UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE BIOCIÊNCIAS

DEPARTAMENTO DE GENÉTICA

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

TAILINI JORDANA REINEHR STOFFEL

AGENTES ESTRESSORES E A MOBILIZAÇÃO SOMÁTICA DOS ELEMENTOS DE TRANSPOSIÇÃO EM *DROSOPHILA*

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Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para a obtenção do grau de Doutor em Ciências (Genética e Biologia Molecular)

Orientador: Élgion Lúcio da Silva Loreto

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Há um prazer nos bosques inexplorados.
Há uma beleza na solitária praia.
Há uma sociedade que ninguém invade.
Perto do mar profundo e da música do seu bramir:
Não que ame menos o homem, mas amo mais a natureza!

- A Peregrinação de Childe Harold (Lord Byron)

SUMÁRIO

LISTA DE ABREVIATURAS

Ago	Proteínas argonauta
APC	*Adenomatous Polyposis Coli*
Arg3	*Argonaute 3*
Aub	Aubergina
cDNA	*Complementary DNA*
CPA	*Cyclophosphamide*
DEGs	*Differentially expressed genes*
dsRNA	*Double strand RNA*
ETs	*Electron transfer system*
gDNA	DNA genômico
GO	*Gene orthology*
HSEs	*Heat shock elements*
HSFs	*Heat shock factors*
IN	Integrase
iRNA	RNA interferente
LINE	Elemento nuclear interdisperso longo
LTR	*Long terminal repeats*
MB	*Mushroom bodies*
miRNA	microRNA
Obp	*Odorant binding proteins*
ORF	*Open reading frame*
OXPHOS	*Oxidative phosphorylation*
piRNA	*Piwi-interacting RNA*
PPO	*Prophenoloxidase*
RING	*Rapid interactive negative geotaxis*
RISC	*RNA-induced silencing complex*
ROS	*Reactive oxygen species*
RT	*Reverse transcriptase*
SE	*Somatic excision*

SINE	Elemento nuclear interdisperso curto
siRNA	*Small interferent RNA*
SP	*Serine proteases*
TD	*Targeting domain*
TE	Elemento transponível
TIR	*Terminal inverted repeats*
Tot	*Turandot-related genes*
TS	Transposição somática
TSD	*Target site duplications*
UV	Ultravioleta
Wpch	*White-peach*
αβ-KCs	*αβ-Kenyon Cells*
4-OH-Cy	*4-hydroxicyclophosphamide*

RESUMO

Elementos transponíveis (TEs) são sequências móveis dos genomas que podem se transpor tanto em células germinativas, quanto em células somáticas, mecanismo conhecido como transposição. A transposição pode causar diversos efeitos nos genomas hospedeiros, que podem ser benéficos, como contribuir para a diversificação do material genético, ou podem ser negativos e, no caso da transposição somática (TS), pode levar inclusive ao desenvolvimento de câncer. Ao longo dos últimos anos, evidências da ativação de TEs por agentes estressores, como em resposta ao choque térmico e à exposição a agentes citotóxicos têm se acumulado em diversos organismos. O elemento *mariner-Mos1* de *Drosophila* promove a transposição em células somáticas e é um excelente modelo para estudos relacionados às consequências biológicas da TS. Nessa tese, o objetivo geral foi verificar se ocorre a ativação de TEs, em especial *mariner-Mos1*, em *Drosophila simulans* e *Drosophila melanogaster* expostas a diferentes agentes estressores – temperatura e alguns quimioterápicos (ciclofosfamida, cisplatina, dacarbazina e daunorubicina). Verificamos que a excisão somática (ES) de *mariner-Mos1* se acumula durante o ciclo de vida de *Drosophila*, mas não é constante durante todo desenvolvimento. Além disso, os diferentes estágios do desenvolvimento não são impactados de maneira semelhante pelo estresse térmico. Verificamos ainda que a ES pode ser prejudicial, conforme sugerido pela correlação entre o nível de ES e redução de atividades comportamentais e viabilidade embrionária. Os tratamentos com a ciclofosfamida (CPA) alteraram os padrões de transcrição de alguns TEs, mas não de *mariner*. Além disso, a CPA afetou genes envolvidos em várias funções biológicas, em particular, o tratamento agudo promoveu uma redução na expressão de genes envolvidos com os constituintes estruturais da mitocôndria, induzindo uma disfunção mitocondrial. Observamos também uma diferença significativa na expressão relativa de *mariner* nas moscas tratadas com a cisplatina. O mesmo não foi observado para os tratamentos com a dacarbazina. Já os experimentos com a daunorubicina estão em andamento.

Palavras-chave: Elementos transponíveis (TEs); Transposição somática (TS); *mariner-Mos1;* agentes estressores.

ABSTRACT

Transposable elements (TEs) are mobile sequences of genomes that can transpose in germ cells and somatic cells, a mechanism known as transposition. Transposition may cause several effects on host genomes, which may be beneficial, such as contributing to the diversification of genetic material, or may be negative, and in the case of somatic transposition (ST) may even lead to the development of cancer. Over the past few years, evidence of the activation of TEs by stressors, such as in response to heat shock and exposure to cytotoxic agents has accumulated in several organisms. The *mariner-Mos1* element of *Drosophila* promotes somatic cell transposition and is an excellent model for studies related to the biological consequences of ST. In this thesis, the objective was to verify if the activation of TEs, especially *mariner-Mos1*, occurs in *Drosophila simulans* and *Drosophila melanogaster* exposed to different stressors - temperature and some chemotherapeutic drugs (cyclophosphamide, cisplatin, dacarbazine and daunorubicin). We found that somatic excision (SE) of *mariner-Mos1* accumulates during the *Drosophila* life cycle, but is not constant throughout development. Moreover, the different stages of development are not similarly impacted by heat stress. We also verified that SE can be harmful, as suggested by the correlation between SE level and reduction of behavioral activities and embryonic viability. The cyclophosphamide (CPA) treatments altered the transcription patterns of some TEs, but not *mariner*. In addition, CPA affected genes involved in various biological functions, in particular, acute treatment promoted down-regulation in expression of genes involved with the structural constituents of mitochondria, inducing mitochondrial dysfunction. We also observed a significant difference in *mariner* relative expression in cisplatin-treated flies. The same was not observed for dacarbazine treatments. Experiments with daunorubicin are in progress.

Keywords: Transposable Elements (TEs); Somatic Transposition (ST); *mariner-Mos1*; stressors.

1. INTRODUÇÃO

1.1 Os elementos transponíveis

Os elementos transponíveis (TEs) são sequências de DNA capazes de mudar sua localização dentro dos genomas hospedeiros, mecanismo conhecido como transposição (McCullers e Steiniger, 2017). Inicialmente, a existência desses elementos que foram descobertos em 1940 pela geneticista Barbara McClintock (1902 – 1992), não foi totalmente aceita pela comunidade científica (Varani et al., 2015). Isso porque essa descoberta demonstrava que os genomas são dinâmicos e possuem plasticidade, contrariando os conceitos da época de estabilidade do material genético. Entretanto, atualmente eles são reconhecidos como importantes agentes do processo evolutivo, são fonte de variabilidade genética e de novos genes, doam regiões regulatórias ou codificadoras, auxiliam na formação de novas redes reguladoras, nas alterações epigenéticas por metilação e RNA interferente (iRNA) (Brown e Bachtrong, 2014; Hua-Van et al., 2011; Kung et al., 2013).

O sequenciamento de genomas em grande escala mostrou que os TEs podem representar grande parte do genoma de praticamente todos os organismos (Kazazian, 2011), constituindo aproximadamente 15% do genoma de *Drosophila melanogaster*, 47% do genoma humano e 60% do genoma do milho (Biémont, 2010; Kidwell e Lisch, 2001).

1.2 Classificação dos TEs

David Finnegan foi o pioneiro na sistemática dos TEs, isto no final dos anos de 1980 (Finnegan, 1989). Sua concepção era de que os TEs poderiam, em sua base filogenética, ser classificados em duas classes de acordo com o seu mecanismo de transposição, que se distingue pelo intermediário que o TE utiliza para a mobilização: RNA – classificados como de Classe I, ou DNA – classificados como de Classe II.

Os TEs de Classe I, também conhecidos como retrotransposons, retroelementos ou transposons de RNA, sofrem transcrição reversa para a sua transposição. Ou seja, assim como ocorre com os retrovírus, ocorre à síntese de moléculas de DNA a partir de um molde

de RNA. As enzimas envolvidas na transposição desses retrotransposons são a transcriptase reversa (r*everse transcriptase* - RT) e a integrase (IN), que são codificadas pelo TE e produzidas pela maquinaria celular.

Já os TEs de classe II, também são conhecidos como tranposons de DNA ou simplesmente transposons. As enzimas responsáveis pela transposição desses elementos são as transposases que atuam na quebra das ligações fosfodiéster da molécula de DNA promovendo a clivagem do transposon do seu local de origem e sua inserção em um novo local do genoma.

Como parte importante da estrutura molecular dos transposons está à presença de regiões terminais invertidas (*terminal inverted repeats* – TIRs) em suas extremidades. Essas podem variar de 9 a 40 pares de bases (pb) de extensão e atuam como sítios de reconhecimento para a ação das transposases (Muñoz-López e García-Pérez, 2010). Além disso, alguns transposons podem ainda apresentar repetições flanqueando as TIRs. Essas sequências são chamadas de sítio alvo de duplicação (*target site duplications* – TSDs), são geradas durante a inserção no sítio receptor e são únicas para cada tipo de transposon (Muñoz-López e García-Pérez, 2010).

A proposta de Finnegan foi aceita por grande parte da comunidade científica e foi a base de duas atualizações subsequentes da taxonomia dos TEs conhecidas como a proposta de Wicker e a do Repbase (Kapitonov e Jurka, 2008; Wicker et al., 2007). A ambição dessas atualizações foi delinear um sistema de classificação unificado com uma série de critérios mais detalhados do que aqueles usados na proposta original do Finnegan. Ambas as atualizações mantiveram a noção de que todos os transposons eucarióticos poderiam ser classificados como retrotransposons ou transposons de DNA, mas eles diferem em sua classificação, sistemas de nomes e número de classes de TEs (Piégu et al., 2015).

Na proposta do Repbase (Figura 1A), o critério mais básico é o mecanismo de transposição dividindo todos os TEs em elementos do Tipo 1 (transposons de DNA) e elementos do Tipo 2 (retrotransposons). A divisão por esse critério inicial é então seguida por critérios relacionados aos tipos de enzimas envolvidas na transposição ou retrotransposição, às semelhanças estruturais e às semelhanças de sequências (Jurka et al., 2005; Kapitonov e Jurka, 2008). O primeiro critério de classificação (mecanismo de transposição) levou os autores a proporem sete classes de TEs.

Já a proposta de Wicker (Figura 1B) está mais alinhada com o esquema original de Finnegan. Ela preserva sua estrutura básica, que divide os TEs em duas classes com base no intermediário de transposição (ou seja, classe I ou retrotransposons – intermediário de RNA e classe II ou transposons de DNA – intermediário de DNA). A classe I é dividida em cinco ordens, com base em características mecanicistas, organização e filogenia da RT. Os elementos da classe II são divididos em duas subclasses, a subclasse 1 são elementos que usam um mecanismo de transposição "recorta e cola", enquanto os elementos da subclasse 2 se transpõem usando um mecanismo de transposição "copia e cola".

Além desses sistemas de classificação, os TEs podem ser divididos em autônomos e não autônomos (Wicker et al., 2007). TEs autônomos são aqueles que codificam as enzimas necessárias para sua transposição, como a RT, em TEs de Classe I, e a transposase nos TEs de Classe II. Elementos não autônomos, apesar de não codificarem suas próprias enzimas necessárias para a transposição, podem ser mobilizados por enzimas produzidas por um elemento autônomo. Um bom exemplo disso são os elementos SINEs, cuja transposição é feita por enzimas RTs produzidas por LINEs (Wicker et al., 2007).

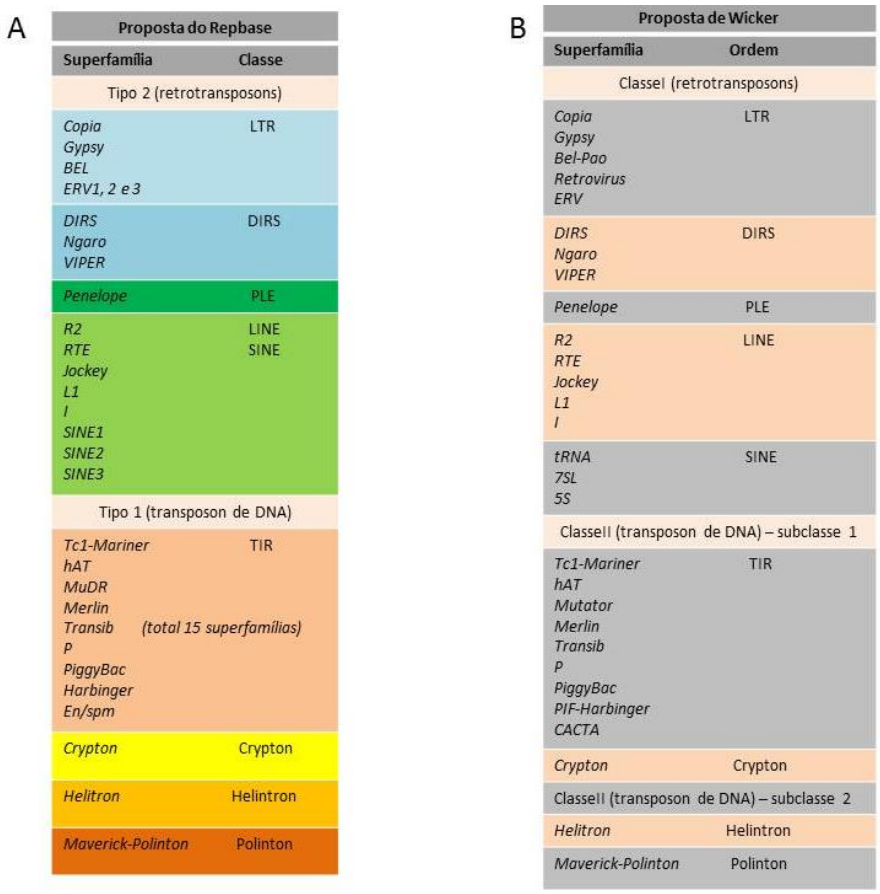

Figura. 1. Comparação de duas propostas para a classificação de TEs eucarióticos. A. Proposta do Repbase. B. Proposta de Wicker. Ambas dividem os TEs em dois grupos: os retrotransposons e os transposons de DNA. Essa divisão basal é chamada de 'tipo' na proposta Repbase e de 'classe' na proposta de Wicker. Esses dois tipos ou classes são subdivididos em 'classes' na proposta do Repbase ou em 'ordem' na proposta de Wicker. No geral, as 'classes' do Repbase e as 'ordens' de Wicker são muito semelhantes e cada grupo contém as mesmas superfamílias de TEs. Fonte: Piégu et al. (2015).

1.3 Transposon *mariner* e a mutação w*hite-peach* em *Drosophila*

O TE *mariner* é um transposon que pertencente à Classe II, Subclasse I, Ordem TIR, Superfamília *Tc1-mariner* e Família *mariner* (Wicker et al., 2007). A família *mariner* é uma das famílias de Classe II mais bem conhecidas, além de ser o grupo de TEs mais distribuído na natureza, com representantes nos mais variados táxons, como rotíferos, fungos, nematoides, insetos, peixes e mamíferos (Muñoz-López e García-Pérez, 2010; Sinzelle et al., 2006).

O TE *mariner* foi inicialmente descoberto no genoma de *Drosophila mauritiana* por causar uma mutação no gene *white* no cromossomo X, causando uma alteração fenotípica

na cor dos olhos das moscas (Jacobson et al., 1986). A proteína codificada pelo gene *white* atua no transporte dos precursores dos pigmentos que dão cor aos olhos de *Drosophila* (Ewart e Howells, 1998; Sullivan, 1975). Essa mutação é resultante da inserção de *mariner* na região promotora do gene *white* o que leva a uma redução drástica na expressão desse gene, gerando indivíduos com olhos cor de pêssego, denominados *white-peach* (Jacobson et al., 1986).

O primeiro transposon *mariner* descrito possui 1286 nucleotídeos de extensão, com TIRs de 28 pb flanqueando uma única ORF - quadro aberto de leitura - (do inglês, *open reading frame*) que codifica uma transposase de 346 aminoácidos (Jacobson et al., 1986; Medhora et al., 1991).

A cópia do elemento *mariner* que originou os mutantes *white-peach*, é conhecida como cópia *"peach"* e produz uma transposase não funcional, ou seja, o elemento permanece estável no genoma (Bryan et al., 1987; Jacobson et al., 1986). Entretanto, na presença de outras cópias de *mariner* autônomas, o elemento inserido no gene *white*, não autônomo, pode utilizar essas transposases para se mobilizar em algumas células, causando uma reversão parcial da mutação a condição selvagem. Quando ocorre a transposição do elemento em células somáticas dos olhos, ocorre um padrão mosaico de pigmentação (Bryan et al., 1987; Jacobson et al., 1986; Medhora et al., 1988; Medhora et al., 1991). Essas moscas possuem olhos *white-peach* com manchas de coloração vermelha onde ocorreu a reversão ao fenótipo selvagem devido a excisão do *mariner peach* (Figura 2).

