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Câmpus de São José do Rio Preto

Cecília Artico Banho

**Desregulação de genes e elementos de transposição e incompatibilidade
híbrida entre subespécies de *Drosophila mojavensis* e *D. arizonae***

São José do Rio Preto
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Tese apresentada como parte dos requisitos para a obtenção do título de Doutor em Biociências, junto ao Programa de Pós- Graduação em Biociências, Área de Genética e Biologia Evolutiva, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Câmpus de São José do Rio Preto, em cotutela com a Université Claude Bernard – Lyon 1.

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Orientadora: Prof^a. Dr^a. Claudia Marcia Aparecida Carareto

Orientadora: Prof^a. Dr^a. Cristina Vieira

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IDEX LYON

Eiffel Program (Campus France)

Comissão Examinadora:

Prof^ª. Dr^a. Claudia Marcia Aparecida CARARETO (UNESP – Câmpus de São José do Rio Preto)

Prof^ª. Dr^a Cristina VIEIRA Orientadora (Université Claude Bernard – Lyon 1)

Prof^ª. Dr^a. Lilian MADI-RAVAZZI (UNESP – Câmpus de São José do Rio Preto)

Prof. Dr. Elgion LORETO (UFSM)

Prof^ª. Dr^a. Laurence MOUTON (Université Claude Bernard – Lyon 1)

Dr. Marcelo Mendes BRANDÃO (UNICAMP)

Prof. Dr. Alessandro VARANI (UNESP – Câmpus de Jaboticabal)

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**Dérégulation des gènes et élément transposables et incompatibilité hybride
entre les sous-espèces *Drosophila mojavensis* et *D. arizonae***

Devant le jury composé de:

CARARETO, Claudia Marcia Aparecida Professeure Université d'état Paulista São José do
Rio Preto: Directrice de thèse

GARCIA GUERREIRO, Maria del Pilar Professeure Université Autonome de Barcelone
(Espagne): Rapporteur

LORETO, Elgion Professeur Université Fédérale de Santa Maria (Brésil):
Rapporteur/Examineur

BRANDÃO, Marcelo Mendes Chercheur Université de Campinas (Brésil):
Rapporteur/Examineur

MADI-RAVAZZI, Lilian Professeure Université d'état Paulista São José do Rio Preto:
Presidente du jury/ Examinatrice

MOUTON, Laurence Maître de Conférences, Université Lyon 1 (France) : Examinatrice

VARANI, Alessandro Chercheur Université d'état Paulista Jabotical (Brésil): Examineur

VIEIRA, Cristina Professeure Université Lyon1: Directrice de thèse

Este trabalho foi realizado sob convenção de co-tutela entre Universidade Estadual Paulista (UNESP) - Brasil e l'Université Claude Bernard (Lyon1 - UCBL) – França, no laboratório de Evolução Molecular, do Instituto de Biociências, Letras e Ciências Exatas (IBILCE/UNESP) – São José do Rio Preto/SP, e no Laboratoire de Biométrie et Biologie Évolutive (LBBE/Lyon1).

Universidade Estadual Paulista (UNESP – IBILCE) Laboratório de Evolução Molecular
Departamento de Biologia
Rua Cristóvão Colombo, 2265, Jardim Nazareth 15054-000 São José do Rio Preto-SP
Brasil

Université Claude Bernard – Lyon 1
Laboratoire de Biométrie et Biologie Evolutive CNRS UMR 5558
43 Boulevard du 11 novembre 1918
69622 Cedex Villeurbanne

Cecilia ARTICO BANHO
cecilia.artico-banho@etu.univ-lyon1.fr
ceci.abanho@gmail.com

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e exemplo de força e perseverança.*

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RESUMO

Hibridização interespecífica é uma condição de estresse que pode levar à esterilidade ou à inviabilidade devido à desregulação de genes e elementos de transposição (TEs), particularmente em espécies do gênero *Drosophila* com grande tempo de divergência. Contudo, a extensão dessas anormalidades em híbridos de espécies com tempo de divergência recente ainda não é bem compreendida. *Drosophila mojavensis* e *D. arizonae* são um bom sistema biológico para investigar essa questão uma vez que a divergência entre elas foi relativamente recente (~1,5 milhões de anos atrás), apresentam diferentes graus de isolamento reprodutivo, e são capazes de produzir híbridos em laboratório. A fim de verificar a ocorrência e o grau da incompatibilidade híbrida, neste estudo, foram realizadas análises fenotípicas para estimar parâmetros da história de vida de descendentes de cruzamentos intra e interespecíficos, bem como análises de expressão diferencial de genes e TEs que possam estar envolvidos no isolamento reprodutivo, por meio de análises de RNA-Seq, de parentais e híbridos. Todos os híbridos apresentaram menor viabilidade em comparação aos descendentes de cruzamentos intraespecíficos, como também todos os híbridos machos dos cruzamentos entre fêmeas *D. mojavensis* vs machos *D. arizonae* apresentaram espermatozoides móveis, enquanto que nos cruzamentos recíprocos, 75% dos híbridos apresentaram espermatozoides imóveis. As análises fenotípicas mostraram ausência de disgenesia gonadal em machos e fêmeas, bem como fertilidade em 100% das fêmeas híbridas. Contudo, apenas 50% dos híbridos com motilidade espermática foram férteis. As análises do transcriptoma evidenciaram expressão conservativa para a maioria dos genes e famílias de TEs em ovários de híbridos em relação às espécies parentais. Em testículos, por outro lado, menor número de genes e TEs tiveram expressão conservada, sendo observada uma tendência à superexpressão de TEs e subexpressão de genes. Além disso, foi verificado que híbridos sem motilidade espermática, provenientes de cruzamentos entre fêmeas *D. arizonae* vs machos *D. mojavensis* apresentaram maior número de genes desregulados que aqueles com espermatozoides móveis, e que a maioria desses genes estavam subexpressos e apresentaram funções relacionadas à espermatogênese, de acordo com análises de ontologia. O sistema de regulação pós-transcricional parece falhar no controle da expressão de TEs superexpressos em ovários e testículos, mesmo quando piRNAs estão presentes na linhagem materna. Isso evidencia que outros mecanismos podem estar relacionados à desregulação dos TEs nos tecidos

reprodutivos de híbridos entre *D. arizonae* e *D. mojavensis*. Dentre esses mecanismos pode-se sugerir a desregulação de alguns genes envolvidos na via de piRNAs, e de modificação de cromatina, como observado em testículos. Contudo, o mesmo não pode ser sugerido para ovários, uma vez que nessa gônada a expressão desses genes não estava desregulada. Em síntese, este estudo mostra que testículos de híbridos entre *D. arizonae* e *D. mojavensis* apresentam maior expressão diferencial de genes e de TEs em relação aos parentais do que em ovários e que, embora piRNAs estejam presentes para muitos dos TEs desregulados, eles não são capazes de controlar sua expressão, o que pode estar ligado ao fenótipo de esterilidade demonstrado pelos parâmetros de história de vida analisados nestes híbridos.

