 0,002[a]	0,08 ± 0,002[a]
Fetuses weight (g)[2]	1,20 ± 0,01[a]	1,22 ± 0,01[a]	1,20 ± 0,01[a]
AWGA		AWGA	OVGA
Sex ratio[1]	1,84 ± 0,46[a]	1,34 ± 0,20[a]	1,40 ± 0,24[a]

Legend: AWGA: fetuses with an appropriate weight for their gestational age; OVGA: body weight higher than the mean weight of the control group fetuses. Different letters (a and b) statistically significant differences (Test: [1]ANOVA/Tukey; [2] Kruskall-Wallis/Dunn; p < 0,05).

Table 3
Relationship and frequency of external malformations in offspring of females treated with Temephos.

Experimental groups			
Parameters	Control	Gest.D1	Gest.D2
Members			
Analyzed fetuses	127	124	95
Normal fetuses	122	117	89
Retr. post. unilateral	5	7	5
Hematoma	0	0	1
Malf.Freq.	5	7	6
%M.F.	3,94	5,64	6,31
Tail			
Normal fetuses	126	120	89
Rolled up tail	1	4	6
Malf.Freq.	1	4	6
%M.F.	0,79	3,22	6,31
Sistemic malformation and abdomen			
Normal fetuses	125	124	94
Hydrops	2	0	0
Gastroschisis	0	0	1
Malf.Freq.	2	0	1
%M.F.	1,57	0,00	1,05

Legend: Malf.Freq. – Frequency of malformation; %M.F. – average value percentage of malformation; Retr. – Retroversion; Post – posterior. Statically compared witch the control (chi – square test, p > 0,05).

that water in the water tanks is consumed by humans for cooking and hydration, which may cause accidental intoxication.

Despite this information, the literature does not present any data about the effects of this larvicide on teratogenesis models. These assays are important for evaluating the risk of intoxication in pregnant women and their descendants, as pregnancy alters women's metabolisms making them more susceptible to the effects of xenobiotics [10,11]. It is also critical to identify the possible side-effects this larvicide may have on newborns. In light of these facts, the present study evaluated the effects of Temephos larvicide on reproductive performance, embryo-fetal development and induction of DNA damage in pregnant female mice.

2. Materials and methods

2.1. Chemical agents

Temephos Ferson 1G (Fersol Indústria e Comércio S/A - Lot: LT552/FAB/09/DEZ/AN/09/DEZ/00/0198), an insecticide product indicated for the control of *Ae. aegypti* larvae, is composed of Temephos 1% w/w, emulsifiers, solvent, diluent and vehicle 99% w/w. The product was diluted in distilled water.

According to OMS (Word Health Organization) [12], the recommended dose of Temephos is 1 ppm. The Ministry of Health also recommends the use of this concentration.

To calculate the lower dose, we used the commercial dose (0.0043 mg/mL of Temephos when ingested by the mouse via gavage, corresponding to the amount of Temephos that would be present in 3 L of water containing Temephos at 1 ppm) that corresponds to when Temephos is deposited in a water tank and 3 L of water are ingested daily by a 70 kg person; this dose was determined [13] using the following formulation (Temephos in 1 ppm × 30 g)/(70.000 g). The dose was subsequently adjusted to the mouse's weight.

The 0.0043 mg/kg (b.w., a.o.) dose is based on the larvicide dose for commercial use, and the higher dose of 0.043 mg/kg (b.w., a.o.) is 10× higher than the recommended dose [14,15].

2.2. Experimental animals

Swiss mice (*Mus musculus*) (30 females and 15 males) of reproductive age (8–10 weeks) with an average weight of 30 g were obtained from the State Bureau of Animal and Plant Health Protection (Agência Estadual de Defesa Sanitária Animal e Vegetal - IAGRO). This

Table 4
Relation and frequency of visceral malformations in the offspring of females treated with Temephos.

Experimental groups			
Parameters	Control	Gest.D1	Gest.D2
Brain- hydrocephalus			
Analyzed fetuses	64	62	48
Normal fetuses	7	2	6
Mild hydroc.	46	35	33
Severe hydroc.	11	25	9
Malf.Freq.	57	60	42
%M.F.	89,06	96,77	87,50
Region urogenital - hydronephrosis			
Normal fetuses	56	58	36
Mild hydron.	8	4	9
Severe hydron.	0	0	3
Malf.Freq.	8	4	12
%M.F.	12,5	6,45	25

Legend: Malf.Freq. – Frequency malformation; %M.F. – average value percentage of malformation; Hydroc. - Hydrocephalus; Hydron. - Hydronephrosis. Statically with the control (Chi-Square test, $p > 0,05$).