A primeira cópia ativa descrita foi chamada de fator de mosaicismo, ou *Mos1*, por promover o fenótipo mosaico (Medhora et al., 1988; Medhora et al., 1991). O *mariner Mos1* possui a mesma extensão da cópia *peach*, diferindo em apenas 11 nucleotídeos distribuídos ao longo da sequência.

Figura 2. A. *D. melanogaster wpch* com elemento *mariner* estável no genoma. B. *D. melanogaster* em que ocorreu a transposição de *mariner* em algumas células apresentando um padrão mosaico de pigmentação nos olhos (olhos *white-peach* com manchas de coloração vermelha onde ocorreu a reversão ao fenótipo selvagem).

A cópia mutante *peach* foi transferida por hibridização interespecífica para *D. simulans* e por transgenia para *D. melanogaster*, gerando linhagens *white-peach* dessas espécies (Bryan et al., 1987; Garza et al., 1991). O número de cópias ativas de *mariner* é variável entre as espécies de *Drosophila* (Capy et al., 1990; Hartl, 2001; Jacobson et al., 1986; Picot et al., 2008; Russel e Woodruff, 1999). *D. mauritiana* possui de 10 a 20 cópias; *D. simulans* apresenta um número de cópias bem diversificado, podendo ocorrer diferenças intrapopulacionais; e *D. melanogaster* parece não apresentar nenhuma cópia ativa.

A partir do modelo *white-peach* pode-se avaliar a atividade do elemento *mariner* em populações naturais de *Drosophila* (Capy et al., 1990; Jardim e Loreto, 2011). Ao realizar cruzamentos entre machos selvagens e fêmeas *white-peach*, a atividade do elemento pode ser verificada pela progênie de machos mosaicos. Além disso, os mutantes *white-peach* são excelentes para estudos de transposição somática, uma vez que quando ocorre a excisão do elemento *mariner* se reflete no fenótipo mosaico, que é facilmente observável.

1.4 Mecanismos de silenciamento dos TEs

Embora os TEs sejam importantes agentes do processo evolutivo, eles também são uma das principais ameaças para a estabilidade do genoma. Ao sofrerem transposição eles podem se inserir em diferentes regiões, como dentro de um gene ou de sua região promotora, alterando sua expressão gênica ou inativando-o. Ainda, a inserção ou a excisão

de TEs podem gerar outras alterações como deleções, duplicações, inversões, quebras e fusões cromossômicas (Hua-Van et al., 2011). Para controlar a atividade e a expansão desses elementos no genoma e para evitar que tudo isso ocorra, os hospedeiros desenvolveram mecanismos de silenciamento dos TEs.

A repressão dos TEs pode ocorrer através de duas principais estratégias: o controle pré-transcricional e o pós-transcricional. No controle pré-transcricional, pode ocorrer a metilação do DNA, que é essencial para a regulação de TEs em eucariotos e consiste na adição de grupos metila (CH_3) ao carbono 5 de citosinas em sequências promotoras, impedindo a ligação de fatores de transcrição e a produção de transcritos (Law e Jacobsen, 2010). Além da metilação, pode ocorrer também a modificação das histonas que são proteínas ligadas à molécula de DNA e que atuam na sua compactação. Essas proteínas possuem uma cauda N-terminal que pode sofrer acetilação ou metilação alterando o estado da compactação da cromatina, regulando assim a transcrição de TEs (Volpe e Martienssen, 2011).

Já o controle pós-transcricional de TEs é feito pela interação entre pequenos RNAs e a família de proteínas Argonauta (Ago). De um modo geral, esses pequenos RNAs são divididos em três tipos: os pequenos RNAs de interferência (*small interferent RNA* – siRNAs) que se ligam às proteínas Ago2 e atuam no silenciamento de TEs nas células somáticas; os piRNAs (*Piwi-interacting RNA*) que se ligam às proteínas PIWI e fazem a repressão de TEs em células germinativas; e, por fim, os microRNAs (miRNAs), que se ligam às proteínas Ago1 e não participam do silenciamento de TEs mas estão envolvidos na regulação de genes endógenos (Czech e Hannon, 2011).

Quando um elemento invade um genoma que não possui defesas contra ele, sua atividade é muito alta, ocorrendo um grande número de eventos de transposição (Blumenstiel, 2011). Isso aumenta a probabilidade de produzir transcritos defeituosos, como mRNA sem cauda poli-A ou mRNA fita dupla (*double strand RNA* – dsRNA).

Em células somáticas, o silenciamento começa quando é detectado esse RNA defeituoso produzido por um novo TE. Os dsRNAs são reconhecidos por uma endonuclease chamada Dicer e são clivados em pequenos RNAs de 21 a 24 nucleotídeos. Esses pequenos RNAs são processados para a produção de siRNAs (Castel e Martienssen, 2013) que se ligam às proteínas Ago2 formando um complexo de silenciamento chamado

RISC (*RNA-induced silencing complex*) (Czech e Hannon, 2011). O complexo RISC utiliza a sequência do siRNA na busca por transcritos dos TEs, que são clivados e em seguida degradados.

Em células germinativas, o controle dos TEs é feito através de um sistema que utiliza os piRNAs associados a uma classe de Argonautas chamada PIWI, que inclui as proteínas Ago3, Piwi e Aubergina (Aub). Os piRNAs são produzidos a partir de lócus conhecidos como "clusters de piRNAs", que contêm sequências de diferentes TEs, a maioria em orientação antisenso. A transcrição desses lócus gera longos precursores de piRNAs, que são clivados pela Ago3 em pequenos RNAs de 24 a 35 nucleotídeos. Esses pequenos RNAs passam por um processamento e formam os piRNAs (Castel e Martienssen, 2013). Os piRNAs antisenso produzidos ligam-se as Piwi e Aub e as direcionam para os transcritos dos TEs, que são clivados para a produção de piRNA senso. Esses piRNAs senso são capturados pela Ago3 e usados para a produção de novos piRNAs antisenso, reiniciando o ciclo. Por isso, esse mecanismo é chamado de "modelo ping-pong" (Castel e Martienssen, 2013; Zamore, 2010).

Assim que um novo TE invade o genoma, o hospedeiro não possui os piRNAs complementares a sua sequência. Esse TE ocasionalmente irá se transpor para um "cluster de piRNA" e sua sequência será usada para a produção de novos piRNAs (Blumenstiel, 2011).

1.5 Ativação de TEs por agentes estressores

Hoffmann e Parsons (1997) propuseram uma definição para estresse que inclui "qualquer mudança ambiental que reduz drasticamente o fitness de um organismo". Já Bijlsma e Loeschcke (2005), discutem definições mais precisas de estresse, em vários níveis biológicos, que vão de moléculas a populações, de acordo com a intensidade das mudanças ambientais e o organismo ou a população envolvida. Além disso, esses autores identificaram duas classes de estresse definidas de acordo com a resposta do organismo: aquelas que evocam uma resposta fisiológica (em um contexto individual) e aquelas que evocam uma resposta fenotípica ou genética (contexto evolutivo).

Ao longo dos últimos anos, evidências da ativação de TEs por agentes estressores têm se acumulado em diversos organismos. No final de 1990, muitos estudos demonstraram a ativação de transposons em resposta ao choque térmico, irradiação e infecções virais em *Drosophila* (Anikeeva et al., 1994; Chakrani et al., 1993; Jouan-Dufournel et al., 1996; Ratner et al., 1992).

O modelo *white-peach* foi utilizado para verificar o efeito da temperatura na transposição somática (TS) de *mariner* (Capy et al., 1992; Russel e Woodruff, 1999). Garza et al. (1991) relataram um aumento na transposição desse elemento em linhagens de *D. melanogaster white-peach*. Chakrani et al. (1993) realizaram cruzamentos a 29°C e encontraram um efeito da temperatura nas taxas de TS em algumas linhagens naturais de *D. simulans*. Ainda, os efeitos do estresse térmico na ativação de transposons têm sido demonstrado em outros organismos, como fungos patógenos de arroz *Magnaporthe oryzae* (Ikeda et al., 2001) e fungos causadores da grafiose em árvores, *Ophiostoma ulmi* e *Ophiostoma novo-ulmi* (Bouvet et al., 2008).

Outro tipo de estresse capaz de induzir um aumento na atividade de TEs é a radiação ultravioleta (UV). No genoma do milho verificou-se um aumento na atividade de transposons da família *Mutator* em resposta a radiação UV-B (Qüesta et al., 2013). Além disso, também houve uma resposta à ação da UV nos elementos LINEs, em cultura de células humanas e SINEs, em cultura de células de ratos (Morales et al., 2003; Myakishev et al., 2008). Embora a UV não tenha ativado o elemento *mariner-Mos1* em *D. simulans* (Jardim et al., 2015).

A ligação entre estresse e TEs pode ser exemplificada pelo comportamento de um retrotransposon específico de levedura, o Ty5 (Dai et al., 2007). Esse TE produz uma integração com um domínio alvo (*targeting domain* – TD). Quando esse TD está fosforilado, o TE interage com os componentes da heterocromatina e sua integração ocorre em genes silenciados ou regiões genômicas pobres em genes. Sob condições de estresse, como a falta de nutrientes, a fosforilação é reduzida e o TE se integra em regiões codificadoras do genoma, podendo causar mutações. Essa fina regulação do Ty5 parece formar um mecanismo para iniciar a remodelagem do genoma em condições críticas, permitindo uma possível adaptação (Dai et al., 2007).

Além desses já estudados, alguns outros agentes também foram mostrados como causadores de estresse em diferentes organismos, como os quimioterápicos em seres humanos (Gonçalves et al., 2009). Esses agentes citotóxicos são usados para o tratamento do câncer, tratamento de doenças autoimunes e como imunossupressores precedendo o transplante de órgãos (Dollery, 1999; Binotto et al., 2003). Embora ainda não tenham sido mostrados, esses agentes estressores também podem causar o aumento nas taxas de transposição de TEs causando diversas consequências para os organismos hospedeiros.

1.6 Transposição somática e suas consequências para os hospedeiros

Devido ao fato de que os eventos de transposição em células somáticas não são herdáveis e possuem efeito apenas sobre o organismo hospedeiro, sem contribuir para os processos evolutivos, durante muito tempo, pouco interesse foi dado à TS (Eickbush e Eickbush, 2011). Entretanto, evidências encontradas nos últimos anos indicam que os TEs podem ser mais ativos em tecidos somáticos do que se imaginava, causando uma série de consequências para os genomas hospedeiros.

Uma das consequências positivas da TS é que ela pode gerar mosaicismo por provocar variação no material genético entre células do mesmo tecido (Vijg, 2014). Isso pode ser observado no sistema nervoso em *Drosophila*, onde retrotransposons LTR, LINEs e transposons de DNA são ativos em estruturas neuronais conhecidas como *mushroom bodies* (MB), que são relacionadas à memória olfativa (Perrat et al., 2013). Um fenômeno similar também pode ser observado no sistema nervoso em seres humanos, onde a TS de retrotransposons LINE-1 parece contribuir para a diversificação e plasticidade neuronal. Essa variabilidade produzida pela atividade de TEs pode ter um papel importante no desenvolvimento cognitivo e diferenças comportamentais entre indivíduos (Evrony, 2016; Richardson et al., 2014; Upton et al., 2015).

O cérebro exibe plasticidade em resposta às experiências ambientais, particularmente durante as primeiras semanas de vida (Bedrosian et al., 2016; Muotri et al., 2005). Parte dessa plasticidade pode ser atribuída às modificações do DNA através de alterações epigenéticas como a metilação ou o remodelamento da cromatina. Entretanto,

análises de sequências de DNA neuronal sugerem um papel para a mobilização de retrotransposons.

Outro ponto positivo é que o aumento nas taxas de transposição de TEs sob condições de variabilidade ambiental, cria efeitos rápidos na atividade gênica e na estrutura geral dos genomas, permitindo adaptações a novos ambientes (Casacuberta e Gonzalez, 2013).

Embora a TS contribua para a diversificação do material genético, ela também pode estar envolvida em consequências negativas para os organismos hospedeiros (Helman et al., 2014). Como os TEs podem gerar instabilidade genética, sua ativação em células somáticas pode estar envolvida com o envelhecimento celular (Sedivy et al., 2013). A ativação de TEs durante o envelhecimento aumenta as taxas de mutações e danos à molécula de DNA, levando a perturbação de todo o funcionamento celular. Isso sugere que a TS desses elementos pode ser um fator determinante no envelhecimento e no desenvolvimento de doenças associadas a esse processo (Sedivy et al., 2013; Sturm et al., 2015). A TS de TEs, particularmente os LINE-*like* e *Gypsy* também tem sido relacionada ao declínio neural e cognitivo idade-dependente em moscas (Bundo et al., 2014; Krug et al., 2017; Li et al., 2013).

Além disso, diversas doenças são relacionadas à TS. Transtornos psiquiátricos em seres humanos são um exemplo. A atividade anormal de TEs foi detectada no cérebro de pacientes com esquizofrenia e em pacientes com distúrbios de bipolaridade, autismo e depressão profunda (Guffanti et al., 2014). Análises computacionais sugerem que a TS pode afetar a expressão de genes neurotransmissores, como dopamina, serotonina e glutamato (Abrusán, 2012). A variação na biossíntese desses neurotransmissores pode afetar o metabolismo do sistema nervoso, prejudicando as sinapses nervosas desencadeando essas desordens neurológicas.

As inserções somáticas individuais de TEs podem ser difíceis de detectar (Gawad et al., 2016). Isso porque para a extração de DNA genômico (gDNA), um pool de células aumenta a quantidade de DNA total, mas diminui o poder de detectar inserções únicas em células individuais. Logo, os núcleos de interesse precisam ser separados do restante do material biológico. Embora as abordagens baseadas em *single-cell* forneçam uma maior sensibilidade teórica para detectar inserções raras, a porcentagem do genoma que é coberto

por cada célula e o número total de células que podem ser testadas com essa abordagem é limitada.

Em *D. melanogaster*, MBs são estruturas do cérebro importantes para a memória olfativa, como já citado anteriormente. Os neurônios MB são divididos em α'β', γ e αβ pela sua morfologia e pelos papéis que desempenham no processamento da memória (Blum et al., 2009; Trannoy et al., 2011; Yu et al., 2006). Estudos sugerem que os transposons são expressos e se mobilizam em particular na porção αβ, chamada αβ-Kenyon Cells (αβ-KCs) (Li et al., 2013; Perrat et al., 2013). Cada mosca possui ~1800 αβ-KCs (Aso et al., 2014).

Treiber e Waddell (2017) desenvolveram um método para extrair e sequenciar gDNA com alta cobertura dos ~1800 αβ-KCs de uma mosca individual e de células do restante do cérebro da mesma mosca. Através dessa abordagem, eles analisaram as taxas de novas TSs. Verificaram que as sequências de transposons não se acumularam com a idade. Além disso, embora alguns transposons tenham sido mais expressos em αβ-KCs, quando comparados com o resto do cérebro, esse aumento não levou a um número muito maior de inserções no gDNA nesses neurônios. Além disso, eles fizeram uma reanálise dos dados com 'elementos genéticos imóveis', ao invés de transposons, que revelou que os dados do sequenciamento continham sequências inesperadas indicativas de translocações de exons. Um novo pipeline de análises revelou que essas translocações e a maioria das novas inserções de transposons resultam de artefatos formados durante o preparo das bibliotecas para o sequenciamento.

Essas descobertas destacam uma possível falha nas abordagens atuais para detectar e avaliar as inserções somáticas de TEs e desafiam algumas hipóteses de que a transposição desempenha um papel importante na geração de diversidade celular e no declínio neuronal dependente da idade.

1.7 Transposição somática e câncer

Apesar de todas as consequências causadas pelas inserções dos TEs, muitas vezes esses passam imperceptíveis pelos seus hospedeiros por serem pouco ativos para terem alguma relevância. Entretanto, o câncer apresenta uma exceção importante. Estudos

realizados no genoma humano apontam uma forte correlação entre diversos tipos de cânceres e a ativação de retrotransposons, como o LINE-1 e o *Alu*.

Embora silenciados em tecidos somáticos, os TEs podem se tornar ativos em células tumorais devido à hipometilação do DNA que parece ser comum nessas células (Hon et al., 2012; Kulis e Esteller, 2010). A hipometilação dos promotores de LINE-1 parece estar correlacionada com um aumento na sua expressão e na sua retrotransposição (Scott et al., 2016; Tubio et al., 2014).

Além disso, LINE-1 possui uma sequência de 6kb e codifica duas ORFs, a ORF1 (Holmes et al., 1992; Moran et al., 1996) e a ORF2 (Dombroski et al., 1991). Sabe-se que a ORF2 possui os papéis de endonuclease e RT para esse TE se transpor (Feng et al., 1996; Mathias et al., 1991), já a função exata da ORF1 é incerta. Entretanto, a super-expressão da ORF1 é uma característica marcante em alguns tipos de cânceres (Ardeljan et al., 2017; Rodic et al., 2014). Foi detectada sua super-expressão através de imuno-histoquímica em mais de 90% dos cânceres de mama e ovário, próximo a 90% dos cânceres pancreáticos, além de, em uma menor porcentagem, cânceres no trato gastrointestinal, de pulmão e de próstata (Rodic et al., 2014).