Palavras-chave: Híbridos, Isolamento pós-zigótico, Fenótipo, Expressão Diferencial, Grupo *repleta*

ABSTRACT

Interspecific hybridization is a stress condition that can lead to sterility and/or inviability, by misregulation of genes and transposable elements (TEs) in *Drosophila* species with high divergence time. However, the extent of these anomalies in hybrids of recently diverged species is not clear. *Drosophila mojavensis* and *D. arizonae* are a useful biological system for such investigation, once they diverged recently (~1.5 m.y.a), have variable degrees of reproductive isolation and can produce hybrids in laboratory. In order to verify the occurrence and degree of hybrid incompatibility, in this study, the life history parameters of offspring of intra and interspecific crosses were estimated, as well as the differential expression of genes and TEs that may be involved in reproductive isolation, through RNA-Seq analyses of parental and hybrids. All hybrids had a decrease in viability compared to intraspecific offspring as well as all hybrid males from crosses between *D. mojavensis* females and *D. arizonae* males presented motile sperm, while in the reciprocal crosses 75% of the hybrids had immotile sperm. Phenotypic analyses showed no gonad dysgenesis and fertility in 100% of hybrid females, however, only 50% of males with mobile sperm were fertile. The analyses of the transcriptome showed that most of the genes and TE families had conservative expression related to the parental lines in hybrid ovaries. In testes, on the other hand, a less conservative gene and TE expression was found, since a bias for TE overexpression and gene underexpression was observed. Moreover, it was verified that hybrids presenting immotile sperm, from crosses between *D. arizonae* females and *D. mojavensis* males, have more misexpressed genes than those with motile sperm, and most of the deregulated genes were underexpressed, having spermatogenesis-related functions, according to GO enrichment. The post-transcriptional regulation system seems to fail to control the expression of overexpressed TEs in ovaries and testes, even when piRNAs are present in the maternal lines and hybrids, which suggests that other factors could be underlying TE up- regulation in reproductive tissues of hybrids between *D. arizonae* and *D. mojavensis*. Among these mechanisms, divergent expression of genes involved in the piRNA pathway, as well as, chromatin modification genes, in the hybrid testes, should be highlighted. However, the same cannot be suggested for ovaries, since in this gonad the expression of these genes was not

deregulated. In summary, this study shows that testes of hybrids between *D. arizonae* and *D. mojavensis* have greater differential expression of genes and TEs in relation to parental species than in ovaries. In addition, it shows that although piRNAs are present for many of the unregulated TEs, they are not able to control their expression, which may be linked to the sterility phenotype shown by the life history parameters estimated in these hybrids.

Keywords: Hybrids, Postzygotic isolation, Phenotype, Differential expression, *repleta* group

RÉSUMÉ

L'hybridation interspécifique est une condition de stress qui peut conduire à des hybrides stériles ou non viables, en raison de la dérégulation des gènes et des éléments transposables (ET), en particulier chez les hybrides entre espèces du genre *Drosophila* ayant un long temps de divergence. Cependant, l'ampleur de ces anomalies chez les hybrides d'espèces ayant un temps de divergence récent n'est pas encore bien comprise. *Drosophila mojavensis* et *D. arizonae* constituent un bon système biologique pour étudier cette question car le temps de divergence est relativement récent (~1,5 m. a.). En plus, ces espèces présentent différents degrés d'isolement reproducteur et sont capables de produire des hybrides en laboratoire. Afin de vérifier l'occurrence et le degré d'incompatibilité des hybrides, nous avons mesuré les traits d'histoire de vie des descendants de croisements intra et interspécifiques, ainsi que l'expression différentielle des gènes et des ET qui peuvent être impliqués dans l'isolement reproducteur. Les analyses phénotypiques ont montré que tous les hybrides présentaient une viabilité inférieure aux descendants des croisements intraspécifiques. Les analyses de la motilité des spermatozoïdes ont montré que tous les hybrides mâles issus de croisement entre femelle *D. mojavensis* et mâle *D. arizonae* croisés avaient des spermatozoïdes mobiles, alors que dans le croisement réciproque, 75% des hybrides avaient des spermatozoïdes immobiles. Les analyses de fertilité des hybrides n'ont montré aucune dysgénésie gonadique chez les mâles et les femelles et une fertilité de 100% chez les hybrides femelles. Cependant, seulement 50% des hybrides mâles ayant la motilité des spermatozoïdes étaient fertiles. Les analyses RNASeq ont montré que la plupart des gènes et des familles d'ET présentaient une expression conservée par rapport aux espèces parentales dans les ovaires des hybrides. Dans les testicules, en revanche, moins de gènes et de ET ont une expression de type conservé et on observe une tendance à la surexpression des ET et à la sous-expression des gènes. En outre, il a été observé que les hybrides sans motilité des spermatozoïdes (issus de croisements entre les femelles *D. arizonae* et les mâles *D. mojavensis*) présentaient un nombre plus élevé de gènes dérégulés que ceux avec des spermatozoïdes mobiles, et que la majorité de ces gènes étaient sous-exprimés et présentaient des fonctions liées à la spermatogenèse. Le système de régulation post-transcriptionnel semble ne pas être efficace dans le contrôle de l'expression des ET qui sont surexprimées dans les ovaires et les testicules, même quand des piRNA sont présents dans la lignée maternelle. Cela montre que d'autres mécanismes peuvent être liés à la dérégulation des ET dans les tissus reproducteurs des hybrides entre *D. arizonae* et *D.*

mojavensis. Parmi ces mécanismes, on peut suggérer la dérégulation de certains gènes impliqués dans la voie des RNAi, et la modification de la chromatine, telle qu'observée dans les testicules. Cependant, on ne peut pas en dire autant des ovaires, car dans ce tissu, l'expression de ces gènes n'a pas été dérégulée. En résumé, cette étude montre que les testicules des hybrides entre *D. arizonae* et *D. mojavensis* présentent une expression différentielle des gènes et des ET plus importante par rapport à celle des parents que les ovaires et que, bien que des piARN soient présents pour un grand nombre des ET dérégulés, ils ne semblent pas être capables de contrôler leur expression. Ceci qui peut être associé au phénotype de stérilité observé pour les traits d'histoire de vie analysés chez les hybrides.

Mots-clés: Hybrides, Isolement postzygotique, Phénotype, Expression différentielle, groupe *repleta*

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1 INTRODUÇÃO GERAL

Como novas espécies surgem e quais são os fatores genéticos envolvidos com o processo de especiação são duas das grandes questões em Biologia Evolutiva que, até os dias atuais, não são completamente esclarecidas. O conceito biológico define espécie como grupos de populações naturais intercruzantes que são reprodutivamente isoladas de outros grupos semelhantes (MAYR, 1942; 1963). Sendo assim, especiação pode ocorrer quando o fluxo de informação genética entre populações é inibido pela formação de barreiras que levam ao isolamento reprodutivo (DOBZHANSKY, 1937; 1940). Dessa forma, a magnitude e a taxa com que as barreiras de isolamento reprodutivo evoluem, em diferentes grupos de espécies, pode ser um fator chave na origem de novas espécies (TURISSINI *et al.*, 2018).

1.1 Mecanismos de incompatibilidade híbrida

Sabe-se, atualmente, que o processo de hibridização interespecífica, que já foi considerado um evento raro, é relativamente comum na natureza, ocorrendo em cerca de 25% das espécies vegetais e em 10% das animais (MALLET, 2005) e que, em espécies incipientes, as barreiras que levam à restrição ao fluxo gênico podem ser incompletas, levando à produção de descendentes híbridos. De modo geral, os mecanismos de isolamento reprodutivo podem ser classificados em diferentes tipos, dependendo do momento em que ocorrem durante o ciclo reprodutivo, podendo ser pré-zigótico, pré-zigótico pós-cópula, ou pós-zigótico. O isolamento pré-zigótico resulta no impedimento de cruzamentos interespecíficos, e pode ocorrer em decorrência de especificidade de nicho, de preferências de habitats e no período reprodutivo, como também devido a fatores comportamentais (padrões de corte e preferência de acasalamento) e mecânicos (morfologia das genitálias externas) (TURISSINI *et al.*, 2018). Os mecanismos de isolamento pré-zigótico pós-cópula, por sua vez, são aqueles que envolvem incompatibilidades entre os gametas, ou mesmo, entre proteínas do trato reprodutivo feminino e fluido seminal masculino (MARKOW; HOCUTT, 1998; KNOWLES; MARKOW, 2001;

COYNE; ORR, 2004; REED; MARKOW, 2008; TURISSINI *et al.*, 2018). Por outro lado, mecanismos de isolamento pós-zigóticos, incluem incompatibilidades que levam à redução do valor adaptativo de híbridos interespecíficos em relação às espécies puras, podendo ocasionar anormalidades no desenvolvimento (inviabilidade) e na reprodução (esterilidade) dos híbridos F_1 ou F_2 , e mesmo no comportamento, como o padrão de corte dos híbridos (ORR; PRESGRAVES, 2000; PRESGRAVES, 2010; MCBRIDE; SINGER, 2010; TURISSINI *et al.*, 2018).