Table 5
Relationship and frequency of skeletal malformations in the offspring of females treated with Temephos.

Experimental groups				
Parameters		Control	Gest.D1	Gest. D2
Members				
Analyzed fetuses		63	62	47
Normal fetuses		0	0	0
Phalanges	Absence	55	53	41
	R.O.	3	1	0
Metac.Metat.	Absence	5	8	6
Malf.Freq.		63	62	47
%M.F.		100	100	100
Sternum				
Normal fetuses		56	58	32
Sternum centers	Absence	1	0	1
	R.O.	0	4	4
	Irregular	4	0	3
Xiphoid process	Absence	1	0	0
	Irregular	1	0	6
Manubrium	Absence	0	0	1
	Irregular	0	0	0
Malf.Freq.		7	4	22
%M.F.		11,11	6,45	46,81*
Head and Jaw				
Normal fetuses		40	45	29
Pal.Pres.	Absence	0	0	0
Occiptal	R.O.	0	0	1
Malf.Freq.		23	17	18
%M.F.		36,51	27,42	38,30

Legend: Malf.Freq. – Frequency Malformation; %M.F. – average value percentage pf malformation; Metac. - Metacarpus; Metat. - Metatarsus; Pal. - Palate; Presf. - Presphenoid; R.O. - Reduced Ossification. *Different statistically significant in relation to control. (Chi-Square test, $p < 0,05$).

study was approved by the Ethics Committee for Animal Experimentation of the Federal University of Mato Grosso do Sul (No. 808/2016).

The animals were maintained in propylene boxes; males were housed in isolation, while the females were housed in pairs. The mice underwent an adaptation period of seven days. Light and temperature were controlled with a 12-hour light cycle (12 h of light, 12 h of dark) and temperature maintained at 22 ± 2 °C in an ALESCO® ventilated cabinet. The mice were provided commercial feed (Nuvital ®) and filtered water ad libitum.

Table 6
Head measurements of offspring of females treated with Temephos.

Experimental groups			
Parameters (mean in mm)	Control	Gest.D1	Gest.D2
Measure 1 - Anterior/ Posterior[2]	9,73 ± 0,20[a,b]	10,21 ± 0,05[a]	10,48 ± 0,06[b]
Measure 2 - Glabella and external occipital protuberance[2]	7,58 ± 0,20[a]	8,00 ± 0,07[a]	8,35 ± 0,10[b]
Measure 3 - Binaural[2]	5,19 ± 0,20[a]	6,00 ± 0,02[b]	6,06 ± 0,04[b]
Measure 4 - Head circunference[2]	3,05 ± 0,03[a]	3,11 ± 0,03[a]	3,10 ± 0,03[a]
Measure 5 - Area of head circunference[2]	0,74 ± 0,01[a]	0,77 ± 0,01[a]	0,77 ± 0,02[a]

Legend: Measure 1 - anterior/posterior- glabella and external occipital protuberance passing over the sagittal suture and bregma; 2 glabella and external occipital protuberance; 3 binaural-reference point to the superior insertion of the ears; 4 head circumference; 5 area of the head circumference. Different letters (a and b) indicate statically significant differences (Test: [1]ANOVA/ Tukey; [2]Kruskall-Wallis/Dunn; $p<0,05$).

Overnight mating was performed at a ratio of 1 male: 2 females, and detection of pregnancy was performed based on the observation of vaginal plug formation (considered day zero of gestation) [16–20].

2.3. Experimental design

The pregnant females were divided into three experimental groups (n = 10). In the control group, – the animals received drinking water at the proportion of 0.1 mL/10 g body weight (b.w.) administered orally (a.o. – gavage) during all days of gestation (1st to 18th). In the gestational groups, animals received Temephos (a.o. - gavage) at a dose of either 0.0043 mg/kg (Gest.D1) or 0.043 mg/kg (Gest.D2) daily throughout gestation (Gestational Group Dose 0.0043 – Gest.D1) and 0.043 mg/kg during all days of gestation (Gestational Group Dose 0.043 – Gest.D2).