Inserções somáticas de LINE-1 ocorrem em muitos tipos de cânceres humanos, e essas inserções podem ser mutagênicas. Métodos de sequenciamento de nova geração identificaram 27 inserções somáticas de LINE-1 em um paciente com câncer colorretal. A maioria dessas inserções estava distribuída em regiões intrônicas e espaços intergênicos do genoma sem efeitos funcionais óbvios. Entretanto uma inserção somática ocorreu no éxon do gene *Adenomatous Polyposis Coli* (APC), gene que age como supressor tumoral no cólon, o que comprometeu sua função (Scott et al., 2016) e pode ter sido uma das causas do desenvolvimento do câncer.

Uma pesquisa realizada com 43 tumores submetidos ao sequenciamento genômico mostrou uma maior retrotransposição em cânceres colorretais do que em câncer de próstata e ovário, com um caso de câncer colorretal apresentando 106 inserções somáticas (Lee et al., 2012). Além disso, também foram observadas inserções somáticas de LINE-1 em cânceres de pescoço e cabeça (Lee et al., 2012; Tang et al., 2017). Já a leucemia mielóide aguda, gliomas do sistema nervoso central e câncer de mama apresentam níveis relativamente baixos de TS de LINE-1 (Achanta et al., 2016; Helman et al., 2014; Lee et

al., 2012; Tubio et al., 2014). Os mecanismos para entender essa predileção de LINE-1 em certas neoplasias não são bem compreendidos.

Alguns TEs também podem atuar como promotores crípticos de oncogenes para direcionar sua expressão no câncer, um processo conhecido como onco-exaptação (Babaian et al., 2016; Wolff et al., 2010). Hyo et al. (2019) analisaram 7.769 tumores de 15 tipos de cânceres e identificaram 129 eventos onde os TEs agiram como promotores de 106 oncogêneses. Um TE importante encontrado foi o *AluJb-LIN28B*, os autores verificaram que a deleção desse TE eliminou a expressão de alguns oncogêneses, ilustrando a necessidade e suficiência de um TE para a ativação desses. Esses resultados destacam esse fenômeno de onco-exaptação como um mecanismo importante para a ativação de oncogêneses e para a tumorogênese.

Ainda existem várias questões não compreendidas sobre o desenvolvimento do câncer, mas está claro que ele é um processo multifatorial (Hoeijmakers, 2009; Vogelstein et al., 2013). Mais estudos são necessários para determinar se a ativação de retrotransposons como o LINE-1 ou *Alu*, pode ser uma das causas dessa doença (Chénais, 2013).

Ainda, devem ser considerados os efeitos dos quimioterápicos usados nos tratamentos das diferentes neoplasias, uma vez que esses, além de causarem estresse oxidativo, afetam a expressão de diversos outros genes e, portanto podem também causar um aumento na expressão de certos TEs.

2. JUSTIFICATIVA

Os TEs podem representar grande parte do genoma de praticamente todos os organismos (Kazazian, 2011) e evidências encontradas nos últimos anos indicam que os TEs podem ser mais ativos em tecidos somáticos do que se imaginava, causando uma série de consequências positivas e principalmente negativas para os genomas hospedeiros (Helman et al., 2014). Como os TEs podem gerar instabilidade genética, sua ativação em células somáticas pode estar envolvida com o envelhecimento celular (Sedivy et al., 2013); além disso, a TS dos TEs LINE-*like* e *Gypsy* também têm sido relacionada ao declínio neural e cognitivo idade-dependente em moscas (Bundo et al., 2014; Krug et al., 2017; Li et al., 2013); e diversas doenças têm sido relacionadas à TS, como transtornos psiquiátricos em seres humanos (Guffanti et al., 2014) e o desenvolvimento do câncer (Chénais, 2013).

Apesar das diversas evidências da ativação de TEs por agentes estressores, como em resposta ao choque térmico, irradiação, infecções virais (Anikeeva et al., 1994; Chakrani et al., 1993; Jouan-Dufournel et al., 1996; Ratner et al., 1992), e a UV (Qüesta et al., 2013), ainda muitas questões permanecem em aberto sobre a ativação da TS dos TEs, bem como se ela é constante ou variável ao longo da vida dos organismos.

O presente estudo possibilitou, através do modelo *white-peach*, que é excelente para estudos de transposição somática, verificar se ocorre a ativação de TEs, em especial *mariner-Mos1*, em linhagens de *D. simulans* e *D. melanogaster* expostas a diferentes agentes estressores, como choque térmico e diferentes quimioterápicos, bem como avaliar as consequências da TS desse TE no comportamento e na expectativa de vida desses indivíduos.

3. OBJETIVOS

3.1 Geral

Verificar se ocorre a ativação de TEs, em especial *mariner-Mos1*, em moscas *D. simulans* e *D. melanogaster* expostas a diferentes agentes estressores.

3.2 Específicos

1. Testar a hipótese de que a excisão somática ocorre e se acumula ao longo do ciclo de vida das moscas da linhagem *D. simulans white-peach*.

2. Analisar a associação entre a transposição de *mariner-Mos1* e alterações no comportamento das moscas e na viabilidade dos ovos em linhagens de *D. melanogaster white-peach* com diferentes taxas de transposição de *mariner*.

3. Comparar a expressão diferencial de genes e transposons em moscas da linhagem *D. melanogaster wpchM-* expostas a ciclofosfamida.

4. Verificar se ocorre uma diferença nas taxas de transposição de *mariner* após expor moscas da linhagem *D. simulans white-peach* a três quimioterápicos: cisplatina, dacarbazina e daunorubicina.

CAPÍTULO 1

The somatic mobilization of transposable element *mariner-Mos1* during the *Drosophila* lifespan and its biological consequences.

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The somatic mobilization of transposable element *mariner-Mos1* during the *Drosophila* lifespan and its biological consequences

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ABSTRACT

Transposable elements (TEs) are mobile DNA sequences on genomes. Some elements are able to transpose in somatic cells, a process known as somatic transposition (ST), which has been associated with detrimental biological effects. The *mariner-Mos1* element of *Drosophila* promotes transposition in somatic and germline cells and is an excellent model for studies related to the biological consequence of somatic excision (SE). In this work, we used temperature stress to induce increasing transposition of *mariner-Mos1* during different stages of the development of *D. simulans*, aiming to quantify SE during lifespan. Furthermore, strains of *D. melanogaster* exhibiting differential expression of *mariner-Mos1* were employed for estimating some biological consequences of *mariner* mobilization. It is shown that SE of *mariner-Mos1* was not constant during development; the larval phase had the highest rates while the pupal stage exhibited lower rates, and in the embryonic stage, no difference was detected. SE can be detrimental, as suggested by correlation in SE level and reduction in behavioral activities and embryonic viability. This study showed that *mariner-Mos1* SE accumulates during the *Drosophila* life cycle, and can be involved in detrimental effects.

1. Introduction

Until recently, there has been little interest in somatic transposition (ST) events as they are not heritable, and also because, normally, organisms have strong ST-silencing mechanisms to minimize their detrimental biological effects (Haig, 2016). However, accumulated evidence in the last decades has shown that ST is more frequent than previously thought, with a broad range of biological effects, ranging from acutely detrimental to adaptive (Haig, 2016; Loreto and Pereira, 2017).

Drosophila is one of principal model organisms for TE studies. Pioneer works showed that genetic damage caused by the ST of *P* and *mariner* elements can reduce lifespan and be involved in aging (Woodruff, 1992; Nikitin and Woodruff, 1995). The development of new approaches to study ST can aid in clarifying TE participation in the phenomena as aging, phenotypic plasticity and the other biological parameters. The *mariner-Mos1* TE and the *white-peach* mutation of *Drosophila* constitute an excellent model to study ST. The *mariner* DNA transposon was initially described in *D. mauritiana* (Medhora et al., 1988). This element belongs to the *mauritiana* subfamily of *mariner* elements (Robertson et al., 1997) and is present in several species of the *melanogaster* complex. It is a class II element, transposing by a DNA intermediary, using cut-and-paste mechanisms. This *mariner* element is 1286-bp long, has TIRs with 28-bp and an open reading frame encoding a transposase. The active copies can continuously mobilize in germ and somatic cells (Hartl, 2001). The insertion of this TE in the promoter region of the *white* gene produces flies with peach eyes, referred as *white-peach* (Jacobson and Hartl, 1985; Haymer and Marsh, 1986; Jacobson et al., 1986). The *mariner* copy (named *peach*) causing the

Abbreviations: TEs, Transposable elements; ST, somatic transposition; SE, somatic excision; MOS1, mariner active copy, produce mosaic eyes; qPCR, quantitative polymerase chain reaction; bp, base pairs; kbp, Kilo-base pair; °C, Celsius degree; M-MLV, Moloney Murine Leukemia Virus Reverse Transcriptase; RING, rapid iterative negative geotaxis behavioral assay

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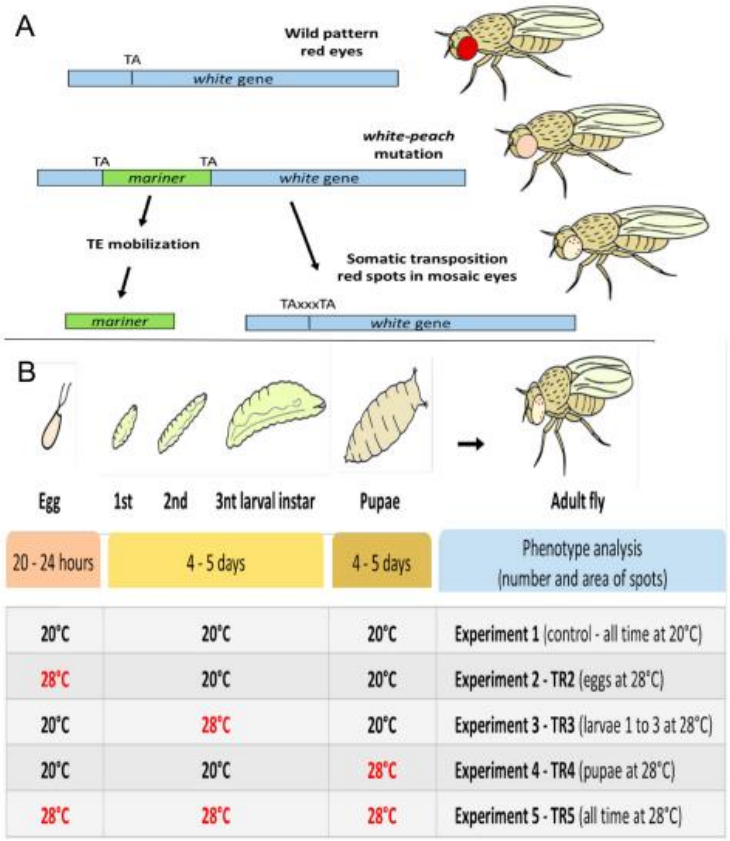

Fig. 1. Relationship between genotype (white gene condition) and phenotype (eye color) and scheme of the experiment.
A) At the top of figure we have the wild condition of the *white* gene and its respective phenotype, a fly with red eyes. The *white-peach* mutation is caused by the insertion of the *mariner* element in the promoter region of the *white* gene, generating flies with peach-colored eyes. In the presence of autonomous copies, the *mariner* inserted in the white gene is able to transpose in some cells, reversing the mutation and resulting in flies with genotype and mosaic phenotype. These flies have white-peach eyes with red spots. B) Scheme of the experiment: individuals were exposed to thermal stress (28 °C) during different stages of development and maintained at 20 °C in the other phases. After adult eclosion, 100 flies of each treatment were analyzed for the number and area of the spots in the eyes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mutation is non autonomous, but the presence of autonomous copies (*Mos1*) in the same genome can mobilize it, leading to reversion of the mutation. Therefore, when mutation reversions occur in eye tissues, flies exhibit a mosaic phenotype *i.e. white-peach* eyes with red spots corresponding to ommatidia in which the *peach* copy was excised (Fig. 1-A; Supplementary Fig. 1 and Haymer and Marsh, 1986; Jacobson et al., 1986; Bryan et al., 1987; Bryan et al., 1990; Medhora et al., 1988; Medhora et al., 1991).

The *Drosophila* eye consists of an array of approximately 800 hexagonal ommatidia, which contains 22 cells, making the total of cells in each eye over 16,000. The eye is derived from the eye-antennal disc, which arises from approximately 20 cells in the embryonic blastoderm. During larval stages occur an intense cell division and the eye-antennal disc at the end of third instar larva contains about 2000 cells. The pupal phase is marked by cell differentiation for tissue formation (Brody, 1999).

The activity of the *mariner-Mos1* element (that will be named *Mos1* thereafter) can be stimulated by temperature increases. The activation of *Mos1* under heat stress could be attributed to the existence of conserved *cis* sequences called heat-shock elements (HSEs), which are regulatory sequences that respond to transcription factors known as heat-shock factors (HSFs) (Chakani et al., 1993; Jardim et al., 2015).

The activation of *mariner-Mos1* under thermic stress and the "*white-peach* model" are appropriate tools for investigating somatic excision (SE) under thermal stress during the *Drosophila* development. The number of red spots in eyes can estimate the excision rate, as each spot is assumed to reflect one independent excision event of *mariner-Mos1* element. Excisions, by themselves, have biological impacts, producing chromosome breaks and deletions (Adams and Sekelsky, 2002; Huang et al., 2013). Moreover, as shown for several transposable elements, the excision rate is correlated to the transposition rate (Liu et al., 2004).

Natural populations of *D. melanogaster* do not have *mariner* elements. However, Garza et al. (1991) produced a transgenic *white-peach* (w^{pch}) line, stable in the absence of active *mariner* elements. Furthermore, they produced other lines carrying active *Mos1* elements and demonstrated that the mobilization rate of this element is six-fold higher in *D. melanogaster* than in *D. simulans*. Therefore, the *white-peach* strains of *D. simulans* and *D. melanogaster* are exceptional material with variable rates of somatic mobilization, allowing comparative studies regarding this phenomenon.

In this study, we used thermic stress to activate *Mos1* transposition at different developmental stages of a *D. simulans white-peach* strain, aiming to test the hypothesis that SE occurs and accumulates throughout the fly's life cycle. The *D. melanogaster white-peach* strains were utilized to analyze the association between *Mos1* transposition and alterations in fly's behavior and eggs viability. In addition, a new qPCR approach was developed to estimate the amount of SE in comparison to that estimated based on the number of eye spots.

2. Material and methods

2.1. Fly stocks

For temperature-shift experiments, we used a *D. simulans* strain, named *Dswp test*, established in our laboratory which is characterized by a low *mariner* activity. In this strain, all individuals have the *peach* copy in the *white* gene and, therefore, present a *white-peach* phenotype. The total number of *mariner* copies was estimated by qPCR at 1.84 (± 0.83 − SD). This means that all individuals had the *peach* copy and most of them had one active *Mos1* copy (Jardim et al., 2015). Due to the low eyes mosaic expression, this strain is suitable for SE quantification, since the eye spots are scattered and each of them is assumed to represent a single SE event.

To estimate the biological effects of SE, we established three *D. melanogaster* isolines with different rates of somatic mobilization of the *peach* copy - median, high and very high. These lines were obtained from crosses between the *D. melanogaster white-peach* strain (w^{Pch}) and the *D. melanogaster* M-19 strain (Robillard et al., 2016), which has many *mariner-Mos1* active copies. w^{Pch} females were crossed with M19 males and isolines were made in the F1. The females chosen for isoline were classified according to the number of eye spots (low - medium - high). After, in the 4*th* generation, we chose three isolines, one for each category. These flies were used to behavioral, biological and molecular assays. All assays were performed in this generation. The obtained lines were called $w^{Pch}M-$, exhibiting median *mariner* activity; $w^{Pch}M+$ had high *mariner* activity and $w^{Pch}M++$ had very high *mariner* activity as estimated by counting eyes spots. As a control in these experiments, we used the w^{Pch} strain, which only contains a single copy of stable *peach* inserted into the *white* gene and no active copy. These strains were molecularly characterized herein (see results).

2.2. Temperature-shift experiments and phenotypic analysis

Five treatments were used (Fig. 1-B). For experiment 1/control (EXP1), flies were maintained throughout the life cycle at 20 °C. In experiments 2 to 4, the flies were maintained at 20 °C, however they were moved to 28 °C at different stages depending on experiment: embryo stage (EXP2), larval stages (EXP3) and during the pupae stage (EXP4). Experiment 5 (EXP5) flies were maintained at 28 °C across the whole life cycle.

The experiments were also performed combining some developmental phase under thermal stress aiming to test whether the SE is cumulative (Suppl. Fig. 2). The treatments were divided in eight categories. For experiment 1 (control) the flies were maintained during all development at 20 °C. In experiments 2 to 7, the flies were maintained at 20 °C, however they were moved to 28 °C, during the embryo stage (EXP2), embryo and larvae stages (EXP3), 1*st* and 2*th* larval instar (EXP4), all larval stages (EXP5), pupae stages (EXP6), all larval instars plus the pupae stage (EXP7). In experiment 8 (EXP8) the flies were maintained at 28 °C thought the life cycle.

The number of red spots in both eyes was counted from 100 adult flies randomly collected after each treatment and observed under a stereomicroscope. The flies exhibiting mosaic eyes were photographed and the area occupied by red spots as well as the total area of eyes was measured using the BEL View 7 software. The number of reds spots was counted, and it was assumed that each spot corresponded to a single excision event. The comparisons among treatments were done by the Kruskal-Wallis test followed by Dunn's correction for multiple comparisons, using the GraphPad Prism 6 software.