Embora os mecanismos de isolamento pós-zigótico sejam extensivamente estudados, principalmente em espécies de *Drosophila*, ainda não se tem amplo conhecimento dos fatores genéticos que influenciam a fertilidade e/ou viabilidade híbrida. Coyne *et al.* (1997) mostraram, pela análise de dados de 171 cruzamentos interespecíficos em *Drosophila*, que os mecanismos de isolamento pós-zigótico evoluem primeiramente em machos que em fêmeas, e que o grau da inviabilidade ou esterilidade está diretamente relacionado à distância genética entre as espécies. Além disso, ao analisarem o tempo de divergência das espécies, os autores constataram que hibridizações entre espécies com tempo de divergência recente produziam principalmente híbridos machos inviáveis ou estéreis, ao passo que apenas intercruzamentos entre espécies com maior tempo de divergência produziam fêmeas inviáveis. Estes resultados corroboram a regra de Haldane (1922), que postula que em eventos de hibridização, quando há esterilidade híbrida, essa afetará primeiramente o sexo heterogamético.

Com o avanço das técnicas de Biologia Molecular e sequenciamento genômico, diversos estudos têm buscado compreender as bases genéticas da especiação. Esses estudos têm mostrado que mudanças na expressão gênica são importantes fontes de variação em características morfológicas adaptativas (MCGIRR; MARTIN, 2019), e, em especial para o processo de especiação. A incompatibilidade decorrente da divergência genética entre os parentais pode causar desregulação de genes específicos em híbridos, os quais podem ser expressos em nível superior ou inferior ao das espécies parentais, resultando na redução do valor adaptativo do híbrido, aumentando, portanto, as barreiras de isolamento pós-zigótico.

Diversos autores vêm pesquisando genes candidatos à especiação, principalmente utilizando como modelo espécies de *Drosophila* (WU *et al.*, 1996; TING *et al.*, 1998; MICHALAK; NOOR, 2003; MICHALAK; NOOR, 2004; HAERTY; SINGH 2006; MA; MICHALAK, 2011; GOMES; CIVETTA, 2014). Dentre os genes identificados que apresentam associação direta com o fenótipo de incompatibilidade híbrida destacam-se os genes listados na Tabela 1.

Tabela 1. Genes associados diretamente com incompatibilidade e/ou esterilidade em *Drosophila*.

Gene	Fenótipo de incompatibilidade e/ou esterilidade	Referências
<i>Odysseus (Odsh)</i>	Híbridos de <i>D. mauritiana</i> - <i>D. simulans</i>	TING <i>et al.</i> 1998
<i>Acylphosphatase (Acyp)</i>	Híbridos de <i>D. mauritiana</i> - <i>D. simulans</i> e híbridos de <i>D. p. pseudoobscura</i> - <i>D. persimilis</i>	NOOR, 2005; MICHALACK; MA, 2008; GOMES; CIVETTA, 2014
<i>Jyalpha</i>	Híbridos de <i>D. melanogaster</i> - <i>D. simulans</i>	MASLY <i>et al.</i> 2006
<i>Zygote hybrid rescue (Zhr)</i>	Híbridos de <i>D. melanogaster</i> - <i>D. simulans</i>	SAWAMURA; YAMAMOTO, 1997
<i>Nucleoporin 153kD (Nup153) e</i>	Híbridos de <i>D. melanogaster</i> - <i>D. simulans</i>	PRESGRAVES <i>et al.</i> , 2003; PRESGRAVES;
<i>Nucleoporin 96kD (Nup96)</i>		STEPHAN, 2007
<i>Hybrid male rescue (Hmr) e</i>	Híbridos de <i>D. melanogaster</i> - <i>D. simulans</i>	BARBASH <i>et al.</i> , 2003; BARBASH <i>et al.</i> , 2004;
<i>Lethal male rescue (Lmr)</i>		BRIDEAU <i>et al.</i> , 2006; FERREE; BARBASH, 2009; SATYAKI <i>et al.</i> , 2014
<i>Gfzf</i>	Híbridos de <i>D. melanogaster</i> e <i>D. simulans</i>	PHADNIS <i>et al.</i> , 2015; COOPER; PHADNIS, 2016.
<i>Overdrive (Ovd)</i>	Híbridos de <i>D. p. pseudoobscura</i> e <i>D. p. bogotana</i>	PHADNIS; ORR, 2008; PHADNIS, 2011
<i>gonadal (gdl)</i>	Híbridos de <i>D. simulans</i> - <i>D. mauritiana</i> e híbridos de <i>D. simulans</i> - <i>D. sechellia</i>	MOEHRING <i>et al.</i> 2006
<i>don juan (dj)</i>	Híbridos de <i>D. simulans</i> - <i>D. mauritiana</i> e híbridos de <i>D. simulans</i> - <i>D. sechellia</i>	MOEHRING <i>et al.</i> 2006
<i>always early (aly)</i>	Híbridos de <i>D. p. pseudoobscura</i> - <i>D. persimilis</i> e em mutantes de <i>D. melanogaster</i>	NOOR, 2005; GOMES; CIVETTA, 2014; WHITE-COOPER, 1998; JIANG; WHITE-COOPER, 2003
<i>cannonball (can)</i>	Mutantes de <i>D. melanogaster</i>	WHITE-COOPER, 1998
<i>meiosis I arrest (mia)</i>	Mutantes de <i>D. melanogaster</i>	WHITE-COOPER, 1998
<i>Spermatocyte arrest (sa)</i>	Mutantes de <i>D. melanogaster</i>	WHITE-COOPER, 1998
<i>twine (twe)</i>	Mutantes de <i>D. melanogaster</i>	WHITE-COOPER, 1998
<i>cookie monster (comr)</i>	Mutantes de <i>D. melanogaster</i>	JIANG; WHITE-COOPER, 2003
<i>janusB (janB)</i>	Mutantes de <i>D. melanogaster</i>	YANICOSTAS; LEPESANT, 1990
<i>gonadal (gdl)</i>	Híbridos de <i>D. simulans</i> - <i>D. mauritiana</i> e híbridos de <i>D. simulans</i> - <i>D. sechellia</i>	MOEHRING <i>et al.</i> 2006
<i>don juan (dj)</i>	Híbridos de <i>D. simulans</i> - <i>D. mauritiana</i> e híbridos de <i>D. simulans</i> - <i>D. sechellia</i>	MOEHRING <i>et al.</i> 2006

Fonte: Elaborado pelo autor

Embora grande quantidade de genes envolvidos com isolamento reprodutivo tenha sido identificada em híbridos de diferentes espécies, ainda é difícil encontrar uma associação entre a desregulação de genes específicos em diferentes híbridos e o processo de isolamento pós-zigótico, isso porque, diversos genes codificantes de proteínas e reguladores podem estar envolvidos nesse processo. Além disso, a desregulação de muitos genes pode ser espécie-específica, devido ao maior ou menor grau de divergência das espécies, ou mesmo pode haver a influência de outras sequências genéticas nesse processo, como os elementos de transposição (do Inglês, *Transposable Elements* (TEs)).