3. Biological assays

3.1. Reproductive performance and embryonic and fetal development (teratogenicity)

On the 18th day of gestation, the animals were euthanized, followed by laparotomy, hysterectomy and oophorectomy. The spleen, heart, liver, lungs and kidneys were collected and weighed. The fetuses and placentas were also weighed. An external systematic analysis of the fetuses was performed to detect possible external malformations. Subsequently, the sex of each fetus was identified. The number of implantations was recorded; when there was doubt, the Salewski [21] technique was used. The number of resorptions and live and dead fetuses were recorded. Based on these data, fetal viability (number of live fetuses/number of implantations × 100), the post-implantation loss rate (number of implantations − number of live fetuses × 100/number of implantations), the resorption rate (number of resorptions × 100/ number of implantations), the placental index (placental weight / fetal weight) and the sex ratio (number of male fetuses / number of female fetuses) were obtained [16–18,20]. Then, the suitability of the observed fetal weight for the gestational age was determined according to Oliveira et al. [16], with the fetuses being classified as follows: fetuses with an appropriate weight for their gestational age (AWGA), showing a body weight within the mean weight of the control group fetuses plus or minus the standard deviation; fetuses with a low weight for their gestational age (LWGA), showing a body weight lower than the mean weight of the control group fetuses minus the standard deviation of the same group; or fetuses overweight for their gestational age (OVGA),

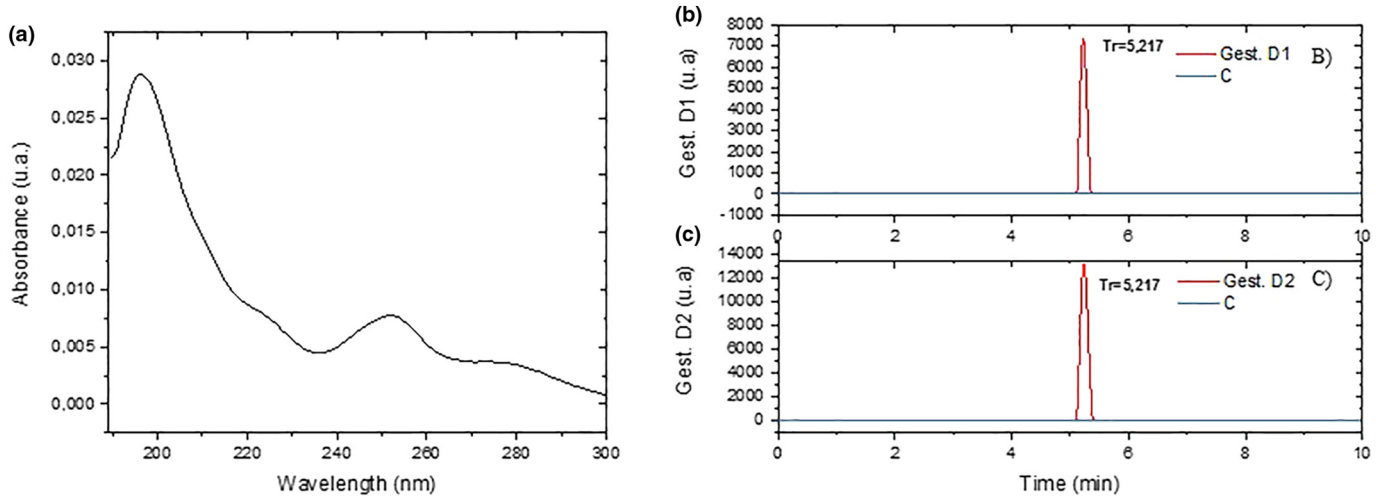

Fig. 1. Figure 1A: UV Scan of Temephos in mobile phase Figure 1B,C: Chromatograms obtained by the method for relation Dose x Control: B) Gest. D1 x Control; C) Gest. D2 x Control

Table 7
Micronucleus assay.

Experimental groups	Micronucleus		
	24 h	48 h	72 h
Control	5,70 ± 1,32[a]	5,20 ± 1,06[a]	8,90 ± 1,35[a]
Gest. D1	10,00 ± 2,16[a]	9,20 ± 1,64[a]	7,30 ± 1,22[a]
Gest. D2	13,70 ± 2,03[a]	13,70 ± 2,10[b]	7,67 ± 1,35[a]

Legend: Different letters (a and b) indicate statically significant differences. (Test: ANOVA/Tukey p<0,05).

showing a body weight higher than the mean weight of the control group fetuses plus the standard deviation of the same group.