2.3. Quantification of SE by qPCR

A qPCR approach was designed to quantify the *mariner* excision rate from the *white* gene in flies having the *white-peach* mutation (Supplementary Fig. 3). The rationale was that primers in the region of the *white* gene with a *mariner* insertion did not amplify this sequence when a very short period of the extension was used in the qPCR reaction. This amplification occurred in the wild-type *white* gene producing an amplicon of 107 bp. A *D. simulans* (strain *DsBrasilia*) wild type for the *white* gene was used as control. The amplification curve of these samples served as a reference to estimate the percentage of reversion of the *white-peach* mutation of the treatments at 20 and 28 °C. Normalization was performed with reference gene L17-RPL17q2, as described by Saint-Leandre et al. (2017). For relative quantification, the $2^{-\Delta\Delta Ct}$ method was used (Livak and Schmittgen, 2001). Thus, the qPCR samples were normalized using a unique copy gene and the amplification rate for wild-type *white* gene, resulting in somatic mobilization of the treated *white-peach* strains being compared to those observed in the strain carrying the wild-type *white* gene (expecting 100% amplification). DNA was isolated from a pool of 20 flies for it treatments. The reagents, conditions and primers of the qPCR assays are described in the Supplementary Material (Supplementary Tables 1, 2 and 3).

Our aim was to estimate the overall excision rate of the copy inserted into the *white* gene of the *Dswp test* strain. Three comparisons of SE rates were conducted: (i) between flies maintained from eggs to the second larval instar at 20 °C and those maintained at 28 °C; (ii) between flies kept during all development, from eggs to adults, at 20 °C and 28 °C; (iii) between adult flies that developed at 20 °C soon after their metamorphosis and maintained at 20 °C or moved to 28 °C for a period of 10 days before DNA extraction. The experiments were carried out with three biological and three technical replicates. For each sample, 20 flies were used for DNA extraction.

The relative SE values obtained for treatments at 20 °C and 28 °C were compared with those observed in the reference strain using a one-way analysis of variance (ANOVA) followed by Dunnett's test that compares each treatment to the control. The treatments at 20 °C and 28 °C were compared to each other using a *t*-test. Differences were considered statistically significant if $p < 0.05$.

2.4. Mariner copy number in D. melanogaster strains

Genomic DNA was isolated from 20 flies following the protocol described by Sassi et al. (2005). DNA integrity was verified by an agarose gel (0.8%) electrophoresis and quantified in a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). qPCR was performed in a StepOnePlusTM Real-Time PCR System (Applied Biosystems, USA) device. For the estimation of the *mariner* copy number in the *D. melanogaster* $w^{Pch}M-$, $w^{Pch}M+$ and $w^{Pch}M++$ strains, qPCR of a unique copy reference gene (ribosomal protein, L17-RPL17q2) was conducted and compared with the *mariner-Mos1* amplification by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using the Ct values. The reagents, conditions and primers of the qPCR assays are described in the Supplementary Material (Supplementary Tables 1, 2 and 3).

2.5. Mariner transposase gene expression

Total RNA in the *D. melanogaster* strains was extracted from 20 flies with TRIzol® reagent (Invitrogen, USA). The quality of the RNA samples was assessed by 1% agarose gel electrophoresis and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Afterwards, the samples were treated with Dnasel (Promega, USA) and complementary DNA (cDNA) synthesis was performed with the M-MLV reverse transcriptase enzyme (Invitrogen, CA, USA) and oligo-dT primers.

The relative expression of the *mariner* transposase gene was estimated using the L17-RPL17q2 as a reference. The reaction and primers employed are as described in Supplementary Tables 1, 2 and 3. The relative gene expression was based on $2^{-\Delta\Delta eq}$ (Livak and Schmittgen, 2001) and the efficiency of the PCR reactions was computed with LinRegPCR (Ruijter et al., 2009).

The values obtained for treatments were using a one-way analysis of

variance (ANOVA) followed by a Dunnett's test. Differences were considered statistically significant if $p < 0.05$.

2.6. Larval crawling assay

The larval crawling experiments followed the protocols described by Nichols et al. (2012). Third instar larvae were collected, rinsed in PBS and placed in a Petri dish containing agar 2%. The plate was placed on graph paper. The total distance travelled by the larvae, for 1 min, was recorded and a comparison was performed among the strains. The values were compared using a one-way ANOVA followed by a Tukey's test for multiple comparisons. A total of 45 larvae of each strain were observed.

2.7. Rapid iterative negative geotaxis (RING) assay

The negative geotaxis and climbing ability of *D. melanogaster* with and without active *Mos1* were estimated by the RING assay as described by Nichols et al. (2012). Five-days old flies were placed in a tube 13 cm long and 2.5 cm in diameter. After an acclimation period of 20 min, the tubes were shaken so that the flies fell to the bottom of the tubes. The climbing of the flies was filmed, and the frame corresponding to 4 sec from the beginning of the experiments was used for the calculation of the average climbing height. In each experiment, 20 flies of each strain were used. Males and females were analyzed separately. The final value corresponded to an average of 25 independent experiments. These values were compared using a one-way ANOVA followed by a Tukey's test.

2.8. Oviposition rates and egg viability

The oviposition rates and viability of eggs were estimated for *D. melanogaster* strains with and without active *Mos1*. Virgin females and males, five-days old, were put together, in couples, into a vial with culture medium. Then, the couples were transferred to a new vial after every 24 h, and the total of eggs oviposited were counted at 24, 48 and 72 h from the beginning of the experiment. Eight couples were evaluated for each strain.

For the estimation of the viability of eggs, 10 recently oviposited eggs (two-hour intervals) were placed in a vial with culture medium. Thirty hours after, the proportion of eggs emerged were counted. Six independent experiments were performed for each strain and data analyzed using one-way ANOVA followed by a Tukey's test.

3. Results

3.1. SE during Drosophila development

3.1.1. Temperature-shift experiments and phenotypic analysis

Heat-shock experiments consisted to expose flies of the *D. simulans Dswp test* strain to the thermal stress at different stages of their development. The selected temperature was 28 °C, which had been shown able to increase significantly the mobilization of the *mariner* element in the *Dswp* test strain (Jardim et al., 2015).

Fig. 2-A shows that heat stress leads to an increase of the number of SE events regardless of stage of development, except when applied to eggs. Comparisons with the control (all times at 20 °C) are significant. The stronger effects are observed on the larvae and when flies were maintained during all their life cycle at 28 °C. The comparison between these two situations is not significant, as well as the comparison between eggs and pupae. Moreover, a cumulative effect was evident. More precisely, when several more developmental phases are exposed to the thermal stress, an increase of eye spot number is observed. The larval stage shows a significant increase in spot number; suggesting a major temperature effect in this period, mainly during the third instar larvae (Fig. 2-A). While significantly different from the control, the impact of temperature on the pupae remains small suggesting that the transposition rate at this stage is low.

The estimate of the total surface of red spots is summarized in Fig. 2-B. The main objective of this analysis was to verify whether the size of the spots was larger if transposition occurred early during development. The red spot surface showed variation between the different treatments. Lower values were found when flies were maintained all the time at 20 °C or at 28 °C exclusively in the pupal phase. For these treatments, the average percentages of the red area were 0.15(± 0.4)% and 0.27(± 0.4)% respectively. The higher percentage of the red area were observed in flies maintained at 28 °C at all times or transferred to this temperature during larvae stages 4.59 (± 8.7) % and 1.29% (± 1.9), respectively. When maintained only in egg stage at 28 °C the average was 1.18 (± 3.2). Statistical analysis showed that the null hypothesis (similar percentage of red surface for all treatments) was rejected. More precisely, pairwise comparisons evidence several statistically significant differences. The total surface of red spots on egg and larvae phases differed from the controls (all time at 20 °C), while no differences were found between egg and pupae phases or between larvae and those maintained always at 28 °C.

Analysis joining several developmental phases showed the higher values were those involving the third instar larvae (Fig. 3-B). Nevertheless, how these phase is related with production of higher spot number, we decide compare the ratio between area with red spots/number of spots, aiming estimate differences the number of "big spots" (Fig. 4). These comparisons showed that treatments in any phase differ from the controls, although no differences were found between eggs and pupae, eggs and larvae or larvae and all time at 28 °C. It is worth highlighting few outliers points observed when eggs were heat-shocked, indicating very big spots.

3.1.2. Analysis of SE rate by qPCR

The SE of *white-peach* copy of the *mariner* element, in all cells of the body, was estimated by the percentage of mosaicism in the *white* gene generated by the excision of the copy inserted in the *white* gene (Fig. 5; Supplementary Table 4).

Whatever the stages of the life cycle, the excision rates were statistically different between the two treatments (20 °C *versus* 28 °C). In all cases, the excision rate was higher at 28 °C than at 20 °C. In condition 3 where individuals are kept at 20 °C during all their development followed by a 10 day transfer to 20 °C or 28 °C, the higher value of excision rate observed in adult flies transfer at 28 °C (1.78 ± 0.17 *versus* 4.63 ± 0.32) suggests that *mariner* can transpose in adults.

Since the different stages here considered have different durations, normalization was required to compare the excision rate of *mariner* at different stages of the life cycle. It was chosen to estimate a daily excision rate. From eggs to second instar larvae, the daily excision rate is about 0.24% at 20 °C and 0.58% at 28 °C. From eggs to emergence, this rate is 0.3% at 20 °C and 0.78% at 28 °C; and 0.18% at 20 °C and 0.43% at 28 °C, adults.

3.1.3. Estimate of mariner copy number and expression in D. melanogaster strains

The copy number of *mariner* was estimated by qPCR and the strain $w^{pch}M-$ has 6.1 ± 2.0 copies, $w^{pch}M+$ 12.1 ± 5.4 and $w^{pch}M++$ 16.2 ± 5.7. The w^{pch} strain was used as a control and presents a single copy of *mariner* (Fig. 6-A; Supplementary Table 6).

The relative expression of the mariner transposase gene was estimated by RT-qPCR (Fig. 6-B; Supplementary Table 5) and the expression rate was in agreement with the copy number. The $w^{pch}M++$ shows a higher expression (considered 100%) followed by $w^{pch}M+$ (55% ± 0.21) and $w^{pch}M-+$ (34% ± 0.11). All these values were significantly different ($p < 0.05$).

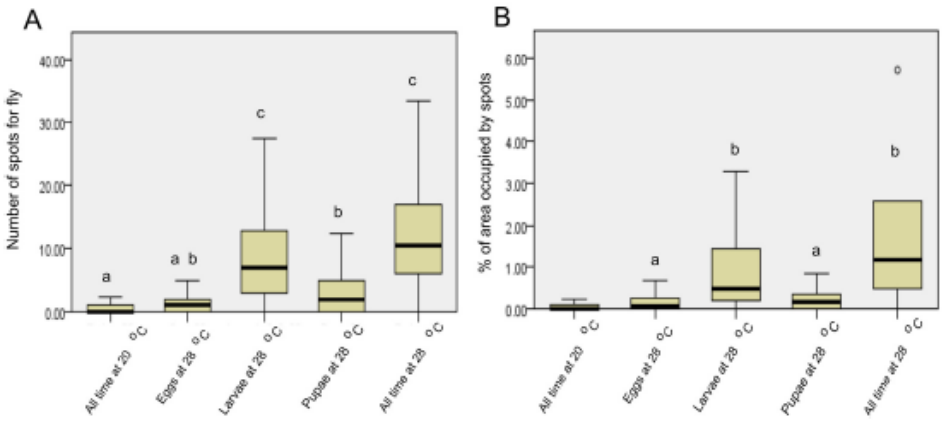

Fig. 2. Heat-shock experiments in different developmental stages.
Box plots showing the difference in (A) median of number of spots per fly in each experiment of temperature shift, (B) Median of percentage of eye area occupied by spots in each experiment of temperature shift. Groups indicated by the same letter do not differ statistically from each other ($\alpha < 0.05$).

3.2. Biological effects of SE

Two behavioral traits were estimated in *D. melanogaster* isolines that had different somatic expression of the *mariner* element: larval crawling ability and adult negative geotaxis. A statistically significant reduction of larval crawling ability is observed only for the $w^{Pch}M++$ strain (Fig. 7-A, $p < 0.0001$). The RING assay shows an inverse relationship between climbing ability and the *Mos1* copy number and expression. Climbing ability is higher in the w^{Pch} strain, median for the $w^{Pch}M-$ and $w^{Pch}M+$ strains and lower for the $w^{Pch}M++$ strain, both for males (Fig. 7-B, $p < 0.0001$) and females (Fig. 7-C, $p < 0.0072$).

Two lifespan features were estimated for strains with different *mariner* expression: oviposition rates, viability of eggs (Fig. 8). Although no difference is observed for oviposition rates among strains, the viability of eggs is lower in strains with active *mariner-Mos1* (Fig. 8, $p < 0.0083$).

4. Discussion

Although there is evidence that ST can be involved in either detrimental or adaptive biological effects for organisms, many questions remain. One of them is whether ST is constant or variable across the lifetime. Although it can be specific for each TE of each organism, dependent on the evolutionary "momentum" of a particular TE in each genome, number of potentially active copies, mechanisms that control transposition and possibility to escape this control (reviewed by Loreto and Pereira, 2017).

The *mariner-Mos1* element is a solid model for studying the biological consequences of somatically active class II TEs, as it has been shown that this element promotes ST (Haymer and Marsh, 1986; Jacobson et al., 1986; Bryan et al., 1987; Medhora et al., 1988; Medhora et al., 1991). Moreover, there are great variations in *mariner* activity are observed among populations, in particular along latitudinal gradients or migration routes (Giraud and Capy, 1996; Russel and Woodruff, 1999; Picot et al., 2008).

The results presented in this study showed, for the first time, that the SE of the *mariner* element can occur during all stages of the life cycle, and it is cumulative. However, SE rate is not constant, exhibiting variations along development. Moreover, the different stages are not similarly impacted by thermal stress. Larvae seem to be more sensitive than pupae, and embryos are not affected. Saint-Leandre et al. (2017) demonstrated that *Mos1* transcription is always high in the testes and

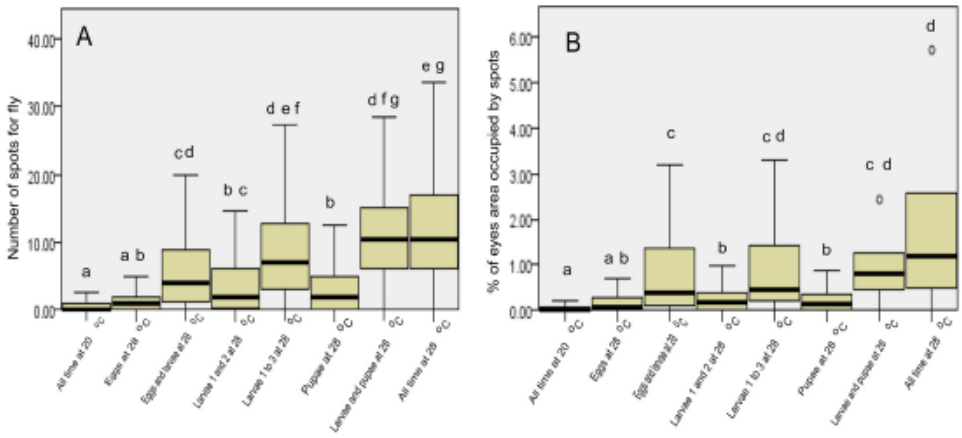

Fig. 3. Heat-shock experiments in joining different developmental stages.
Box plots showing the difference in (A) median of number of spots per fly in each experiment of temperature shift, (B) Median of percentage of eye area occupied by spots in experiments in that some developmental phase were submitted to thermal stress, aiming test if the SE is cumulative. Groups indicated by the same letter do not differ statistically from each other ($\alpha < 0.05$).