1.2 Elementos de Transposição e seus mecanismos de regulação

Os elementos de transposição são sequências de DNA repetitivas capazes de se movimentar de um local para outro no genoma, replicando-se a si mesmos, sendo essa capacidade a essência de seu sucesso evolutivo. Essas sequências estão presentes em grandes proporções e diversidade nos genomas de quase todos os eucariotos (exceto em *Plasmodium falciparum*) (WICKER *et al.*, 2007), sendo que em fungos 3 a 20% do genoma é composto por TEs, ao passo que em metazoários essa proporção varia de 3 a 45% (DABOUSSI; CAPY, 2003; WICKER *et al.*, 2007). Finnegan (1989) pioneiramente propôs que os TEs fossem classificados em duas classes, com base no seu mecanismo de transposição: elementos de Classe I, que se transpõem por transcrição reversa de um intermediário de RNA usando um mecanismo DNA-RNA-DNA, e elementos de Classe II que se transpõem diretamente de DNA para DNA. A classificação de Finnegan foi objeto de duas grandes atualizações que têm sido debatidas ativamente; uma delas, o sistema hierárquico de classificação de Wicker (WICKER *et al.*, 2007) e a outra a utilizada pelo Repbase, que é o banco de dados de elementos repetitivos de DNA mais comumente usado (JURKA *et al.*, 2005; KAPITONOV; JURKA, 2008).

Como não há consenso para um sistema universal de classificação dos TEs (PIEGU *et al.* 2015), descreve-se a seguir o sistema hierárquico de classificação de TEs em eucariotos proposto

por Wicker *et al.* (2007), que mantém a divisão dos TEs em duas classes, mas aplicando critérios enzimáticos e mecanicistas, e inclui em ordem hierárquica os níveis subclasse, ordem, superfamília, família e subfamília. Na Classe I estão inclusos os elementos que utilizam uma etapa de transcrição reversa, mobilizando-se por meio de um mecanismo denominado *copy and paste* (elementos de RNA, conhecidos como retrotransposons). Essa classe é composta por cinco ordens, sendo denominadas LTR (*Long terminal repeat*), DIRS (*Dictyostelium intermediate repeat sequence*), PLE (*Penelope-like elements*), LINE (*Long Interspersed Nuclear Element*) e SINE (*Short Interspersed Nuclear Element*), as quais são distinguidas pela organização de seus constituintes internos, filogenia da enzima transcriptase reversa, bem como pelo seu mecanismo de transposição (WICKER *et al.*, 2007). Por outro lado, a Classe II é composta por elementos de DNA, ou seja, aqueles que não utilizam uma etapa intermediária de RNA para mobilização, possuindo um mecanismo de transposição conhecido como *cut and paste*, uma vez que ocorre a excisão do elemento do genoma antes de sua reinserção em outra região (FINNEGAN, 1989; WICKER *et al.*, 2007). Esta classe contém duas subclasses, que são distinguidas pelo número de cadeias de DNA que são cortadas durante a transposição. A Subclasse I é composta por elementos das ordens TIR (*terminal inverted repeat*) e Crypton, e a Subclasse II por elementos das ordens Maverick e Helitron (WICKER *et al.*, 2007).

É interessante ressaltar que os elementos de Classe I apresentam cópias adicionais em cada novo evento de mobilização, enquanto que em elementos de Classe II, a transposição é, na maior parte das vezes, conservativa (WICKER *et al.*, 2007). Considerando esses aspectos e o fato de que TEs são entidades dinâmicas, durante eventos de mobilização essas sequências podem ter efeitos relevantes sobre o genoma hospedeiro. Esses efeitos podem ser verificados em nível da linhagem celular germinativa ou em estados precoces de desenvolvimento, assim como em nível somático, resultando em mudanças fenotípicas no hospedeiro. Além disso, eventos transposição podem desencadear modificações estruturais no genoma, como translocações, duplicações segmentais e deleções capazes de induzir profundas alterações genômicas, levando à

sua contração ou expansão, além do estabelecimento de novas relações de ligação entre genes (KIDWELL; LISCH, 2001). Tem-se reportado, também, que os TEs desempenham papel importante na regulação de diversos processos celulares e diversificação das famílias gênicas, sendo capazes de promover a transdução e amplificação de fragmentos de genes do genoma hospedeiro (VAN de LAGEMAAT *et al.*, 2003); bem como, de influenciar a expressão e função gênica, ampliando a variabilidade do repertório transcricional e proteico (CARARETO *et al.*, 2013; LOPES *et al.*, 2008, 2013). Em humanos, estima-se que 25% das regiões promotoras contêm sequências derivadas de TEs, e aproximadamente 20% dos genes em humanos e ratos contêm esses elementos em suas regiões UTRs (*Untranslated Regions*) (WONG; CHOO, 2004).

A relação dinâmica dos TEs com o genoma no qual estão inseridos explica, em alguns pontos, os processos evolutivos que ocorrem nos organismos. Em geral, a intensa mobilização de TEs, em organismos bem adaptados, pode ocasionar efeitos deletérios aos hospedeiros. Sendo assim, o controle da sua expressão é fundamental para estabelecer um balanço de efeitos positivos e negativos, garantindo a viabilidade das funções do genoma nos organismos. Esse controle é realizado por meio do silenciamento pós-transcricional (utilizando RNAs de interferências) e do silenciamento transcricional (metilação do DNA e modificação de histonas) (BRENNECKE *et al.*, 2007; RIGAL; MATHIEU, 2011). Os mecanismos de regulação pós-transcricionais envolvem a maquinaria de RNA de interferência (RNAi). Em células somáticas, os TEs são controlados por meio de siRNAs (*small interfering RNA*), que ao reconhecerem e se ligarem ao mRNA promovem sua degradação (HIRAKATA; SIOMI, 2019; SATO; SIOMI, 2020). Em células germinativas, o mecanismo de regulação pós-transcricional, descrito em *Drosophila* e humanos, possui algumas semelhanças com o que ocorre em células somáticas, contudo, neste caso o RNA de interferência é denominado de piRNA (*piwi-interacting RNAs*), devido ao grupo de proteínas que participam do mecanismo de silenciamento, pertencentes à família das Argonautas, Ago3, Aubergina (Aub) e Piwi (revisado em HIRAKATA; SIOMI, 2019; SATO; SIOMI, 2020).

A produção de piRNAs acontece por duas vias em *Drosophila*. A via de biogênese de piRNAs primários ocorre a partir de transcritos provenientes de *clusters* de piRNAs presentes nos genomas. Esse processo envolve principalmente as proteínas *Piwi* e *Zucchini* (*Zuc*), entre outras (BRENNECKE *et al.*, 2007; IPSARO *et al.*, 2012; SATO; SIOMI, 2020). Por outro lado, a via de piRNAs secundários é produzida apenas em células germinativas, por meio de um mecanismo de amplificação chamado de *ping-pong* (BRENNECKE *et al.*, 2007). Nesse mecanismo, piRNAs primários ou de origem materna são necessários para iniciar a via, uma vez que, piRNAs anti-senso precisam ser ligados às proteínas *Piwi* ou *Aub*. Quando esse complexo (piRNA-proteína) é formado, ele é capaz de degradar transcritos complementares (no sentido senso) de TEs que provavelmente estavam ativos no genoma. Essa clivagem produz, então, transcritos de piRNA senso, os quais são ligados à proteína *Ago3*, que degrada transcritos de TEs anti-senso, levando então ao mecanismo de amplificação *ping-pong* (Figura 1). Esse mecanismo, geralmente, permite uma eficiente resposta contra cópias de TEs ativas nos genomas (BRENNECKE *et al.*, 2007; FABLET, 2014). Adicionalmente, em tecidos germinativos de *Drosophila* foi verificado que os complexos *Piwi*-piRNA são capazes de reprimir a transcrição de TEs por modificação do estado da cromatina (IWASAKI *et al.*, 2016). Em gônadas animais, muitas inserções de TEs são metiladas nos resíduos H3K9 de maneira dependente de *PIWI*-piRNA e problemas nessa via podem resultar em perda seletiva da marca repressiva H3K9me3 em inserções de TEs alvo e, portanto, na sua ativação (SIENSKI *et al.*, 2015).