Subsequently, the fetuses were randomly divided into two subgroups. The first was intended for visceral analysis, for which the fetuses were fixed in Bodian's solution distilled water (142 mL), acetic acid (50 mL), formaldehyde (50 mL) and 95% ethanol (758 mL) for at least seven days. Visceral analysis was performed via microdissection with strategic cuts to examine the chest and abdomen according to Barrow and Taylor [22] and to examine the head according to Wilson [23] as modified by Oliveira et al. [16]. Visceral changes were described based on the studies by Taylor [24], Manson and Kang [25], Damasceno et al. [26] and Oliveira et al. [16]. The second subgroup of fetuses were intended for skeletal analysis using the alizarin red technique proposed by Staples and Schnell [27] as modified by Oliveira et al. [16]. The fetuses were fixed in acetone for at least seven days. For the diaphonization process, the fetuses were eviscerated and placed in a solution of KOH (0.8%) followed by four drops of alizarin. This solution was replaced every 24 h over four days. After this period, the KOH solution was discarded, and the fetuses were placed in a bleaching solution (1 L glycerin,1 L of ethyl alcohol: 0.5 L of benzyl alcohol), which was replaced every 24 h for seven days. Skeletal changes were classified according to Taylor [24], Manson et al. [28], Damasceno et al. [26] and Oliveira et al. [16].

All analyses were performed under a stereomicroscope (NIKON SMZ745T).

3.2. Analysis of head measurements

For head measurement parameters, the child neurology protocol [29] was used as a reference. The first measurement was anterior/posterior (glabella and external occipital protuberance passing over the sagittal suture and bregma - measure 1), the second measurement was made in the glabella and external occipital protuberance (measure 2), and the third measurement was binaural (reference point to the superior insertion of the ears, with the tape measure over the coronal suture and passing through the bregma - measure 3). All measurements were carried out with the aid of a digital caliper (ZAAS Precision®).

Posteriorly, the fourth measurement was made using a tape measure. The head circumference (measure 4) was obtained, and then, the area of the head circumference (measure 5) was calculated using the formula $A = \pi r^2$.

3.3. Design of HPLC method for placenta analysis

An instrumental HPLC procedure coupled with DAD detector was designed to reveal that Temephos reached the fetus by contacting the placenta in the mother. The method aims to identify chemical traces in the placenta at the typical concentration then magnify it times ten utilizing a Shimadzu HPLC Prominence 20A with quaternary pump, autosampler and a DAD detector. The specific conditions for the analyses were determined by test optimization and method validation. The Temephos was extracted from the placenta using the QueCHERS method as described by Anastassiades et al. [30].

Placenta samples were prepared for the usual dose (Gest group. D1), 10 times usual dose (Gest group. D2) and negative control group. The identified ideal conditions for the HPLC-DAD method were mobile phase of acetonitrile – water 80:20 (v/v), chromatographic column Eclipse C18 (100 mm × 5.0 mm, 2.1 µm) and 200 nm wavelength, at room temperature (25 °C) and 10 min retention time.

3.4. Micronucleus assay

The technique used for the micronucleus assay was based on Hayashi et al. [31], as modified by Oliveira et al. [16]. A total of 20 µL of peripheral blood was collected via tail vein puncture, deposited on a slide that was previously stained with acridine orange (1 mg/mL) and then covered with a coverslip. Samples were collected on the 16th, 17th and 18th gestational day to assess whether Temephos had the ability to cause cumulative damage. The slides were stored in a freezer at −20 °C for at least 15 days. A total of 2000 cells/animal were analyzed under an epifluorescence microscope (Motic®; Model BA 410) at a magnification of 400×.

3.5. Statistical analysis

The data are presented as the mean ± standard error of the mean

(SEM) and were evaluated according to the nature of their distribution (parametric: ANOVA/Tukey test; nonparametric: Kruskal - Wallis/Dunn test). To compare frequencies (percentages) between the control and experimental groups, the chi-square test was used. For qualitative data and frequencies, the litter was utilized as the unit basis, as recommended in the literature [32]. However, for quantification of the visceral and skeletal malformations, the fetus was used as the basic unit according to Moreira et al. [33] and Oliveira et al. [16]. The level of significance was set at $p < 0.05$.