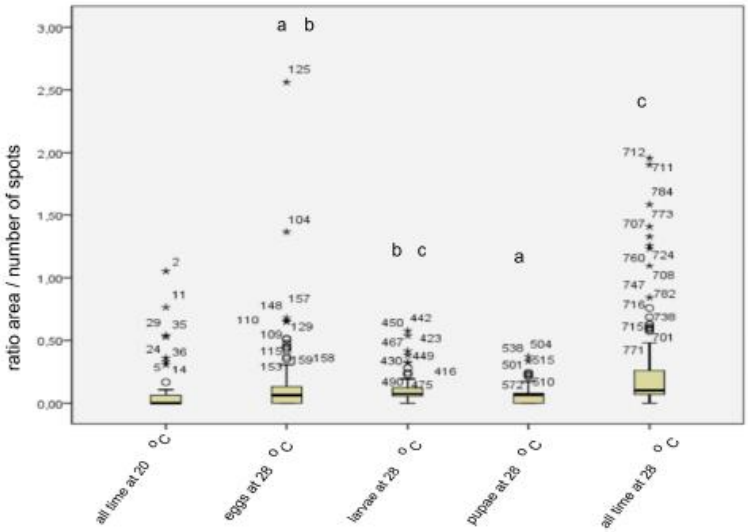

Fig. 4. Heat-shock experiments in different developmental stages (area occupied). Box plots of ratio between areas occupied by red spots/number of spots.
* = outliers values and the number of samples. Groups indicated by the same letter do not differ statistically from each other ($\alpha < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

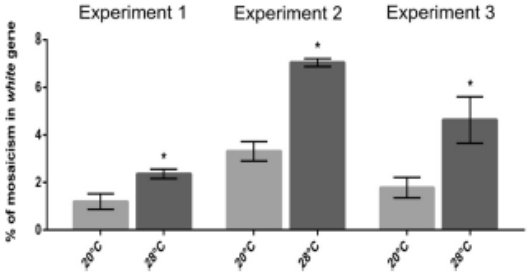

Fig. 5. Quantification by qPCR of the somatic excision of *mariner* w^{pch} element. The SE for all body cells, estimated by the percentage of mosaicism in the *white* gene generated by the excision of *mariner* w^{pch} and consequent reversion to the wild genotype: the individuals remained at 28 °C from the beginning of their development until the 2nd larval instar. Experiment 2: the organisms remained at 28 °C throughout their development to adult stage. Experiment 3: adult flies remained at 28 °C, after they eclosion, for a period of 10 days. * = comparison treatments 20 °C and 28 °C are statistically significant ($\alpha < 0.05$).

very low in ovaries. Inversely, the *argonaute 3* (*Arg3*) gonad transcriptional activity is higher in ovaries than in testes. As *Arg3* is a protein involved in TE silencing *via* the *pi*RNA pathway, the high rate of transcription of this gene in ovaries could produce eggs with high potential for inhibiting *Mos1* activity, leading to the low activity observed in embryos in our assays. It would be interesting to assess whether *si*RNA is involved in the somatic activity of this TE.

The phenotypic approach to quantify ST, based on eyes spots, can be associated with the differential number of ommatidia precursor cells during development. Upon the beginning of embryogenesis, approximately 20 cells are destined to build the adult eye. This number grows to roughly 10,000 cells in the imaginal disc at the end of the third larval instar (Hales et al., 2015).

Other variables that can influence the distinct SE rates observed in different developmental stages are the duration of each phase. The embryonic stage extends to, in average, 24 h. The larval and pupae stages are longer, lasting for approximately four to five days each (Tyler, 2000; Hales et al., 2015). The short embryonic stage can also explain why do not occurred increase of ST events during this stage. Phases with a larger duration, as in the case of the larvae and pupae stages, featured a higher SE rate. However, the pupae phase had a lower SE rate than the larvae stage, and both had approximately the same

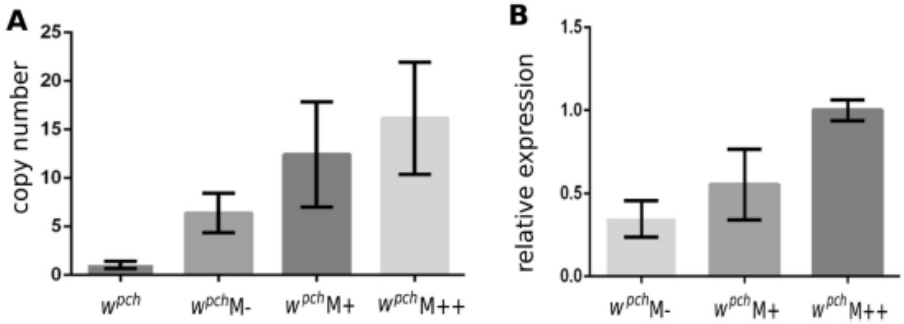

Fig. 6. Copy number and relative expression of *mariner* in *D. melanogaster* strains.
A) *Mos1* copy number estimated by qPCR in the w^{pch}, $w^{pch}M-$, $w^{pch}M+$ and $w^{pch}M++$ *D. melanogaster* strains. B) relative *mariner-Mos1* expression estimated by RT-qPCR for the $w^{pch}M-$, $w^{pch}M+$ and $w^{pch}M++$ *D. melanogaster* strains.

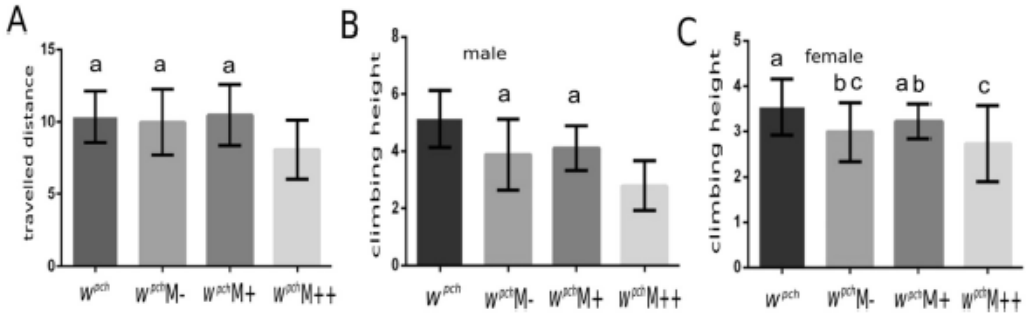

Fig. 7. Behavioral analyses of *D. melanogaster* studied strains.
A) Larval crawling represented by the average number of 0.5 cm2 squares travelled by minute. No statistical differences were observed in comparisons among w^{pch}, $w^{pch}M-$ and w^{pch} $M+$ strains. However, the strain $w^{pch}M++$ showed a significant reduction in the crawling behavior when compared the others. B) RING experiment for males: average height of climbing, in centimeters. C) RING experiment for females: average height of climbing. Groups indicated by the same letter do not differ statistically from each other ($\alpha < 0.05$).

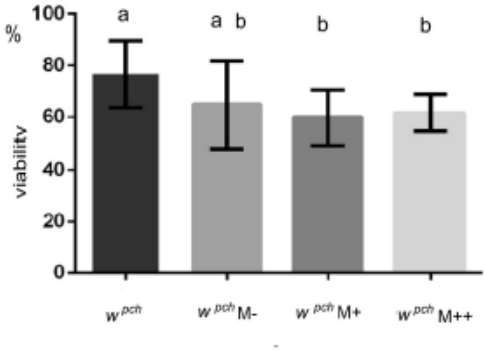

Fig. 8. Eggs viability of *D. melanogaster* studied strains.
Estimate of viability of eggs, in %, among *D. melanogaster* strains analyzed. There are a significant reduction of viability for strains having active Mos1 ($p < 0{,}0083$). Groups indicated by the same letter do not differ statistically from each other ($\alpha < 0.05$).

duration (four days). This result suggests that other factors along with time across stages can be involved in establishing SE rates.

The cell cycle can also be involved in modulating ST. During the first 3 h of embryogenesis, intense cell division occurs over the phases of cleavage, blastoderm formation and gastrulation. After these phases, the cells begin to differentiate to give rise to different tissues and organs until the egg hatches as larvae (Tyler, 2000; Hales et al., 2015). During the larval stages, there is almost no differentiation and the cells pass through successive mitoses, increasing the size of the organism such that molting can occur. The larvae body has structures called imaginal discs, which, during the pupae stage, will differentiate into adult structures, and cells called histoblasts induce the formation of the abdominal epidermis and internal organs (Tyler, 2000). After pupation, the larvae tissues self-destruct whereas imaginary discs and histoblasts differentiate to form the adult body (Tyler, 2000). The larval stage has higher ST rates and is a period with intense cell division, while the pupal phase is marked by cell differentiation for tissue formation. Intensive mitotic processes can be involved with a higher ST while that stages with more cell differentiation can have lower ST rates.

Regarding the area occupied by the spots, our hypothesis was that there is a correlation between the size of the spots and the stage of development during which ST occurred. Events taking place at the beginning of development, such as embryonic phase or the first/second larval instar, could result in larger spots owing to the fact the eye imaginal discs would be growing and passing through successive mitotic processes. Nevertheless, the results do not support this hypothesis once a proportion of the occupied area inhabited by spots do not exhibit a clear relationship with the phases in which the flies were treated.

Few studies have addressed the question related to ST accumulation over the course of an organism's lifespan. In *Drosophila*, Perrat et al. (2013) and Li et al. (2013) have shown that TEs are active in a specific group of neuronal cells, called *αβ-Kenyon Cells* (*αβ–KCs*) within the *mushroom body* of fly nervous system. These cells are related to olfactory memory. The aforementioned studies suggest the ST is cumulative and involved either in neural plasticity or neural decline and aging. More recently, Treiber and Waddell (2017), using a new approach to isolate specific *αβ–KCs* cells and sequencing the genome of them, did not found TE insertions that could suggest higher TE activity in these cells or accumulation of ST with aging.

The qPCR approach used in this work showed the SE, at least in relation to the *mariner-wpch* element, is cumulative during the entire life cycle and occurs either at 20 °C or 28 °C, being higher at elevated temperatures. In addition, the results obtained with experiment 3 demonstrated there to be an increase in *mariner* SE rates in adult flies maintained at 28 °C for a period of 10 days. These data indicate that this element may have activity even in adult organisms even when considering that the fruit fly is mostly composed of post-mitotic cells. Our excision estimates for the *mariner* element on the *white-peach* locus is remarkably high. Flies maintained at 28 °C, from eggs until adult eclosion, have "lost" the *mariner* element in 7% of their *white* gene. Taking into account the excision that occurs at the adult stage, in an elderly fly the loss of mariner of white gene can overpass by 10%. Even considering that only part of these excisions could result in new insertions and mutations, the biological consequences can be significant. The excision per si can be detrimental producing chromosome breaks and deletions (Adams and Sekelsky, 2002; Huang et al., 2013).

Behavioral and lifespan traits estimated for isolines carrying different *mariner* SE rates suggest a harmful consequence due the TE mobilization. Although the experiments do not show definitively the direct action of ST in the phenotypic traits analyzed, the correlation between SE rates and decrease of behavioral abilities as larval crawling and adult negative geotaxis or lifespan features, as viability of eggs are suggestive that ST is a component of the observed phenomena.

Sturm et al. (2015) suggested that the gradual chromatin decondensation and increasing of transposon mobilization could explain why the mortality rate rises exponentially throughout the adult life in most animal species. However, at least for *mariner* in *Drosophila*, we did not observe the exponential increase of somatic mobilization during the adult phase. Instead, we observed a continuous ST during all of the life cycle, in particular a higher rate during the larval stage.

The results obtained in this study shows that *mariner-Mos1* SE under thermal stress is not constant throughout development. The activation of transposition does not depend solely on the time of exposure to stress, but is influenced by other factors, such as the developmental

stage and the cellular processes taking place in that stage. Moreover, some fitness components as behavioral and lifespan traits showed be affected in inverse order with the SE measured, suggesting a detrimental impact of somatic transposition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2018.08.079.

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CAPÍTULO 2

Cyclophosphamide in *Drosophila* promotes genes and transposable elements differential expression and mitochondrial dysfunction.

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Cyclophosphamide in *Drosophila* promotes genes and transposable elements differential expression and mitochondrial dysfunction

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ABSTRACT

Cyclophosphamide (CPA) is an alkylating agent used for cancer chemotherapy, organ transplantation, and autoimmune disease treatment. Here, mRNA sequencing and high-resolution respirometry were performed to evaluate the alterations of *Drosophila melanogaster* gene expression fed with CPA under acute (0.1 mg/mL, for 24 h) and chronic (0.05 mg/mL, for 35 days) treatments. Differential expression analysis was performed using Cufflinks-Cuffdiff, DESeq2, and edgeR software. CPA affected genes are involved in several biological functions, including stress response and immune-related pathways, oxi-reduction and apoptotic processes, and cuticle and vitelline membrane formation. In particular, this is the first report of CPA-induced mitochondrial dysfunction caused by the downregulation of genes involved with mitochondria constituents. CPA treatment also changed the transcription pattern of transposable elements (TEs) from the *gypsy* and *copia* superfamilies. The results presented here provided evidence of CPA mitochondrial toxicity mechanisms and that CPA can modify TEs transcription in *Drosophila* flies.

1. Introduction

Cyclophosphamide (CPA) is an alkylating agent widely used as a chemotherapeutic drug for cancer and autoimmune disease treatment, and as an immunosuppressant preceding organ transplantation (Binotto et al., 2003; Dollery, 1999). CPA is metabolised by the hepatic cytochrome, P450, (Anan et al., 2017) into its primary active metabolite, 4-hydroxycyclophosphamide (4-OH-Cy), followed by conversion to the active form – phosphoramide mustard (Chang et al., 1993; Selvakumar et al., 2005). Phosphoramide alkylates the nitrogen (N-7 position) of the guanine bases in DNA, causing intra-strand cross-linking between guanine and adenine. If the cellular machinery fails to repair this DNA damage, apoptosis can be triggered (El-Serafi et al., 2014). Besides phosphoramide mustard, CPA metabolism produces the reactive aldehyde acrolein (Giraud et al., 2010). Acute exposure to acrolein may impair the normal functioning of the mitochondrial electron transport chain, generating reactive oxygen species (ROS) via lipid and protein damage (Luo and Shi, 2005; Luo et al., 2005).

Most studies addressing gene expression alterations induced by CPA have used a microarray approach. Kubisch et al. (2013) found 212 differentially expressed genes (DEGs) in prostate cancer tumours exposed to CPA (120 mg/kg/day). These genes are involved in coagulation pathway, axon guidance and steroid biosynthesis. El-Serafi et al. (2014) studied the blood cells of patients treated with CPA before undergoing stem cell transplantation and reported the downregulation of 139 genes associated with immune/autoimmune activation and allograft rejection. Upregulated genes found in that study were mainly immune-related receptor genes. Guo et al. (2014) found DEGs involved in DNA replication, transcription and spliceosome, and metabolism of nucleotides and amino acids in the spleens of mice treated with CPA. Van Dycke et al. (2015) found DEGs related to DNA damage response, oxidative stress and immune-related pathways.

RNA-Seq is, in general, more accurate than microarrays to detect DEGs. Using RNA-Seq, Wu et al. (2016) performed a global transcriptome analysis to characterise immune-unresponsive tumours and found that upregulated genes are involved in immune stimulatory signalling, immune effector activation, and inflammatory pathways activated in immune-related diseases. Downregulated genes were primarily involved in survival functions essential to tumour cells.

Besides changes in protein-coding genes, an increasing number of studies suggest that changes in transposable elements (TE) expression and mobilisation are linked to cancer. TEs have been highlighted as

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possible sources of mutation in tumorigenesis (Burns, 2017). TEs are DNA fragments able to change their position within or between the chromosomes they reside in. A considerable portion of eukaryotic genomes is composed of TEs and their remnants. In humans, the most abundant TE, the *Alu* element, is represented by over 1 million copies, and the second most abundant (*L1*) has approximately 500,000 copies. In total, TEs correspond to 47% of the human genome (De Koning et al., 2011; Lander et al., 2001). TEs are responsible for mutations, alterations in gene regulation and chromosome rearrangements, which are typically deleterious (Hua-Van et al., 2011; McCullers and Steiniger, 2017). Lee et al. (2012) found 190 somatic transposition events in samples of prostate, ovary and colorectal tumours – they found that TEs were inserted into tumour suppressor genes, changing their expression. Studies indicate that cellular stress can activate transposition (Capy et al., 2000; Horvath et al., 2017). Several chemotherapeutic drugs induce cell stress and, therefore, may induce somatic transposition (Mourier et al., 2014).

Comparing the transcriptomes of distantly related species under different conditions or treatments can be very useful in identifying common regulators and revealing fundamental principles. *Drosophila melanogaster* and *Caenorhabditis elegans* are the main animal models used and are extensively characterised in the modENCO project (Gerstein et al., 2014). Also, regarding TEs, *D. melanogaster* has one of the most studied eukaryotic genomes, and 30% of their TEs are full length and believed to be active (Kaminker et al., 2002).

In this study, we aim to carry out a deep sequencing analysis of mRNA transcripts of fruit flies subjected to acute and chronic treatments with CPA. Based on the sequencing results, we further study mitochondrial activity in CPA treated and untreated flies.

2. Materials and methods

2.1. Fly strain

Drosophila melanogaster wpch M- was described in Pereira et al. (2018). *D. melanogaster* were maintained in cornmeal, sucrose, and yeast medium. Only female flies were used for RNA extraction because we previously found that it lowers variation between replicates, giving more accurate results.

2.2. Chronic cyclophosphamide treatment

CPA concentrations were determined according to Podratz et al. (2011). However, because of CPA toxicity, drug concentration was lowered until we got a workable number of flies for RNA extraction.

A stock solution of CPA (20 mg/mL) was mixed in the culture medium at a final nominal concentration of 0.05 mg/mL. First, 40 to 50 adult flies were added to flasks with culture medium, with or without CPA, for oviposition. After 24 h, adult flies were removed and the eggs were kept in the medium throughout their development. After 35 days of continuous feeding with CPA, 20 three-day-old female flies were collected for RNA extraction. The experiments were performed in triplicate.

2.3. Acute cyclophosphamide treatment

A stock solution of CPA (20 mg/mL) was added to the culture medium at a final nominal concentration of 0.1 mg/mL. Red food coloring (E163) was also added to the culture medium for visual screening of feeding. Thirty to forty flies were added to the CPA-containing medium and allowed to feed for 24 h. After this period, 20 three-day-old female flies were collected for RNA extraction. The experiments were performed in triplicate.

2.4. Toxic effects of CPA

D. melanogaster were fed CPA for the entire life cycle as described in Section 2.2. Toxicity of CPA was measured as the number of larvae reaching the adult stage ($n = 100$ flies). Treated and untreated groups were compared by non-paired *t*-test. Differences were considered significant if $p < 0.05$ (two-tailed test).