O impacto causado pela instabilidade genética proveniente da hibridização interespecífica pode gerar diversas modificações no genoma hospedeiro. Dentre elas se encontram a desregulação de TEs, bem como de seus elementos regulatórios, podendo influenciar diretamente a expressão gênica. De acordo com Senti *et al.* (2015), o aumento da expressão de TEs no genoma pode gerar maiores quantidades de mRNAs de TEs disponíveis no citoplasma. Esse processo, consequentemente, influencia o complexo nuclear *Piwi*-piRNA, aumentando a repressão em nível transcricional, pelo recrutamento de marcas inativadoras da cromatina, como

H3K9me3. De fato, diversos estudos analisaram a influência de TEs na expressão de genes vizinhos, por meio da análise de marcas repressoras ou ativadoras da cromatina. Sienski *et al.* (2012) observaram que, em pelo menos 88% das regiões genômicas em *D. melanogaster* que continham a marca inativadora H3K9me3 na eucromatina haviam inserções de TEs, com pelo menos 5 kb de distância, à jusante ou à montante de onde estavam localizadas. Essas inserções são capazes de influenciar a expressão de genes vizinhos, como foi verificado para o locus *ex* (*expanded*) no qual o retrotransposon *Gypsy* é normalmente inserido. Nesse loco, *Gypsy* está, aproximadamente, a 1,2 kb à jusante do sítio de iniciação da transcrição (TSS) e, em condições normais, esse TE tem sua expressão controlada pelo espalhamento da marca de inativação da cromatina H3K9me3, em aproximadamente 10 a 12 kb, cobrindo, portanto, o TSS de *ex*. Contudo, em mutantes para a proteína Piwi, a qual participa das vias de silenciamento transcricional e pós- transcricional, foi verificada a perda completa de marcas H3K9me3, desencadeando a expressão desse elemento. Além disso, também foi reportado que em mutantes para essa proteína, 34% dos genes com inserções de TEs próximas a eles foram superexpressos, sendo que 80% desses estavam associados com inserções de TEs com até 5 kb de distância (SIENSKI *et al.*, 2012).

Esses resultados evidenciam o impacto do aumento da expressão de TEs nos processos de regulação e expressão de genes vizinhos, favorecendo indagações sobre a existência de associação desses elementos com genes diferencialmente expressos em tecidos germinativos de híbridos, e consequentemente, com o surgimento de barreiras reprodutivas pós-zigóticas que podem levar ao processo de especiação. Embora atualmente não existam evidências diretas sobre a influência da desregulação dos TEs sobre os genes da especiação, tem sido demonstrado que híbridos interespecíficos apresentam diversos genes desregulados, bem como aumento nas taxas de transposição (KELEHER *et al.*, 2012; SATYAKI *et al.*, 2014; ROMERO-SORIANO *et al.*, 2017; LOPEZ-MAESTRE *et al.*, 2017), contudo, associação de ambos fatores ainda não é completamente entendida.

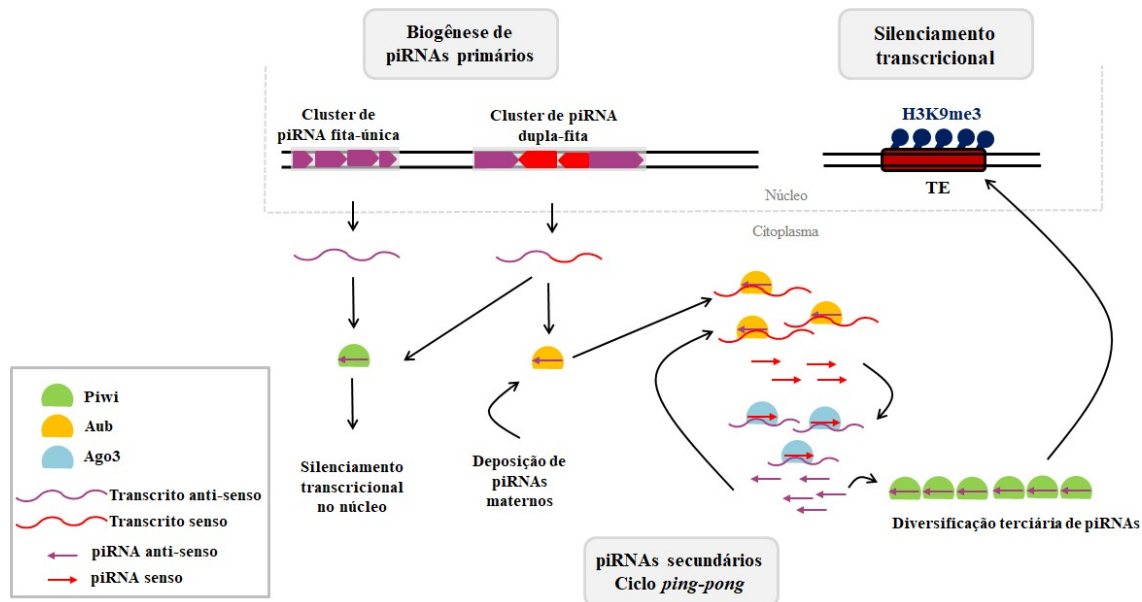


Figura 1. Mecanismo de silenciamento via piRNAs em *Drosophila*. Na biogênese de piRNAs primários, os piRNAs são transcritos de regiões genômicas denominadas *clusters*, os quais podem ser de fita única ou de dupla fita (específico de células germinativas). Posteriormente, os piRNAs são processados e se ligam às proteínas Piwi ou Abu (Aubergina). piRNAs anti-senso ligados à proteína Aub tem como alvo transcritos de TEs no sentido senso. Após o reconhecimento do transcrito por complementariedade o complexo Aub-piRNAs, por meio de atividade endonuclease, degrada o TE originando piRNAs senso, os quais são ligados à Ago3 (Argonauta3), que tem como alvo transcritos de TEs no sentido anti-senso. Este mecanismo é denominado ciclo *ping-pong*, originando piRNAs secundários. Alguns complexos Aub-piRNA são herdados maternalmente e contribuem para o início do ciclo de amplificação de piRNAs secundários (*ping-pong*). As proteínas Piwi ligadas à piRNAs antisenso (primários ou originados por diversificação terciária) se dirigem ao núcleo celular, onde recrutam proteínas de metilação que promovem silenciamento transcricional ao adicionar marcas de histona H3K9me3.

Fonte: Elaborado pelo autor e Adaptado de FABLET *et al.*, 2017 e IWASAKI *et al.*, 2015.