4. Results

4.1. Evaluation of biometric parameters and reproductive performance

4.1.1. Evaluation of biometric parameters

The initial weight, final weight, uterine weight and net weight gain did not differ between groups ($p > 0.05$), and there was a reduction of weight gain in the Gest group. D2 ($p < 0.05$).

In regards to the absolute and relative weight of the groups, there was an increase in relative weight of the kidneys in the Gest group. D2 ($p < 0.05$). There were no significant differences between the other groups ($p > 0.05$) (Table 1).

4.1.2. Evaluation of reproductive performance

The relative parameters of reproductive performance (number of implants, number of live fetuses, number of dead fetuses, fetal viability, rate of post-implantation loss, resorption number, resorption rate, placental weight, placental index, fetal weight and sex ratio) did not present significant differences between the groups ($p > 0,05$) (Table 2).

4.2. Evaluation of embryo-fetal development

4.2.1. External, visceral and skeletal malformations

External malformations occurred in all groups, including the control group. The identified malformations include unilateral hind paw retroversion, comma tail, hydrops and gastroschisis (Table 3). There were no statistically significant differences observed between the groups.

Visceral malformations were found in the brain region (hydrocephalus) and in the urogenital region (hydronephrosis) in all groups. There were no significant differences between the experimental groups and the control group ($p > 0,05$) (Table 4).

Skeletal malformations were observed in all groups, including control. However, only the Gest group. D2 had an increased incidence of sternal malformations ($p < 0.05$) (Table 5). In general, the malformations observed were reduced ossification and/or agenesis of phalanges, metacarpus, metatarsus, sternum centers, xiphoid process, manubrium, palate, presphenoid and the occipital bone.

4.2.2. Evaluation of head measurements

No significant differences were observed for the anterior/posterior measurements of the occipitofrontal circumference and the circumferential area. The glabella measurement and external occipital protuberance measurements were increased in the Gest group. D1, and the binaural measurement was increased in both the Gest. D1 and Gest. D2 groups ($p > 0.05$) (Table 6).

4.2.3. Identification and quantification of temephos in the placenta by HPLC-DAD

The Fig. 1A shows the resultant ultraviolet spectrum from sample scanning, which is compatible with the Temephos structural elucidation described in the literature [34]. The presence of Temephos could be verified. Additionally, the negative control reading differed from the sample readings demonstrating that the chemical indeed reaches the placenta (Fig. 1B,C). Even after its metabolism, Temephos doses of 0.0012 mg/kg (Gest group. D1) and 0.018 mg/kg (Gest group. D2) were found with a retention time of 5.1273.

4.3. Toxicogenic evaluation: micronucleus test

The micronucleus assay showed an increase at 48 h in the Gest group. D2 ($p < 0,05$). There were no significant differences between groups at 24 or 72 h ($p > 0,05$) (Table 7).

5. Discussion

Our study simulated the ingestion of approximately 3 L of water with Temephos by pregnant women, as well as a second situation where the dose was 10× higher than the commercial dose. Our findings demonstrated that the use of this larvicide, even with a dose 10× higher than the recommended dose, did not cause changes to the biometric parameters suggesting that Temephos is not toxic to mammals. This result is corroborated by Danesi; Tacca [35] and Yu et al. [36] as they reported that weight loss in pregnant females is an important indicator of toxicity in the compound test.

The analyzed parameters in relation to reproductive performance reinforced the absence of maternal toxicity, since no differences were found between the treated groups and the control group. There was a weight gain during pregnancy in the Gest group. D1, however, this seemed to be an isolated event without concern as there were no differences in placental weight and index. In general, macrosomic fetuses were also associated with placental alterations and placental index [37].

In regards to embryo-fetal development, the external malformations (limbs, tail, systemic malformation and abdomen) were similar across all groups.

The visceral analysis found malformations in the brain (hydrocephalus) and urogenital system (hydronephrosis), which occurred in the control treated groups. However, they are considered variants of normality since they can regress after birth [17,24]. It is important to emphasize that fetuses were prematurely removed from the uterus, as is typically done in the field, reinforcing the idea that the reported malformations were variants of normality [16,18–20]. The same is true of the skeletal malformations, with the most common reduction or absence of ossification occurring in the phalanges, metacarpus, metatarsus, sternal centers, xiphoid process, manubrium, palate, pterophyte and occipital.