2.5. RNA isolation and transcriptome sequencing

Total RNA was extracted from 20 three-day-old female flies using TRIzol® (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA integrity was analysed using Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA) and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and QuBit 3.0 Fluorometer (Thermo Fisher Scientific).

Dynabeads® mRNA Purification Kit (Invitrogen) was used to isolate mRNA from the RNA samples. Libraries were prepared with the Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific) according to manufacturer's instructions. Resulting library pools were quantified with Qubit™ dsDNA HS Assay Kit (Invitrogen) and quality checked with Ion Sphere™ Quality Control Kit (Life Technologies). Templating of an Ion 540 chip was performed using Ion 540™ Kit - OT2 (Thermo Fisher Scientific) and then sequenced on the Ion S5 sequencer according to manufacturer's instructions. Data were collected using Torrent Suite v4.0 software and resulting reads were exported as raw reads in fastq format.

2.6. Read processing, transcript quantification and differential expression analysis

Galaxy platform (Afgan et al., 2018; https://usegalaxy.org/) was used for bioinformatic analyses. Read quality was checked using FastQC (Galaxy Version 0.72; Andrews, 2010), and low-quality reads and adapters were trimmed using Trim Galore v 0.4.3.1. Reads with a quality threshold of < 25 on the Phred scale, empty reads, and short sequences with a length of < 20 bp were removed.

For read alignments, we used TopHat 2 software (Galaxy Version 2.1.1; Kim et al., 2013). *D. melanogaster* was used as a reference genome (Source: Ensembl Project website, available at https://support.illumina.com/sequencing/sequencing_software/igenome.html). HTSeq-count software (Galaxy Version 0.9.1; Anders et al., 2015) was used to count aligned reads.

For differential gene expression analysis, three different programs were used: (1) DESeq2 (Galaxy Version 2.11.40.2; Love et al., 2014), where genes with a p value ≤ 0.05 were considered differentially expressed; (2) edgeR (Galaxy Version 3.20.7.2; Robinson et al., 2010), where genes that presented an FDR value ≤ 0.1 were considered differentially expressed; and (3) Cuffdiff (Galaxy Version 2.2.1.2; Trapnell et al., 2010) to assemble transcripts and estimate their abundance using Cufflinks software. Genes with a p value ≤ 0.05 were considered differentially expressed.

Differential expression analysis of TEs was performed using TE_{TOOLS} (Lerat et al., 2016). TEs with a p value ≤ 0.05 were considered differentially expressed.

2.7. Functional annotation of transcriptomes

Analyses of the relationships between DEGs and gene orthology (GO) annotation were performed using the Cytoscape 3.6.1 (Shannon et al., 2003). Annotated information in Flybase regarding protein features, molecular function, and biological processes was retrieved and Cytoscape annotation was manually edited using the Flybase information. Manual editing allowed us to improve the classification produced by Cytoscape.

2.8. High-resolution respirometry

The protocol described by Pesta and Gnaiger (2011) was used with an O2k-system high-resolution oxygraph (Oroboros Instruments, Innsbruck, T, Austria). Four flies were homogenised and placed in MIR05 breath buffer (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2 PO_4, 20 mM HEPES, 110 mM sucrose, 0.1 mg/mL of fatty acid-free BSA) and charged in Oroboros chambers. First, basal oxygen flux rate was determined by addition of physiological substrates: 5 mM proline, 5 mM pyruvate, and 2 mM malate (complex I substrates); 4 mM ADP (substrate for ATP synthesis) and 10 mM succinate (complex II substrate). Oxygen flux uncoupled to ATP synthesis was then measured by inhibiting ATP synthase with oligomycin (0.2 µg/mL). 500 nM FCCP, an uncoupler of oxidative phosphorylation (OXPHOS), was further added to measure the electron transfer system (ETS). ETSs related to complex I and II were determined by addition of 0.5 µM rotenone and 5 mM malonate, respectively. Respiration was then inhibited by addition of 2.5 µM antimycin A to determine residual oxygen consumption (ROX). DatLab software (Oroboros Instruments) was used for data acquisition and analysis.

3. Results

3.1. Toxic effect of cyclophosphamide

In the acute treatment, adult flies were fed with CPA for 24 h had normal survival and showed no overt signs of toxicity. In the chronic treatment, flies fed culture medium containing CPA for their entire life-cycle. This led to a significant reduction in the emergence of adult flies, with 83% emergence in the control group vs 28% in the CPA treated group (Fig. 1).

3.2. Differential expression analysis

Transcriptomes of female flies treated with CPA were generated by NGS sequencing in the Ion S5™ System. After the trimming process, 4,926,514 reads were generated for the chronic treatment, 3,612,220 for the acute treatment, and 6,282,918 for the control samples. Reads were analysed using three different methodologies (Cufflinks-Cuffdiff, DESeq2 and edgeR) to identify DEGs. The three approaches identified DEGs for both the acute and chronic treatments when compared to untreated flies. Total number of DEGs generated by each software is described in Fig. 2. For following analyses, data from Cufflinks-Cuffdiff were used because of the highest number of DEGs. Besides, most genes identified by DESeq2 and edgeR were also present in Cufflinks-Cuffdiff analysis. From the 409 DEGs found in the acute treatment, 322 were downregulated and 87 were upregulated (Fig. 2C). For the chronic treatment, Cufflinks-Cuffdiff identified 106 DEGs, 88 genes were downregulated and 18 were upregulated (Fig. 2F).

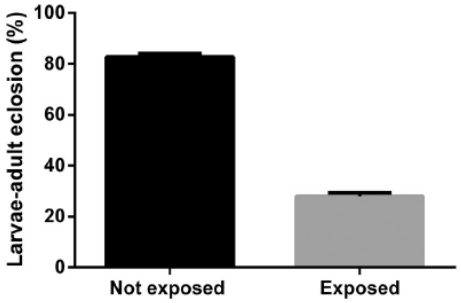

Fig. 1. Larvae to adult eclosion rate of *Drosophila melanogaster* fed with culture medium containing CPA (0.05 mg/g of medium) for the entire life cycle ($p < 0,0001$, two-tailed unpaired *t*-test).

3.3. Functional annotation of transcriptomes

3.3.1. Acute treatment

DEGs from the acute treatment were clustered in 16 classes (Fig. 3 and Suppl. Table 1). Genes involved in the production of mitochondrial components were significantly affected (53 downregulated genes). In the sensory perception of chemical stimuli class five downregulated genes were found, four belonging to the Obp family (Obp56e, Obp99a, Obp99b, and Obp99c). Other classes of downregulated genes found were structural constituent of cuticle (45 genes), oxidation-reduction process (29 genes), carbohydrate metabolism (19 genes), cytoskeleton protein composition (12 genes), calcium-mediated signalling (10 genes), transmembrane transport (9 genes), lipid metabolism (9 genes), response to stress and toxic substances (8 genes), amino acid metabolic process (4 genes), and vitelline membrane proteins (4 genes). Other 34 downregulated genes did not cluster into any functional classes and were called "other" (Fig. 3A and Suppl. Table1).

Upregulated genes were clustered into five functional classes (Fig. 3B and Suppl. Table 1). The proteolysis class has the largest number of DEGs (15 genes), most of them code for serine proteases and belong to the *Jonah* multi-gene family. Ten genes were involved in responses to oxidative stress and to pathogens, two simultaneously involved in both: *TotM* and *PPO2*. Other classes found were regulation of gene expression (8 genes), apoptotic process and autophagy (6 genes), and transmembrane transport (6 genes).

3.3.2. Chronic treatment

Downregulated genes identified in the chronic treatment were clustered into seven main functional classes (Fig. 4A and Suppl. Table 2). The most representative class was vitelline membrane proteins (9 genes). Of the seven genes found in the response to stress class, two belonged to the family of *Turandot-related* genes, TotA and TotC, which were upregulated in the acute treatment. Other classes found were metabolic process (7 genes), ubiquitin-dependent proteolysis (7 genes), ribosome constituent (6 genes), transmembrane transport (4 genes), and mitochondrial components (3 genes). Seventeen genes could not be clustered in any class.

Two functional classes of upregulated genes were observed in the chronic treatment (Fig. 4B and Suppl. Table 2). The oxidation-reduction process is represented by two genes of the cytochrome *p*450 family (Cyp4e3 and Cyp6a17). Two genes involved in pathogen and drug responses were also upregulated. Another seven genes were upregulated but did not cluster in a particular class.

3.4. Differential expression analysis of transposable elements

To test the hypothesis that CPA treatment can lead to the activation of TEs by promoting cellular stress, TEs expression analysis was performed using TE_{TOOLS} after acute and chronic treatment of the flies with CPA. In the acute treatment, four LTR retrotransposons were differentially expressed. Three belonged to the *gypsy* superfamily (*Accord_1*and *Invader2_1*, upregulated; *gypsy_1*, downregulated) and *copia_DM_1* (*copia* superfamily, upregulated). The element *FW*, a non-LTR retrotransposon (*jockey* family), and the DNA transposon *ProtoP_B* were downregulated (Table 1). In the chronic treatment, the LTR retrotransposon elements *Blood_15*, *DM297_15*, *tirant_15* (*gypsy* superfamily) were downregulated, and the non-LTR retrotransposon *copia_DM_15* were upregulated.

3.5. High-resolution respirometry

Gene expression analysis after acute treatment with CPA showed that most DEGs were related to mitochondria, indicating that its function may be disturbed after short-term exposure to CPA. To further investigate this hypothesis, the functionality of fly mitochondria was

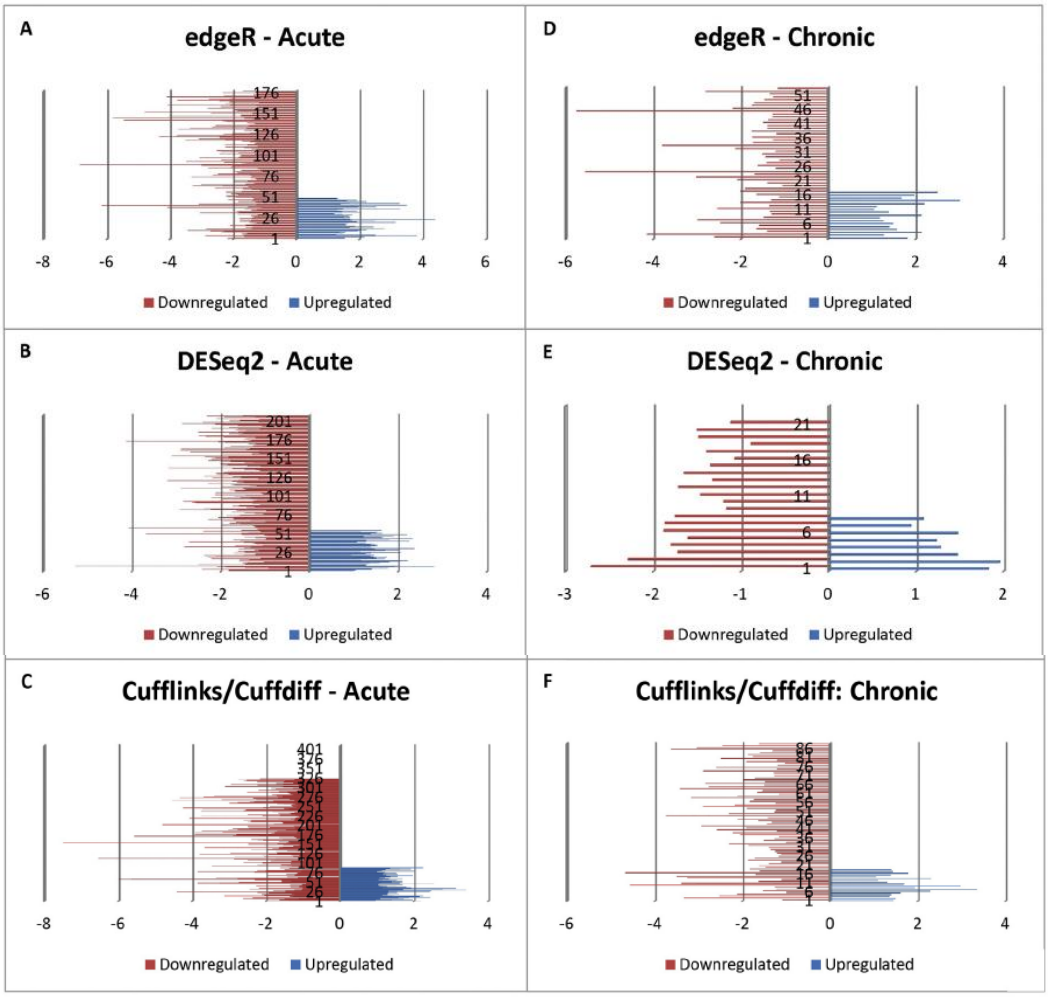

Fig. 2. Number of differentially expressed genes (DEGs) identified by edgeR, DESeq2, and Cufflinks/Cuffdiff. (A) edgeR analysis of the acute treatment resulted in 226 DEGs. (B) DESeq2 analysis of the acute treatment resulted in 263 DEGs. (C) Cufflinks/Cuffdiff analysis of the acute treatment resulted in 409 DEGs. (D) edgeR analysis of the chronic treatment resulted in 70 DEGs. (E) DESeq2 analysis of the chronic treatment resulted in 29 DEGs. (F) Cufflinks/Cuffdiff analysis of the chronic treatment resulted in 106 DEGs.

tested using high-resolution respirometry. Acute exposure to CPA caused significant mitochondrial dysfunction in flies (Fig. 5A and B). Decreased levels of oxygen flux in the PPM stage indicated a decrease in the basal respiratory capacity of mitochondria. CPA treatment also induced a marked reduction in the electron transfer flux dependent on complex II, represented by the ETS CII-Linked (Fig. 5B). Mitochondrial functionality of flies treated chronically with CPA was also tested, however, there was no significant difference when compared to untreated samples (data not shown).

4. Discussion

Studies on human and mouse models found that CPA promotes alterations of gene expression (El-Serafi et al., 2014; Guo et al., 2014; Kubisch et al., 2013; Van Dycke et al., 2015; Wu et al., 2016). Our results show that CPA also promotes DEGs in *Drosophila*. Some biological functions affected in humans and mice, for example, oxidative stress, immune-related pathways, transcription and spliceosomes, nucleotides and amino acid metabolism, were also altered in *Drosophila*. However, DEGs in several functional processes were reported for the first time in this study.

A remarkable aspect of our results is that several genes involved in mitochondria formation were downregulated in flies under acute treatment with CPA, suggesting that CPA could promote mitochondrial dysfunction. High-resolution respirometry revealed mitochondrial impairments and, in particular, a reduction in the basal respiratory capacity and electron transfer flux dependent on complex II. Crouch et al. (2017) showed that rats treated with CPA had a decreased ability to perform exercises. Maximum mitochondrial ATP production (ATPmax) decreased one day post-treatment and remained below baseline for six weeks, suggesting that CPA induces persistent metabolic dysfunction (Crouch et al., 2017). They proposed that the rapid decline in ATPmax may be due to direct inhibition of the mitochondrial electron transport system by the CPA metabolite, acrolein (Luo and Shi, 2005; Luo et al., 2005). However, sustained inhibition of ATPmax is probably caused by persistent mechanisms such as rupture of the mitochondrial structure because of CPA toxicity. Our data suggest that mitochondrial dysfunction associated with CPA treatment could be a consequence of the

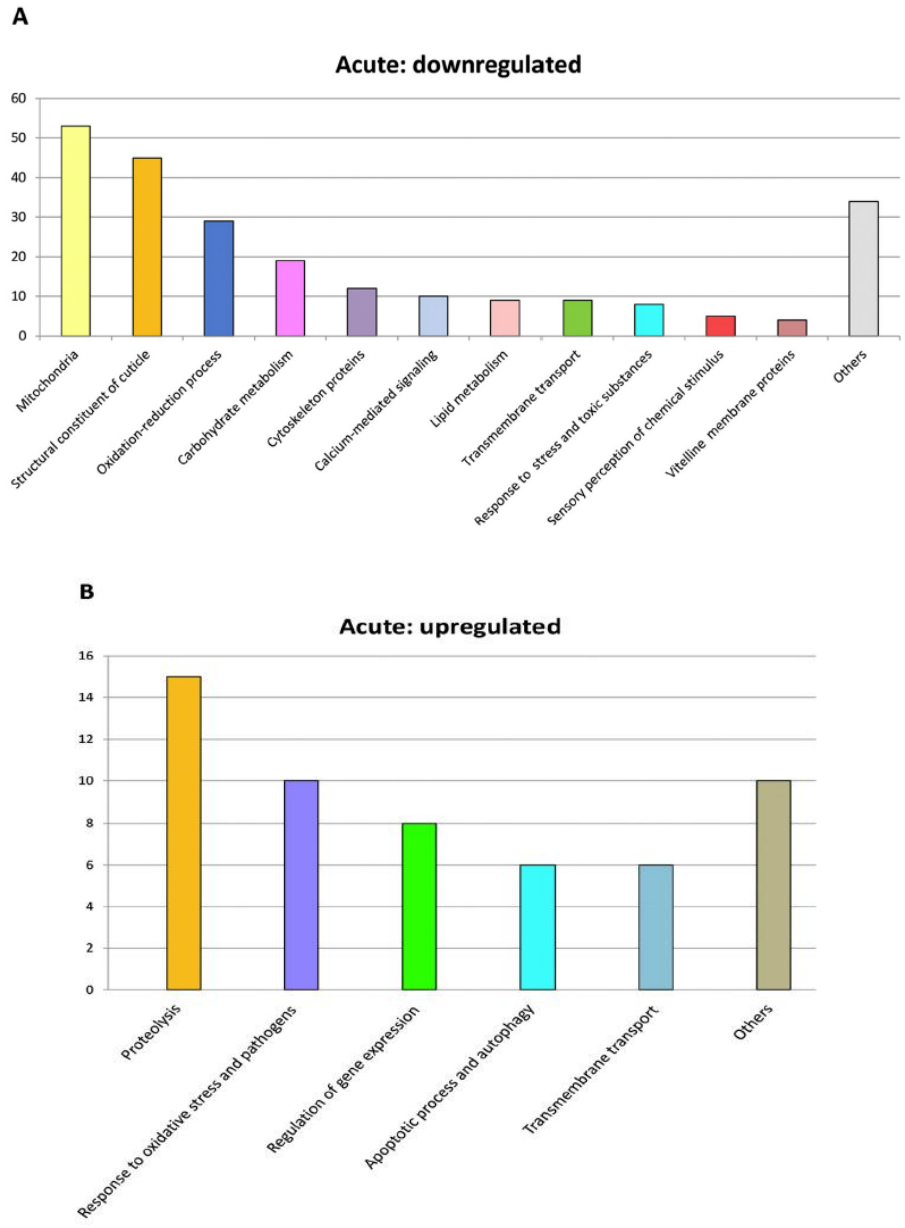

Fig. 3. Functional classes of DEGs identified in the acute treatment with CPA (A) Downregulated genes were clustered into 11 functional classes. Genes that did not clustered are called "others." (B) Upregulated genes were clustered into five functional classes. Genes that did not clustered are called "others" (See Supplementary Table 1 for details).

altered expression of genes related to mitochondrial components.