1.3 Mobilização de TEs em híbridos

Em eventos de hibridização, os genomas de duas espécies ou populações diferentes se encontram no *background* híbrido podendo levar a uma ampla desregulação genômica, do transcriptoma, do epigenoma e podendo também influenciar a mobilização de TEs (SLOTKIN; MARTIENSSSEN, 2007; REBOLLO *et al.*, 2012). De fato, a ativação dos TEs em genomas híbridos tem sido reportada na literatura (CARNELOSSI, *et al.*, 2014; KELLEHER *et al.*, 2012; VELA *et al.*, 2014; GUERREIRO, 2015; SATYAKI *et al.*, 2014; LOPEZ-MAESTRE *et al.*, 2017; ROMERO-SORIANO *et al.*, 2017). Devido à instabilidade genética causada pelo choque

genômico, eventos de transposição foram relatados em híbridos de plantas e animais (KIDWELL *et al.*, 1977; PETROV *et al.*, 1985; BAACK *et al.*, 2005; PARISOD *et al.*, 2010; KAWAKAMI *et al.*, 2011; KELLEHER *et al.*, 2012; VELA *et al.*, 2014; GUERREIRO *et al.*, 2015; HILL *et al.*, 2016; ROMERO-SORIANO *et al.*, 2017; CASTILLO; MOYLE, 2019). Em plantas, explosões de transposição foram registradas em três diferentes híbridos de girassol (gênero *Helianthus*), as quais estavam relacionadas ao aumento do número de cópias de elementos da ordem LTR (*Ty1/copia-like* e *Ty3/gypsy-like*), os quais foram responsáveis pelo aumento do genoma híbrido em aproximadamente 50% (BAACK *et al.*, 2005; KAWAKAMI *et al.*, 2011). Em animais, aumento nas taxas de transposição em decorrência de hibridização foi verificado em híbridos de espécies de cangurus, *Macropus eugenii* e *Wallabia bicolor* (O'NEILL *et al.*, 1998; METCALFE *et al.*, 2007). Nesses híbridos as consequências de eventos de transposição foram detectadas como grandes alterações cromossômicas, cariotípicas e no estado de metilação da cromatina, contribuindo para o menor valor adaptativo desses híbridos (O'NEILL *et al.*, 1998; METCALFE *et al.*, 2007).

Drosophila é um grupo extensivamente estudado quanto aos efeitos de eventos de mobilização de TEs em decorrência de eventos de hibridização. Nessas espécies, a ativação de TEs pode ser verificada em híbridos provenientes de cruzamentos intraespecíficos, bem como interespecíficos. Em nível intraespecífico, diversos estudos mostraram o fenômeno de disgenesia híbrida, caracterizado por atrofia gonadal ou inviabilidade larval, o qual está relacionado à mobilização de elementos específicos em determinadas espécies (PICARD, 1976; BLACKMAN *et al.*, 1987; YANNOPOULOS *et al.*, 1987; KIDWELL *et al.*, 1977; HILL *et al.*, 2016). Como exemplo, o sistema de disgenesia P-M, que ocorre em *D. melanogaster* e *D. simulans*, está relacionado à presença do elemento *P* ativo em apenas uma das populações submetidas a cruzamentos intraespecíficos. Quando populações maternas que não possuem o elemento *P* ativo (denominadas M) se cruzam com machos provenientes de populações que apresentam este elemento ativo em seus genomas (denominados P), as fêmeas híbridas geradas apresentam

diversos efeitos disgênicos como atrofia gonadal, e consequentemente ausência ou redução de fertilidade, aberrações cromossômicas e mutações espontâneas (KIDWELL *et al.*, 1977; BINGHAM *et al.*, 1982; HILL *et al.*, 2016). Contudo, é interessante ressaltar que essas consequências são verificadas em apenas uma direção de cruzamento, na qual as fêmeas não possuem os elementos ativos em seus genomas (KIDWELL *et al.*, 1977; BINGHAM *et al.*, 1982). Estudos posteriores mostraram que a mobilização desses elementos na linhagem germinal, e consequente prejuízo para os híbridos estavam relacionadas à falhas no sistema de regulação pós-transcricional, associado à via de piRNAs (revisado em LUO; LU, 2017). Mais especificamente, quando a linhagem materna não possui em seu genoma elementos que estão ativo na linhagem paterna, piRNAs primários provenientes de *clusters* genômicos e de deposição materna estarão ausentes, e portanto, a via de biogênese de piRNAs secundários, *ping-pong loop*, será prejudicada, resultando na ausência de controle pós-transcricional desse elemento (LUO; LU, 2017).

Em nível interespecífico foi mostrado que em híbridos de *D. buzzatii* e *D. koepferae* (espécies irmãs do grupo *repleta*, subgênero *Drosophila*) houve aumento na transposição do retrotransposon *Oswaldo*, que se encontra reprimido nos genomas parentais (LABRADOR *et al.*, 1994), bem como, uma explosão de transposição associada a três elementos: *Oswaldo*, *Helena* e *Galileo* (GUERREIRO, 2015; VELA *et al.*, 2014). Um estudo mais recente em híbridos desse par de espécies evidenciou que a desregulação, e consequente ativação de determinados TEs estava associada à divergência de genes que participavam do sistema de regulação pós-transcricional, via piRNAs, entre as espécies parentais (ROMERO-SORIANO *et al.*, 2017). Dessa forma, nos híbridos, a regulação desses elementos não era realizada de forma eficiente, resultando no aumento de sua expressão. Similarmente, análises de transcriptomas de híbridos de *D. melanogaster* e *D. simulans* mostraram ativação global de famílias de TEs, herdados tanto maternalmente, quanto paternalmente, e que a desrepressão poderia ter sido ocasionada pela grande divergência adaptativa de genes da via de piRNA ao invés de diferenças espécies-

específicas de piRNAs derivados de TEs (KELLEHER *et al.*, 2012). Assim, a hibridização ou introgressão em populações podem contribuir para eventos de mobilização de TEs e instabilidade genômica que pode ser benéfica ao híbrido ao lhe conferir a capacidade de adaptação e especiação, ou ser prejudicial, podendo levá-lo à extinção (FONTDEVILA, 2005).

Embora, ainda não existam evidências se o aumento de transposição influencia a regulação gênica em híbridos, e vice versa, contribuindo para as barreiras de isolamento reprodutivo (REBOLLO *et al.*, 2010), um estudo em híbridos de *D. melanogaster* e *D. simulans* mostrou uma associação direta de efeitos epistáticos deletérios entre dois genes específicos, *Hmr* (*Hybrid male rescue*) e *Lmr* (*Lethal male rescue*), levando à desregulação de genes de heterocromatina, e culminando no aumento de expressão TEs e sequências satélites, principalmente em regiões centroméricas (SATYAKI *et al.*, 2014). Considerando esses fatores, espécies que divergiram recentemente e que apresentam barreiras de isolamento pré e pós-zigóticas incompletas, que propiciam a produção de híbridos, são bons modelos para o estudo das bases genéticas que influenciam o isolamento reprodutivo, bem como a dinâmica dos TEs nos genomas híbridos e sua influência nos passos iniciais do processo de especiação.

1.4 As espécies *Drosophila mojavensis* e *D. arizonae*

Dentre as espécies do gênero *Drosophila*, *D. mojavensis* e *D. arizonae* (grupo *repleta*, subgênero *Drosophila*) são espécies irmãs, cactofílicas, que constituem populações alopátricas e simpátricas, capazes de produzir híbridos em laboratório (RUIZ *et al.*, 1990; MASSIE; MARKOW, 2005; JENNINGS; ETGES, 2009). Populações de *D. arizonae* são mais generalistas, utilizando como sítios de alimentação e reprodução cactos colunares ou do gênero *Opuntia*, dependendo de sua distribuição geográfica (RUIZ; HEED 1988; REED *et al.*, 2006). Essa espécie é encontrada a partir do sul da Guatemala, México e sudoeste dos Estados Unidos da América, onde podem constituir populações simpátricas com *D. mojavensis* (HEED, 1982; RUIZ; HEED, 1988; REED *et al.*, 2006). *D. mojavensis*, por sua vez, foi dividida em quatro

subespécies, sendo elas *D. m. mojavenensis*, encontrada no Deserto de Mojave, *D. m. baja*, presente na Península de Baja Califórnia, *D. m. sonorensis*, localizada no deserto de Sonora e sul do Arizona e *D. m. wrigleyi*, endêmica da ilha de Santa Catalina, na costa da Califórnia (REED *et al.*, 2006), e em cada uma dessas regiões, as diferentes subespécies utilizam diferentes cactos colunares como sítio de alimentação e reprodução (REED *et al.*, 2006; JENNINGS; ETGES, 2009). Reed *et al.* (2006) também reportaram que as populações pertencentes às quatro principais áreas de distribuição de *D. mojavenensis* apresentam-se estruturadas e com diferenças genéticas significativas, em relação aos haplótipos de DNA mitocondrial. Dados semelhantes foram reportados por Ross *et al.* (2006) utilizando locos microssatélites. Esses dados evidenciam que as populações de *D. mojavenensis* apresentam restrição do fluxo gênico entre as quatro principais regiões geográficas.