Measurements of occipitofrontal circumference (measure 4) and head circumference area (measure 5) were similar for all groups. The anterior/posterior measurements (measure 1), glabella and external occipital prothality (measure 2) and biauricular plate (measure 3) were only increased with an appropriate weight during pregnancy. Thus, once again, we discard the possibility of macrosomic fetuses or macrocephalus. This hypothesis is reinforced by the skeletal analysis that showed no irregularities in the size of the cranium bones. Further, Van Der Linden et al. [38], emphasizes that when newborns do not have weight fluctuations, it is an indication of also not having congenital abnormalities. This corroborates our findings as we also did not observe weight fluctuations within the treated groups.

In summary, the HPLC-DAD method showed that Temephos reaches the placenta, which can be justified by Temephos' high lipid solubility [39] and the fact that the placenta is a rich lipid tissue.

When DNA integrity was assessed, it was found that only the highest Temephos dose showed a significant increase in micronucleus frequency after 48 h. However, this amount declined by 72 h. This finding requires attention and caution in determining the safety of Temephos in pregnant females. This caution is reinforced by Aiub et al. [40], who found that higher doses of Temephos (21.45 μM) delivered to male *Wistar* rats is genotoxic. This same author also demonstrated that the lower commercial dose is genotoxic in the SOS and Ames test systems. Additionally, Benitez-Trinidad et al. [41] reported that through the use of a comet assay, Temephos increases DNA damage in human lymphocytes but does not increase MN frequency. In contrast, Temephos increased the tail length, tail moment and MN frequency in HepG2 cells

compared to control cells. Therefore, Temephos causes stable DNA damage in HepG2 cells but not in human lymphocytes.

The current study shows that Temephos does not induce maternal toxicity, alter reproductive performance, or alter embryo-fetal development, but does cause discrete DNA damage in pregnant females as evaluated by the micronucleus test. This could predispose animals to chronic diseases such as cancer. Thus, Temephos is considered to have a low risk of teratogenicity and genotoxicity.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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References

[1] M.E.B. de Mélo, K.da A.C. Merlo, R.R.deC. Fernandes, C.F. Luna, G.T.N. Diniz, M.T.J.deA. Catanho, et al., Ação mutagênica do inseticida organofosforado temefós em células de medula óssea de camundongos, Rev. Int. Adolfo Lutz. 67 (2008) 196–201.

[2] G. Mathew, M.A. Rahiman, K.K. Vijayalaxmi, In vivo genotoxic effects in mice of Metacid 50, an organophosphorus insecticide, Mutagenesis 5 (1990) 147–149.

[3] A.L. Barros, V.V. Souza, S.D. Navarro, A.S. Oesterreich, R.J. Oliveira, C.A.L. Kassuya, et al., Genotoxic and mutagenic effects of diflubenzuron, an insect growth regulator, on mice, J. Toxicol. Environ. Health A. 76 (2013) 1003–1006, http://dx.doi.org/10.1080/15287394.2013.830585.

[4] N.deC. Ghisi, E.C. Oliveira, A.J. Prioli, Does exposure to glyphosate lead to an increase in the micronuclei frequency? A systematic and meta-analytic review, Chemosphere 145 (2016) 42–45.

[5] D.F. Diniz, M.A. DE Melo-Santos, E.M. Santos, E.B. Beserra, E. Helvecio, D. DE Carvalho-Leandro, et al., Fitness cost in field and laboratory *Aedes aegypti* populations associated with resistance to the insecticide temephos, Parasit. Vectors 30 (2015) 8–662, http://dx.doi.org/10.1186/s13071-015-1276-5.

[6] A. Soltani, H. Vatandoost, M.A. Oshaghi, N.M. Ravasan, A.A. Enayati, F. Asgarian, Resistance mechanisms of anopheles stephensi (Diptera: Culicidae) to temephos, J. Arthropod-Born Dis. 16 (2014) 71–78.

[7] M.G. Chediak, F.J.R. Pimenta, G.E. Coelho, I.A. Braga, J.B. Lima, K.R. Cavalcante, et al., Spatial and temporal country-wide survey of temephos resistance in Brazilian populations of *Aedes aegypti*, Mem. Inst. Oswaldo Cruz 111 (2016) 311–321, http://dx.doi.org/10.1590/0074-02760150409.

[8] L. Grigoraki, V. Balabanidou, C. Meristoudis, A. Miridakis, H. Ranson, L. Swevers, et al., Functional and immunohistochemical characterization of CCEae3a, a carboxylesterase associated with temephos resistance in the major arbovirus vectors *Aedes aegypti* and *Ae. albopictus*, Insect Biochem. Mol. Biol. 74 (2016) 61–67, http://dx.doi.org/10.1016/j.ibmb.2016.05.007.