Only three genes involved in mitochondria formation/metabolism remained downregulated in the chronic treatment. Mitochondrial activity returned to normal, as suggested by high-resolution respirometry assay results. Two theories may explain this: (1) mitochondria were less affected in the chronic treatment because of the lower concentration of CPA used, or (2) after an initial gene expression alteration, continued exposure to CPA resulted in a return to a default pattern of mitochondrial function.

We proposed a model of the major biological changes that may be induced by CPA (Fig. 6). At the organism level, there are alterations in the expression of genes involved in cuticle and vitelline membrane formation, which are specific to *Drosophila*. This has also been observed in formaldehyde-induced stress (Moskalev et al., 2014). In the acute treatment, genes related to cuticle formation were differentially expressed, resulting in changes in the cuticle structure. The cuticle is present on the body surface and in the internal lining of the tracheae, salivary ducts and gut, and forms most of the insect exoskeleton – the first barrier to environmental hazards. Changes in cuticle structure can be an adaptive response against stressor agents (Cinege et al., 2017). Cuticle metabolism is controlled by moulting hormones, the ecdysteroids (Spindler et al., 1990), and we found two genes involved in

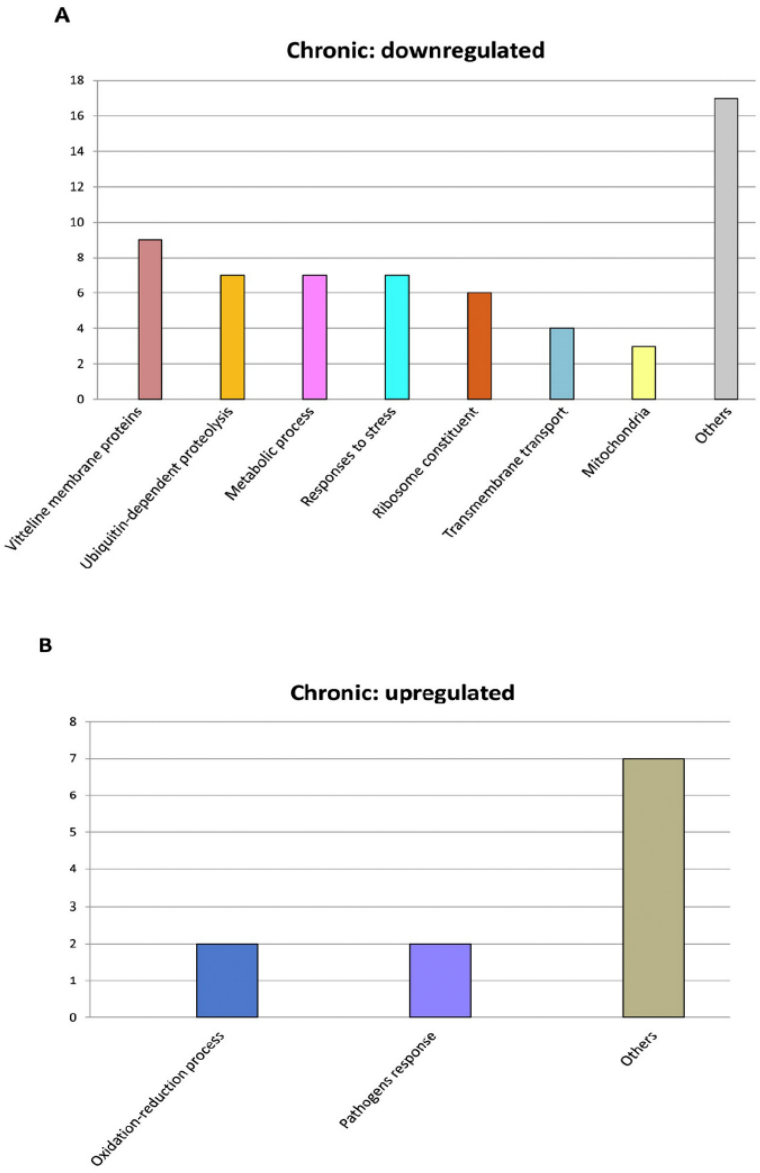

Fig. 4. Functional classes of DEGs identified in the chronic treatment with CPA. (A) Downregulated genes were clustered into seven functional classes Genes that did not clustered are called "others." (B) Upregulated genes were clustered into two functional classes. Genes that did not clustered are called "others" (See Supplementary Table 2 for details).

ecdysteroid metabolism (*CG13360*, *CG11878*) in the acute treatment. As an alternative theory, differential expression of cuticle genes may be a non-adaptive side effect of ecdysteroid genes' mis-regulation promoted by CPA. In the chronic treatment, cuticle genes were not affected, while vitelline membrane genes remained downregulated.

Genes related to chemosensory functions and affected by CPA belong to the *Odorant Binding Proteins* (*Obp*) family. In *D. melanogaster*, this family is composed of 52 genes that encode proteins that bind, solubilise, and transport hydrophobic odorants across the aqueous sensillum lymph to receptors. (Larter et al., 2016). *Obp* genes are abundantly expressed in antenna, including four of the *Obp* genes that we found downregulated in the acute treatment (Larter et al., 2016). These results suggest that downregulation of *Obp* genes could be an adaptive response, reducing the physiological response to CPA as an odorant.

At the cellular level, five main biological functions were affected in the acute treatment (Fig. 6). Metabolic processes involving mainly carbohydrate, but also lipid and amino acid metabolisms, were downregulated. Guo et al. (2014) also observed DEGs for metabolic processes in the spleen of mice exposed to CPA, however, the main genes affected

Table 1
Differentially expressed transposons in adult *Drosophila melanogaster* after acute or chronic treatment with CPA.

Treatment	Transposable element	Superfamily	Class	Expression
Acute	ACCORD_I	*Gypsy*	LTR retrotransposon	Upregulated
Acute	INVADER2_I	*Gypsy*	LTR retrotransposon	Upregulated
Acute	COPIA_DM_I	*Copia*	LTR retrotransposon	Upregulated
Acute	FW	*Jockey*	non-LTR retrotransposon	Downregulated
Acute	GYPSY_I	*Gypsy*	LTR retrotransposon	Downregulated
Acute	PROTOP_B		DNA transposon	Downregulated
Chronic	COPIA_DM_I5	*Copia*	non-LTR retrotransposon	Upregulated
Chronic	BLOOD_I5	*Gypsy*	LTR retrotransposon	Downregulated
Chronic	DM297_I5	*Gypsy*	LTR retrotransposon	Downregulated
Chronic	TIRANT_I5	*Gypsy*	LTR retrotransposon	Downregulated

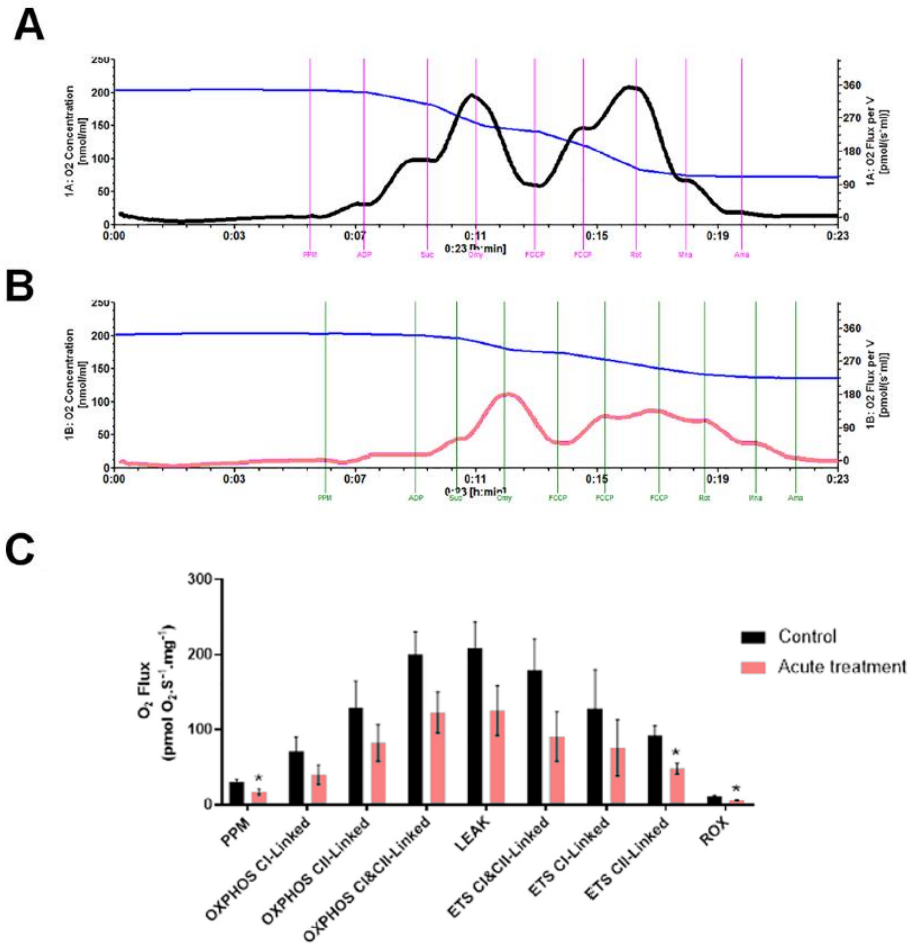

Fig. 5. Effects of acute CPA treatment on mitochondrial functionality of flies. Representative graph of oxygen flux/concentration by high-resolution respirometry in mitochondria from control flies (A) and CPA treated flies (B). Measurements were made in the presence of PPM (pyruvate, proline and malate), ADP (adenosine diphosphate), Suc (succinate), Omy (oligomycin), FCCP (Carbonyl cyanide-4-trifluoromethoxy phenylhydrazone uncoupler), Rot (rotenone), Mna (malonate) and Ama (antimycin). (C) Oxygen flux measured by SUIT protocol from high-resolution respirometry indicating the following mitochondrial events: PPM (oxygen flux rate associated with the substrates pyruvate, proline and malate without phosphorylation); OXPHOS representing the oxygen flux coupled to the oxidative phosphorylation by ADP addition: OXPHOS CI-Linked (oxygen flux dependent on the complex I), OXPHOS CII-Linked (oxidative phosphorylation associated to complex II after succinate addition), OXPHOS CI&CII-Linked (oxidative phosphorylation associated to complexes I and II working together); LEAK (respiration after inhibition of phosphorylation with oligomycin); ETS representing maximum oxygen consumption reached by addition of the uncoupler FCCP: ETS CI-Linked (electron transfer system associated to complex I after inhibition with rotenone) ETS CII-Linked (electron transfer system associated to complex II after inhibition with malonate); ROX (oxygen residual flux). Data are expressed as mean ± S.E.M by *t*-test comparison (n = 5). *Indicates $p < 0.05$ as compared to the control group.

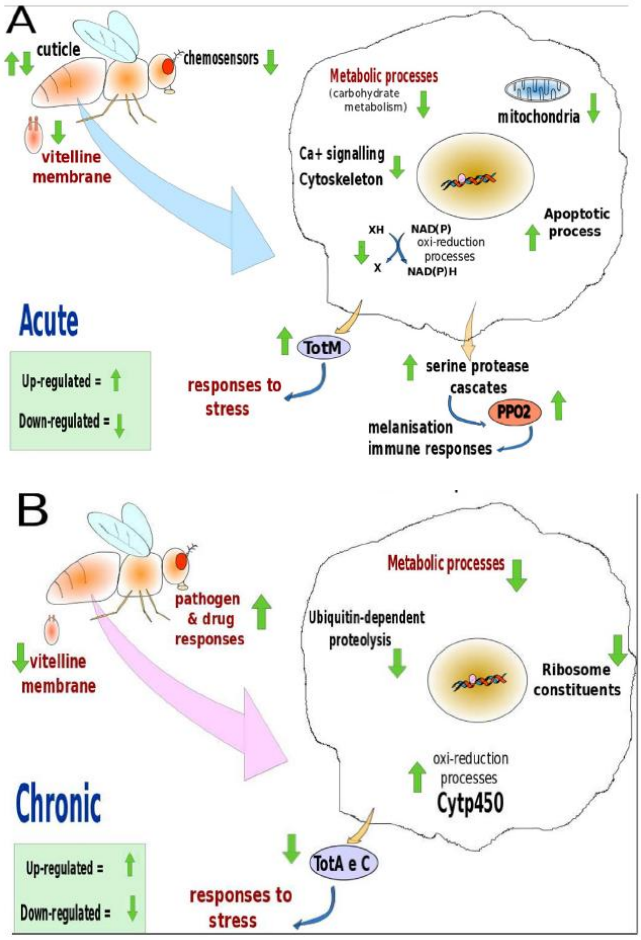

Fig. 6. Main gene expression alterations in *Drosophila* under CPA treatment. A) Acute treatment. At organism level, cuticle and vitelline membrane formation and chemosensory functions are affected. At the cellular level, metabolic processes, mitochondrial function, apoptosis and other processes are affected. B) Chronic treatment. At organism level, vitelline membrane formation and pathogen and drug responses are affected. At the cellular level, the processes affected include oxi-reduction, stress responses and ubiquitin-dependent proteolysis.

were associated with nucleotide and amino acid metabolism. We found that cellular oxidation-reduction processes were also affected, especially the processes involving dehydrogenase enzymes that use NAD(P) as a cofactor. Metabolic and oxi-reduction processes could be suppressed as a consequence of mitochondrial dysfunction, which disrupts cell metabolism in general. Furthermore, the over-expression of genes involved in apoptosis may also be coupled with mitochondrial dysfunction observed in the acute treatment experiment. Mitochondria plays a central role in apoptosis, and mitochondrial dysfunction induces the apoptotic process (Desagher and Martinou, 2000). The reduction of metabolism may also be associated with the downregulation of genes for ribosomal constituents, as observed in chronically treated flies.

In the chronic treatment, metabolic processes were also downregulated, although fewer genes were affected when compared to the acute treatment. Oxi-reduction process in the chronic treatment was marked by an over-expression of genes from the cytochrome p450 family, which are involved in CPA degradation (Chang et al., 1993; Selvakumar et al., 2005). Cytochrome p450 forms a large and diverse family represented by 86 genes in *D. melanogaster*, carrying out many enzymatic reactions, like the synthesis of numerous compounds and the detoxification of xenobiotics (Chung et al., 2009). Flies under CPA chronic treatment presented two cytochrome p450 genes upregulated and one of these genes, *Cyp4e3*, is involved in insecticide resistance (Harrop et al., 2014; Terhzaz et al., 2015). Thus, over-expression of these genes must be associated with CPA detoxification.

DEGs involved in proteolysis were present in both the acute and chronic treatments, although in different contexts. In the acute treatment, upregulated genes were mainly those encoding serine proteases (SP). SP carry out several important roles: dietary protein digestion, blood coagulation, complement activation and, in arthropods, formation of pathways for immune response mediation, haemolymph coagulation, and protoxin processing in insect midguts (Cao and Jiang, 2018). Among the roles played by SP in insects is prophenoloxidase (PPO) activation leading to the melanisation reaction, a major immune

response in arthropods (Binggeli et al., 2014). Most of the SPs upregulated by the acute CPA treatment are part of *Drosophila* immune response to bacteria, fungi, viruses (Carpenter et al., 2009; De Gregorio et al., 2001), and other stress factors like ionising radiation, formaldehyde, toluene, and dioxin (Moskalev et al., 2015). Acute treatment also upregulated *PPO2*, which is involved in events such as cuticle and eggshell hardening, wound healing, and melanotic encapsulation of invading parasites (Liu et al., 2012). SPs and *PPO2* genes involved in pathogen response were also upregulated. Lu et al. (2014) suggested that insect PPO has other functions, not just cellular and humoral defence. The coordinated activation of SP and *PPO2* under CPA exposure suggests that PPO may be involved in xenobiotic detoxification.