Embora existam poucos estudos sobre as barreiras de isolamento reprodutivo pós-zigótico entre *D. arizonae* e *D. m. mojavenensis*, muitos estudos têm evidenciado a complexidade de suas barreiras de isolamento pré-zigótico. Isto é, essas espécies apresentam isolamento pré-zigótico e isolamento pré-zigótico pós-copula variável e dependente da direção de cruzamento, bem como da subespécie de *D. mojavenensis* considerada (PATTERSON, 1946; RUIZ *et al.*, 1990; MARKOW; HOCUTT, 1998; KNOWLES; MARKOW, 2001; REED; MARKOW, 2004, ETGES *et al.*, 2006; KELLEHER; MARKOW, 2007). Alguns estudos reportaram que fêmeas de *D. mojavenensis* provenientes de populações simpátricas apresentam isolamento reprodutivo pré-zigótico quase completo em relação a machos de *D. arizonae*, ao passo que fêmeas de *D. mojavenensis* que vivem em alopatria com *D. arizonae* mostraram menor índice de isolamento reprodutivo (RUIZ *et al.*, 1990; WARSEMAN; KOEPFER, 1977; MASSIE; MARKOW, 2005), o que se ajusta à Teoria do Reforço (DOBZHANSKY, 1937). Por outro lado, Markow *et al.* (1998), ao analisarem fêmeas de *D. mojavenensis* provenientes da península de Baja California, encontraram maiores taxas de isolamento reprodutivo em relação à *D. arizonae* em comparação com populações de *D. mojavenensis* do sul do Arizona (USA), evidenciando que índices de

isolamento pré-zigótico são variáveis entre essas subespécies. Além disso, considerando a barreira de isolamento reprodutivo pré-zigótico pós-cópula, foi mostrado que após cruzamentos interespecíficos de *D. mojavenensis* e *D. arizonae*, uma massa espermática, denominada reação de inseminação (consequência de incompatibilidades entre proteínas presentes no esperma e no trato reprodutivo feminino) é formada no útero das fêmeas. A persistência dessa massa no trato reprodutivo feminino é variável de acordo com as subespécies de *D. mojavenensis*, contudo, em alguns casos ela pode permanecer por dias (KNOWLES; MARKOW, 2001), sendo capaz de esterilizar as fêmeas ou evitar o comportamento de re-cópula nessas espécies, o qual é essencial para seu sucesso reprodutivo (PATTERSON, 1946; MARKOW; ANKNEY, 1984; ALONSO-PIMENTEL, 1994).

As espécies *D. arizonae* e *D. mojavenensis* apresentam isolamento pós-zigótico incompleto e assimétrico, visto que em cruzamentos de fêmeas de *D. arizonae* com machos de *D. mojavenensis* os machos híbridos são estéreis, mas em cruzamentos recíprocos a prole pode ser fértil, dependendo da origem da população materna (RUIZ *et al.*, 1990; REED; MARKOW, 2004). Esse par de espécies apresenta tempo divergência recente, cerca de 1,5 milhões de anos (SANCHES-FLORES *et al.*, 2016); diferindo, portanto, das espécies já estudadas quanto aos efeitos da hibridização na expressão gênica e de TEs, como *D. melanogaster* e *D. simulans* (KELLEHER *et al.*, 2012) e *D. buzzatti* e *D. koepferae* (ROMERO-SORIANO *et al.*, 2017). Nesses dois pares de espécies, em cujos híbridos ocorre intensa desregulação de TEs, o tempo de divergência está em torno de 1,2 a 5 (RUSSO *et al.*, 1995; KLIMAN *et al.*, 2000; TAMURA *et al.*, 2004; CUTTER, 2008) e 4,63 milhões de anos (GOMEZ; HASSON, 2003; LAAYOUNI *et al.*, 2003; OLIVEIRA *et al.*, 2012), respectivamente. Além disso, estudos anteriores evidenciaram que, diferentemente dos híbridos das espécies acima citadas, em híbridos fêmeas de *D. arizonae* e uma subespécie de *D. mojavenensis*, não há intensa desregulação, mas sim a ativação de determinados TEs (LOPEZ-MAESTRE *et al.*, 2017). Mais especificamente, Lopes-Maestre *et al.* (2017), a partir da análise de expressão global de TEs mostraram que três

elementos se apresentaram superexpressos nos híbridos em relação aos parentais (*GTWIN*, *Copia1* e *Frogger*) e que a provável causa dessa desregulação foi a ausência de piRNAs correspondentes na linhagem materna, impedindo, portanto, o controle da transcrição. Os autores também encontraram, por análise global do transcriptoma, que em híbridos haviam alguns genes específicos, relacionados à reprodução e ao desenvolvimento, desregulados em relação aos parentais; contudo, análises para verificar se esses TEs estavam influenciando a desregulação desses genes não foram realizadas. Adicionalmente, Carnelossi *et al.* (2014) analisaram a expressão do elemento *I*, um retrotransposon sem LTR que está associado ao fenômeno de disgenesia híbrida em *D. melanogaster*, em tecidos germinativos de híbridos de *D. m. mojavenensis* e *D. arizonae*. Os autores verificaram que os ovários dos híbridos, independente da direção do cruzamento parental, como também os testículos de híbridos parcialmente férteis, apresentaram níveis de expressão semelhantes aos parentais. No entanto, em testículos dos híbridos do cruzamento recíproco (fêmeas *D. arizonae* e machos de *D. mojavenensis*), que são estéreis, houve maior expressão do elemento *I*, mais especificamente nos espermatoócitos primários, locais onde já foram detectados altos níveis de transcrição de genes codificadores de proteínas responsáveis pela espermatogênese, e de genes específicos da linhagem germinativa masculina (FULLER, 1998). Esses resultados sugeriram que, de alguma forma, a maior expressão desse TE poderia estar relacionada ao fenômeno de esterilidade observado.

Devido ao fato de *D. mojavenensis* e *D. arizonae* estarem em processo recente de especiação, a investigação de mecanismos genéticos que podem levar ao isolamento reprodutivo, e o papel dos TEs nesse processo é relevante. Além disso, analisar descendentes de cruzamentos de *D. arizonae* com diferentes subespécies de *D. mojavenensis*, as quais apresentam diferentes graus de incompatibilidade híbrida pode ampliar o entendimento dos fatores polimórficos que influenciam o surgimento de barreiras de isolamento reprodutivo. Adicionalmente, de acordo com Gomes *et al.* (2015), espécies que apresentam esterilidade híbrida assimétrica, como é o caso de *D. arizonae* e *D. mojavenensis*, são candidatas ideais em estudos de isolamento

reprodutivo, visto que é possível comparar a expressão de sequências genéticas de híbridos estéreis com férteis e seus respectivos parentais.

2 OBJETIVOS

Esta tese foi dividida em três partes principais, sendo que, a primeira delas teve por objetivo estimar os parâmetros da história de vida de híbridos interespecíficos entre *D. arizonae* e quatro subespécies de *D. mojavensis*, com ênfase em componentes relacionados ao isolamento pós-zigótico, com viabilidade, motilidade espermática e atrofia de gônadas. A segunda parte buscou analisar o impacto do fenômeno de hibridização interespecífica na expressão gênica em tecidos reprodutivos de machos e fêmeas híbridas provenientes de cruzamentos entre *D. arizonae* e duas subespécies de *D. mojavensis*. Por fim, na terceira parte, foi investigada a extensão da desregulação de Tes em ovários e testículos de híbridos, e sua associação com os mecanismos de regulação pós-transcricional, por meio da via de piRNAs.