[9] L. George, A. Lenhart, J. Toledo, A. Lazaro, W.W. Han, R. Velayudhan, et al., Community-effectiveness of temephos for dengue vector control: a systematic literature review, PLoS Negl. Trop. Dis. 9 (2015) e0004006, , http://dx.doi.org/10.1371/journal.pntd.0004006.

[10] E.O.F. Sally, L.A. Anjos, V. Wahrlich, Metabolismo basal durante a gestação: revisão sistemática, Ciência e Saúde coletiva 18 (2013), http://dx.doi.org/10.1590/S1413-81232013000200013.

[11] C. Zhang, D. Xu, H. Luo, J. Lu, J. Ping, H. Wang, Prenatal xenobiotic exposure and intrauterine hypothalamus-pituitary-adrenal axis programming alteration, Toxicology (2014) 74–84, http://dx.doi.org/10.1016/j.tox.2014.08.015.

[12] OMS–Organização Mundial da Saúde, Larvicidas Recomendados Pela Organização Mundial Da Saúde Para Utilização Em Água Potável, http://portalarquivos2.saude.gov.br/images/pdf/2014/abril/28/larvicida.pdf (Acesso em 27 de fevereiro de 2018).

[13] Especificidades dos nutrientes, in: M.R. Vitolo (Ed.), Nutrição Da Gestação Ao Envelhecimento, OECD ilibrary, 2008(Rio de Janeiro).

[14] OECD, Guidelines for testing of chemicals section 4: health effects, Test No. 414: Prenatal Development Toxicity Study, 2001.

[15] OECD, Guidelines for testing of chemicals, Draft Proposal for an Extended One-Generation Reproductive Toxicity Study, 28 2009.

[16] R.J. Oliveira, T.Y.N. Kanno, M.J.S. Salles, O.A.C.S. Lourenã, L.R. Ribeiro, G.A. Freiria, et al., Effects of the polysaccharide ß-glucan on clastogenicity and teratogenicity caused by acute exposure to cyclophosphamide in mice, Regul. Toxicol. Pharmacol. Regul. Toxicol. Pharmacol. 53 (2009) 164–173.

[17] C.A. Gonçalves, J.M. Siqueira, C.A. Carollo, M.O. Mauro, N. David, A.L. Cunha-Laura, et al., Gestational exposure to *Byrsonima verbascifolia*: teratogenicity, mutagenicity and immunomodulation evaluation in female Swiss mice, J. Ethnopharmacol. 150 (2013) 843–850, http://dx.doi.org/10.1016/j.jep.2013.09.012.

[18] N. David, M.O. Mauro, C.A. Gonçalves, J.R. Pesarini, R.L.B. Strapasson, C.A.L. Kassuya, et al., *Gochnatia polymorpha* ssp. floccosa: bioprospecting of an anti-inflammatory phytotherapy for use during pregnancy, J. Ethnopharmacol. 154 (2014) 370–379, http://dx.doi.org/10.1016/j.jep.2014.04.005.

[19] C.A. Gonçalves, N.L. Silva, M.O. Mauro, N. David, A.L. Cunha-Laura, S.A. Auharek, et al., Evaluation of mutagenic, teratogenic, and immunomodulatory effects of *Annona nutans* hydromethanolic fraction on pregnant mice, Genet. Mol. Res. 13 (2014) 4392–4405, http://dx.doi.org/10.4238/2014.

[20] R.J. Oliveira, M.S. Mantovani, J.R. Pesarini, M.O. Mauro, A.F. Da Silva, T.R. Souza, et al., 6-Dimethylaminopurine and cyclohexamide are mutagenic and alter reproductive performance and intrauterine development *in vivo*, Genet. Mol. Res. 14 (2015) 834–849, http://dx.doi.org/10.4238/2015.

[21] E. Salewisk, Färbemethoden zum makroskopischen nachweis von implantationsstellen am uterus der ratte, Naunym-Schmiedeberg Arch. Exp. Pathol. Pharmkol. 247 (1964) 367.

[22] M.V. Barrow, W.J. Taylor, A rapid method for detecting malformation in rat fetuse, J. Morphol. 127 (1969) 291–305.