In the chronic treatment, DEGs involved in proteolysis were downregulated and related to ubiquitin-dependent proteolysis. The ubiquitin system promotes the turnover of intracellular proteins under basal metabolic conditions; however, it is also related to intracellular proteins' degradation under stress (Ciechanover, 1994).

The family of *Turandot-related* genes (*Tot*) are induced under stress conditions, like heat shock, paraquat feeding, UV and pathogens exposure, ionising radiation, formaldehyde, toluene, and dioxin (Ekengren and Hultmark, 2001; Moskalev et al., 2015). Tot proteins are humoral factors secreted from body fat, accumulate in body fluids, and are important agents of *Drosophila* stress tolerance (Ekengren et al., 2001). In contrast to flies under acute CPA treatment, flies under chronic treatment showed a downregulation of *Tot* genes, suggesting that Tot proteins could be involved in an initial response to stress factors.

Several studies suggest that stressors can activate the mobilisation of TEs (Capy et al., 2000; Guio et al., 2018; Horvath et al., 2017). Although transcription is merely the initial step for mobilisation, upregulation of transcription is associated with increased mobilisation (Pereira et al., 2018). In this study, we found that CPA could promote the over-expression of TEs belonging to LTR and non-LTR retrotransposon groups. These results suggest that L1, the only active TE in humans, can be activated during chemotherapy.

5. Conclusions

Some parallels can be made between mammalian and *Drosophila* responses to CPA, such as downregulation of genes for essential survival functions and immune signalling (Wu et al., 2016), expression of coagulation pathway–associated genes, steroid biosynthesis (El-Serafi et al., 2014), and oxidative stress (Van Dycke et al., 2015). These similarities between organisms so phylogenetically distant points out to a possible conservation in the regulatory mechanisms of response to CPA toxicity. However, further studies are necessary to confirm such findings. Several responses to CPA treatments are exclusive to *Drosophila*, such as changes in cuticle and vitelline membrane formation and perception of chemical stimulus. This is the first report of mitochondrial dysfunction following CPA treatment, which is associated with the downregulation of genes involved with mitochondrial constituents, and that CPA can change several TEs transcription patterns.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2020.108718.

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CAPÍTULO 3

Mobilização somática do elemento transponível *mariner-Mos1* em *Drosophila simulans* após tratamentos com quimioterápicos.

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Em andamento.

INTRODUÇÃO

A cisplatina é uma droga amplamente utilizada para o tratamento de cânceres de ovário, testículo e coloretal (Garcia et al., 2008). Sua atividade antitumoral é atribuída à ligação ao DNA formando adutos, que causam danos a essas moléculas. Se a maquinaria celular falhar no reparo desses danos, pode ocorrer a apoptose.

Da mesma forma, a dacarbazina também é utilizada como um agente antineoplásico (Pourahmad et al., 2009). Nos mamíferos, a dacarbazina é metabolizada em metildiazônio, que medeia a alquilação do DNA, formando adutos que causam danos às moléculas de DNA, podendo ocorrer a apoptose.

Já a daunorubicina se intercala e se liga ao DNA bloqueando a síntese de DNA, RNA e proteínas (Goebel, 1993). Seu mecanismo de ação consiste em inibir a topoisomerase II, formando um complexo DNA-topoisomerase II, levando a uma eventual quebra do DNA.

Podratz et al. (2011) verificaram que as alterações apoptóticas e comportamentais induzidas pela cisplatina em *Drosophila* se assemelham às observadas em mamíferos, o que faz de *Drosophila* um modelo adequado para estudar os mecanismos da toxicidade da cisplatina. Além disso, alguns estudos destacaram a forte conservação nas vias de sinalização entre *Drosophila* e seres humanos, o que torna essas moscas excelentes modelos para se estudar a ação de diferentes drogas de "humanos" em moscas (Aritakula e Ramasamy, 2008; Edwards et al., 2011; Vidal et al., 2005).

Uma fração significativa de quase todos os genomas eucarióticos consiste em DNA móvel, conhecidos como elementos transponíveis (TEs) (Hua-Van et al. 2011). Sua capacidade de se mover e sua natureza repetitiva fazem dos TEs uma importante fonte de mutações nos genomas. Eles podem gerar mutações quando se deslocam de um local para outro do genoma, pois podem produzir quebras no DNA (Izsvak et al., 2009). Além disso, quando se inserem em um novo local, podem interromper uma sequência funcional existente ou adicionar sequências reguladoras que podem afetar genes próximos (Chuong et al., 2016; Elbarbary et al., 2016; Rebollo et al., 2012).

O transposon *mariner* é um TE que pode se mobilizar continuamente, tanto em células germinativas quanto em células somáticas (Hartl, 2001). A inserção desse TE na região promotora do gene *white* em *Drosophila* produz moscas com olhos cor de pêssego, denominadas *white-peach* (Jacobson et al., 1986).

A cópia de *mariner* causadora dessa mutação é não autônoma, ou seja, permanece estável no genoma. Na presença de uma cópia autônoma, ele pode se mobilizar, levando a reversão da mutação. Quando ocorre essa reversão da mutação nos olhos das moscas, as mesmas exibem um fenótipo em mosaico, ou seja, olhos cor de pêssego com manchas vermelhas (Jacobson et al., 1986; Bryan et al., 1987; Medhora et al., 1988; Medhora et al., 1991).

O objetivo desse trabalho foi verificar, através de qPCR e da contagem do número de manchas nos olhos, se ocorre uma diferença nas taxas de transposição de *mariner*, após a exposição de moscas da linhagem *D. simulans white-peach* a três quimioterápicos: cisplatina, dacarbazina e daunorubicina.

MATERIAIS E MÉTODOS

Linhagens utilizadas e tratamentos com os quimioterápicos

Moscas da linhagem *D. simulans* Dswp test, que foi estabelecida em nosso laboratório as quais possuem uma baixa atividade de *mariner* (conforme descrito no primeiro artigo – capítulo um) foram tratadas com dois quimioterápicos: cisplatina e dacarbazina. Já os tratamentos com a daunorubicina ainda estão em andamento.

Os tratamentos ocorreram por um período de 24 horas (tratamento agudo) ou durante toda a fase de desenvolvimento das moscas, desde ovos até adultos (tratamento crônico). No tratamento agudo, moscas adultas com três dias de vida foram mantidas por um período de 24 h em meio de cultura para *Drosophila* (com farinha de milho, sacarose e levedura) contendo duas concentrações de cisplatina, 50 µg/mL e 100 µg/mL. Os tratamentos com a dacarbazina também foram realizados nas mesmas condições e nestas mesmas concentrações. Além disso, foram adicionados ao meio de cultura corante alimentar vermelho (E163), para ter certeza que as moscas haviam comido o meio com o quimioterápico. As moscas fêmeas com o abdômen corado foram usadas para posterior extração de RNA.

Para os tratamentos crônicos, as moscas foram mantidas, desde ovos até adultas em meio contendo 50 µg/mL dos mesmos fármacos, cisplatina e dacarbazina. Moscas adultas, com três dias de vida pós eclosão foram coletadas e analisadas em um estereomicroscópio para a análise fenotípica.

Quantificação relativa das taxas de transposição de *mariner*

O RNA total de 20 moscas fêmeas expostas ao tratamento agudo com a cisplatina e com a dacarbazina foi extraído com o reagente TRIzol® (Invitrogen, USA). As amostras foram quantificadas usando um espectofotômetro NanoDrop 2000 (Thermo Scientific). Em seguida, as amostras foram tratadas com DNaseI (Promega, USA) e foi realizada a síntese de cDNA com a enzima transcriptase reversa M-MLV (Invitrogen, CA, USA) e primers oligo-dT.

A quantificação da expressão relativa de *mariner* foi realizada por qPCR em um StepOnePlus Real-Time PCR (Applied Biosystems, USA). Para estimar a expressão relativa foram usados os *primers Mos*, que amplificam *mariner* e, como referência, o *primer L17–RPL17q2*, que codifica uma proteína ribossômica. Os cálculos foram feitos baseados no método $2^{-\Delta\Delta cq}$ (Livak and Schmittgen, 2001) usando os valores de Ct. Os *primers* utilizados estão descritos no material suplementar do primeiro artigo apresentado (capítulo 1).

Após, foi realizado um teste t comparando o grupo controle (moscas que não foram expostas aos medicamentos) com o grupo das moscas tratadas com a cisplatina e com a dacarbazina.

Análise fenotípica do número de spots nos olhos

As moscas expostas ao tratamento crônico da cisplatina e da dacarbazina foram coletadas e analisadas em um estereomicroscópio onde o número de spots vermelhos em ambos os olhos foi contado e assumiu-se que cada spot corresponde a um único evento de transposição. Todos os experimentos foram realizados em triplicata.

Após a contagem, foi realizado um teste t comparando o grupo controle (moscas que não foram expostas aos medicamentos) com o grupo das moscas tratadas.

RESULTADOS PARCIAIS

Estimativa da expressão relativa de *mariner* e análise fenotípica do número de spots nos olhos

Houve uma diferença significativa na expressão relativa de *mariner,* avaliado por qPCR, nas moscas tratadas com a cisplatina em ambas as concentrações de 50 µg/mL ($p = 0,0075$) e de 100 µg/mL ($p = 0,0002$) (Figura 1). Além disso, parece haver uma dose-dependência, uma vez que houve um aumento da expressão relativa desse TE quando comparados o controle (média = 1); e as concentrações, 50 µg/mL (média = 3,91) e 100 µg/mL (média = 4,35).

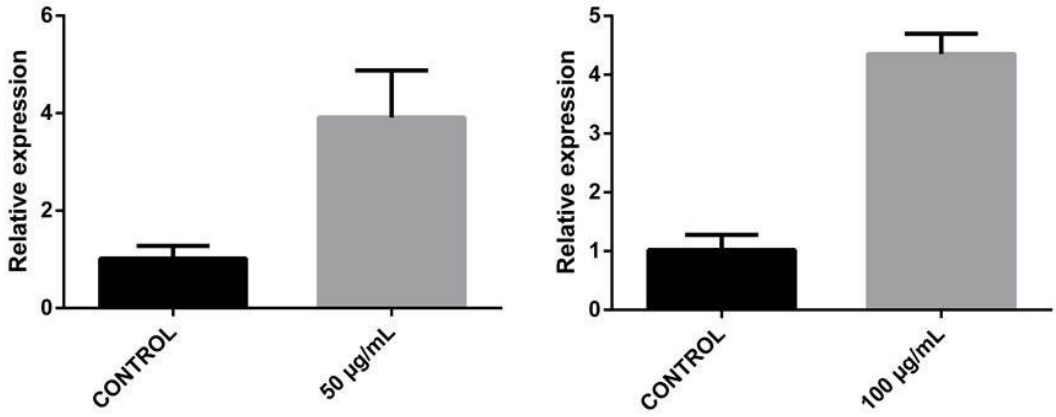

Figura 1. Expressão relativa de *mariner* em moscas expostas ao tratamento agudo da cisplatina. A. Na concentração de 50 µg/mL (p = 0,0075). B. Na concentração de 100 µg/mL (p = 0,0002). Teste t.

Não foi possível realizar a análise fenotípica do número de spots nos olhos das moscas expostas ao tratamento crônico da cisplatina até o momento, pois assim como ocorreu com as moscas da linhagem de *D. melanogaster* wpchM- expostas a ciclofosfamida (como mostrado no segundo artigo desta tese – capítulo dois), também houve uma redução no número de moscas que eclodiram de pupa para adulto nesse tratamento com a cisplatina.

Já nas moscas expostas ao tratamento agudo com a dacarbazina não foi observado uma diferença significativa na expressão relativa de *mariner* em nenhuma das concentrações analisadas (50 µg/mL p = 0,6446 e 100 µg/mL p = 0,2646). Além disso, também não foi observada uma diferença significativa no número de spots dos olhos das moscas expostas ao tratamento crônico. Ainda, parece que esse medicamento não afetou o desenvolvimento das moscas, uma vez que o número de moscas que eclodiram de pupa para adultos parece não ter sido afetado.

4. DISCUSSÃO GERAL

Muitas questões ainda permanecem em aberto sobre a TS dos TEs, uma delas é se ela é constante ou variável ao longo da vida dos organismos, e se diferentes agentes estressores podem aumentar as taxas de TS ou não.

Nossos resultados mostraram pela primeira vez, que a excisão somática (ES) do elemento *mariner* pode ocorrer durante todos os estágios do ciclo de vida de *Drosophila*, e é cumulativo. Entretanto, não é constante, exibindo variações ao longo do desenvolvimento. Além disso, as larvas se mostraram mais sensíveis ao estresse térmico do que pupas e os embriões parecem não ser afetados. Isso pode estar relacionado à atividade transcricional da argonauta nas gônadas, que é maior em ovários do que em testículos (Saint-Leandre et al., 2017). Essa alta taxa de transcrição desse gene nos ovários pode produzir ovos com um alto potencial de inibir a atividade de *mariner-Mos1*.

Ainda, a abordagem fenotípica para quantificar a TS com base nos spots dos olhos pode estar associada com o número de células precursoras dos omatídeos durante o desenvolvimento, uma vez que no início da embriogênese, aproximadamente 20 células são destinadas a formação do olho adulto, já no final do terceiro instar larval esse número cresce para aproximadamente 10.000 células no disco imaginal (Hales et al., 2015). O ciclo celular também pode influenciar, uma vez que o estágio larval apresentou as maiores taxas de TS e é um período com intensa divisão celular, já a fase de pupa é marcada pela diferenciação celular para a formação dos tecidos. Intensos processos mitóticos podem estar envolvidos com um aumento da TS, enquanto estágios com maior diferenciação celular podem ter taxas mais baixas.

Através de qPCR foi possível observar que a ES de *mariner-wpch* é cumulativa durante todo ciclo de vida das moscas e ocorre tanto em 20°C quanto em 28°C, sendo maior em temperaturas elevadas. Além disso, foi observado um aumento nas taxas de ES de *mariner* em moscas adultas mantidas a 28°C durante 10 dias. Isso indica que esse elemento pode ser ativo em organismos adultos, mesmo considerando que a mosca da fruta é composta principalmente de células pós-mitóticas. Mesmo considerando que apenas parte dessas excisões pode resultar em novas inserções e mutações, as consequências biológicas podem ser significativas. Os experimentos de comportamento e de expectativa de vida

estimadas para as linhagens com diferentes taxas de ES sugerem uma consequência prejudicial devido à mobilização de *mariner*.

Diferentemente do que acontece em temperaturas elevadas, parece que os tratamentos com a ciclofosfamida (CPA) não alteram a expressão de *mariner*, uma vez que não encontramos nem uma sobre-expressão e nem uma diminuição da expressão deste transposon. Entretanto, observamos que a CPA promoveu a sobre-expressão de alguns TEs pertencentes aos grupos de retrotransposons LTR e não-LTR, sugerindo que o LINE-1, único TE ativo em seres humanos, pode ser ativado durante a quimioterapia. Estudos mostram que em algumas neoplasias, como no adenocarcinoma ductal pancreático, no início da doença apenas 11% das lesões histológicas expressam a ORF1 do LINE-1, já neoplasias mais desenvolvidas, 90% tem uma super-expressão da ORF1(Rodic et al., 2014). Ou seja, com o avanço da doença há um aumento na expressão da ORF desse TE, e isso pode estar relacionado ao início dos tratamentos com quimioterápicos.

Além disso, também encontramos vários genes diferencialmente expressos (DEGs) após os tratamentos com a CPA. Uma característica marcante dos nossos resultados foi a *downregulation* de vários genes envolvidos na formação da mitocôndria em moscas expostas ao tratamento agudo, sugerindo que a CPA poderia promover disfunção mitocondrial. Essa hipótese foi corroborada por análises de respirometria de alta resolução, que revelaram que a mitocôndria realmente estava comprometida nessas moscas. No tratamento crônico, apenas cinco genes relacionados à formação ou ao metabolismo da mitocôndria foram *dowregulated*. Além disso, a atividade mitocondrial retornou ao normal, como visto pela respirometria de alta resolução.

Já ao contrário do observado em moscas tratadas com a CPA, moscas expostas a cisplatina apresentaram uma diferença significativa na expressão relativa de *mariner* tanto na concentrações de 50 µg/mL ($p = 0,0075$) quanto de 100 µg/mL ($p = 0,0002$). Além disso, parece haver uma dose-dependência, uma vez que há um aumento da expressão relativa desse TE na maior concentração de cisplatina, 100 µg/mL (média = 4,35), quando comparado a menor concentração, 50 µg/mL (média = 3,91).

Moscas expostas ao tratamento agudo com a dacarbazina não apresentaram uma diferença significativa na expressão relativa de *mariner* em nenhuma das concentrações analisadas (50 µg/mL $p = 0.6446$ e 100 µg/mL $p = 0.2646$), e também não foi observada

uma diferença significativa no número de spots dos olhos das moscas expostas ao tratamento crônico.

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