[23] J.G. Wilson, Methods for administering agentes and detecting malformations in experimental animals, in: J.G. Wilson, J. Waekany (Eds.), Teratology: Principles Snd Thecniques, The University of Chicago Press, Chicago, 1965.

[24] P. Taylor, Pratical Teratology, Academic Press, New York, 1986.

[25] J.M. Manson, Y.J. Kang, Test methods for assessing female reproductive and developmental toxicology, in: A.W. Hayes (Ed.), Principles and Methods of Toxicology, Raven Press, New York, 1994.

[26] D.C. Damasceno, W.G. Kempinas, G.T. Volpato, M. Consonni, M.V.C. Rudge, F.J.R. Paumgartten, Anomalias congênitas: estudos experimentais, Editora Média, Belo Horizonte, 2008.

[27] R.E. Staples, V.L. Schenell, Refinements in rapid clearing technic in the KOH-alizarin red method for fetal bone, Stain. Technol. 39 (1964) 61–63.

[28] J.M. Manson, H. Zenich, D. Costlowr, Teratology test methods for laboratory animal, in: A.W. Hayes (Ed.), Principles and Methods of Toxicology, Raven Press, New York, 1982.

[29] M.A. Montenegro, M.M. Guerreiro, Protocolos de Neurologia Infantil, Universidade Estadual De Campinas–UNICAMP. S.D, https://www.fcm.unicamp.br/fcm/sites/default/files/paganex/protocolos_neurologia_infantil.pdf.

[30] M. Anastassiades, S. Lehotay, D. Stajnbaher, F. Schenck, J. Assoc. Off. Anal. Chem. Int. 83 (2003) 412–431.

[31] M. Hayashi, T. Morita, Y. Kodama, T. Sofundi, M. Ishidate Junior, The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides, Mutat. Res. 245 (1990) 245–249.

[32] J.K. Hanseman, M.D. Hogan, Selection of the experimental unit in teratology studies, Teratology 12 (1995) 165–172.

[33] C.Q. Moreira, M.J.S.S. Faria, J.E. Baroneza, R.J. Oliveira, E.G. Moreira, Developmental exposure to fenproporex: reproductive and morphological evaluation, Hum. Exp. Toxicol. 24 (2005) 397–402.

[34] L. Berg Gordon, Farm Chemicals Handbook, Meister, Willoughby, 1989, p. C5.

[35] R. Danesi, M. Del Tacca, Teratogenesis and immunosuppressive treatment, Transplant. Proc. 36 (2004) 705–707.

[36] Y. Yu, Y. Yang, X. Zhao, X. Liu, J. Xue, J. Zhang, et al., Exposure to the mixture oforganophosphorus pesticides is embryotoxic and teratogenic on gestational ratsduring the sensitive period, Environ. Toxicol. 32 (2017) 139–146, http://dx.doi.org/10.1002/tox.22219.

[37] K.E. Brett, Z.M. Ferraro, J. Yockell-Lelievre, A. Gruslin, K.B. Adamo, Maternal–fetal nutrient transport in pregnancy pathologies: the role of the placenta, Int. J. Mol. Sci. 15 (2014) 16153–16185, http://dx.doi.org/10.3390/ijms150916153.

[38] V. Van der Linden, A. Pessoa, W. Dobyns, A.J. Barkovich, H.V. Júnior, E.L. Filho, et al., Description of 13 infants born during october 2015-january 2016 with congenital zika virus infection without microcephaly at birth-brazil, Morb. Mortal. Wkly Rep. 2 (2016) 1343–1348, http://dx.doi.org/10.15585/mmwr.mm6547e2.

[39] S. Oga, M.M.A. Camargo, J.A.O. Batistuzzo, Fundamentos de Toxicologia, 3rd ed., (2008), p. 474.

[40] C.A. Aiub, E.C. Coelho, E. Sodré, L.F. Pinto, I. Felzenszwalb, Genotoxic evaluation of the organophosphorous pesticide temephos, Genet. Mol. Res. 1 (2002) 159–166.

[41] A.B. Benitez-Trinidad, J.F. Herrera-Moreno, G. Vázquez-Estrada, F.A. Verdín-Betancourt, M. Sordo, P. Ostrosky-Wegman, et al., Cytostatic and genotoxic effect of temephos in human lymphocytes and HepG2 cells, Toxicol. in Vitro 29 (2015) 779–786, http://dx.doi.org/10.1016/j.tiv.2015.02.008.