3 CAPÍTULO 1

Life-history traits in hybrids of *Drosophila arizonae* and *Drosophila mojavensis* subspecies

Cecilia Artico Banho^{1,2}, Felipe Santa Rosa do Amaral¹, Cristina Vieira², Claudia Marcia Aparecida Carareto¹

¹UNESP - São Paulo State University, Department of Biology, São José do Rio Preto, São Paulo State (SP), Brazil

² Université de Lyon, Université Lyon 1, CNRS, Laboratoire de Biométrie et Biologie Evolutive UMR 5558, F-69622 Villeurbanne, France

ABSTRACT

Drosophila arizonae and *D. mojavensis* are recently diverged (~1.5 million years ago (m.y.a)) species widely used in speciation studies due to their ability to produce hybrids in the laboratory; however, no evidence of introgression has been found in nature, despite favourable conditions for the hybridization of the species due to their overlapping habitats. The prezygotic isolation of these species has been well characterized, but few studies have demonstrated the consequences of interspecific hybridization on their postzygotic isolation. To verify the occurrence and degree of hybrid incompatibility in *D. arizonae* and *D. mojavensis*, we evaluated the life-history parameters of their inter- and intraspecific offspring. Phenotypic analyses showed that all hybrids presented a decrease in viability compared to intraspecific offspring. Sperm motility analyses showed that all hybrid males of one cross direction presented mobile sperm, while in reciprocal crosses, 75% of the hybrids presented immobile sperm. The fertility analyses showed 100% of fertile hybrid females, which had no gonad dysgenesis, however, only 25% of the male hybrids with mobile sperm were fertile. These findings suggest the presence of polymorphic factors dependent on the *D. mojavensis* subspecies that can influence the fitness of F₁ hybrids and that several genetic mechanisms can influence the reproductive isolation between these sibling species.

Keywords: male sterility, repleta group, reproductive isolation

INTRODUCTION

Drosophila mojavensis and *D. arizonae* are recently diverged (approximately 1.5 m.y.a) sibling species (SANCHEZ-FLORES *et al.*, 2016) that belong to the *mulleri* complex of the *repleta* group. The area of *D. mojavensis* distribution extends from Arizona to southern California, Sonora, Sinaloa, and the peninsula of Baja California, while *D. arizonae* has a more diffuse population distribution in Central America, with populations reported as far south as Guatemala, through Mexico and in the USA, where *D. arizonae* can be found in California and Arizona. Although widely distributed, *D. arizonae* does not have populations differentiated enough to be classified as subspecies. On the other hand, *D. mojavensis* is classified into four subspecies based on morphological features (METTLER, 1963), allozymes (ADH) (MATZKIN; EANES, 2003), genetic population differences (ROSS; MARKOW, 2006), chromosomal inversions and the degree of reproductive isolation (WARSEMAN; KOEPFER, 1977; RUIZ *et al.* 1990; REED; MARKOW, 2004). *Drosophila arizonae* and *D. mojavensis* are sympatric populations in an area that includes northern Sinaloa and the whole of Sonora, where both species use a specific columnar cactus (*Stenocereus sp.*) as a host; in contrast, in the other areas, the species constitute allopatric populations (MASSIE; MARKOW, 2005; JENNINGS; ETGES, 2009). The four geographically isolated subspecies of *D. mojavensis* are found in the Mojave Desert (*D. m. mojavensis*), Baja California peninsula (*D. m. baja*), Sonoran Desert (*D. m. sonorensis*) and Catalina Island on the coast of southern California (*D. m. wrigleyi*). In each of these regions, *D. mojavensis* uses a specific host cactus for feeding and breeding sites, with no evidence of gene flow, and thus comprises well-structured populations (KNOWLES; MARKOW, 2001; PITNICK *et al.*, 2003; REED; MARKOW, 2004; MASSIE; MARKOW, 2005; REED *et al.*, 2007; ETGES *et al.*, 2010).

These sibling species are widely used in speciation studies due to their ability to produce hybrids in the laboratory; however, no evidence of introgression has been found in nature, despite the favourable ecological conditions for hybridization between *D. arizonae* and *D. mojavensis*, mainly because of their sympatric populations (JENNINGS; ETGES, 2009). As they are incipient species, some reports have shown incomplete and asymmetric postzygotic isolation in their interspecific hybrids. It was demonstrated that in crosses between *D. arizonae* mothers and *D. mojavensis* fathers, the male offspring are often sterile, while in reciprocal crosses, the

offspring are fertile, depending on the source of the *D. mojavenensis* populations (RUIZ *et al.*, 1990; REED; MARKOW 2004; CARNELOSSI *et al.*, 2014).

It is important to emphasize that despite several studies analysing the degree of prezygotic and postmating prezygotic reproductive isolation in *D. arizonae* and *D. mojavenensis*, few studies have evaluated the levels of postzygotic isolation in hybrids of these species, particularly between the four *D. mojavenensis* subspecies and *D. arizonae*, probably because of the difficulty in obtaining a large number of offspring due to the well-characterized mechanisms of prezygotic isolation between them (WARSEMAN; KOEPFER, 1977; RUIZ *et al.*, 1990).

Therefore, in this study, we aimed to evaluate components of the life-history traits in hybrids from reciprocal crosses between *D. arizonae* and four subspecies of *D. mojavenensis*. Despite obtaining a low offspring number, we were able to record the productivity, viability, sperm motility, fertility and gonadal dygenesis in descendants of all intra- and interespecific crosses, which allows us to better understand the complexity of the mechanisms involved in the postzygotic reproductive isolation process between these sibling species.

MATERIAL AND METHODS

Stock flies

The studied populations of *D. mojavenensis* and *D. arizonae* are distributed in the southwest of the United States of America and in Mexico. Four representative strains of *D. mojavenensis* subspecies, which were obtained in the UC *San Diego Drosophila Stock Center* [*D. m. mojavenensis*: Anza (01), from Anza Borrego Desert, California, USA, Stock Center n°: 15081-1352.01; *D. m. wrigleyi*: CI (22), from Catalina Island, California, USA, Stock Center n°: 915081-1352.22; *D. m. baja*: BC (20), from Cape Region, Santiago, Baja California Sur, Mexico, Stock Center n°: 15081-1352.20 and *D. m. sonorensis*: AG (26), from Agiabampo Bay, Sonora, Mexico, Stock Center n°: 15081-1352.26] and one strain of *D. arizonae* [HI (17), from Metztitlan, Hidalgo, Mexico, Stock Center n°: 15081-1271.17] allopatrically related to all *D.*

mojavensis lines, were used (Figure 1).



Figure 1. Geographic distribution of the four subspecies of *D. mojavensis* and *D. arizonae* analysed. Source: prepared by the author.

Fecundity and viability analyses

Virgin males and females of each strain were separated by sex 10 hours after eclosion and stored separately in yeasted cactus-banana vials, with 10 flies per vial, until they were sexually mature (9 days of age); then, they were used in mating experiments. Mature virgin females from each *D. mojavensis* line were mated to males of *D. arizonae*, and reciprocal crosses were performed. Hybrids from *D. mojavensis* mothers are referred to as Hybrid A, while hybrids from *D. arizonae* mothers are referred to as Hybrid B (Figure 2). As a control, *D. mojavensis* and *D. arizonae* females were mated to males of their own strain.

The interspecific and control crosses were carried out in five replicates, with 10 couples in each vial. The matings were performed for 72 h under constant temperature (23°) and light/dark (10/14 h) conditions. The crosses were carried out in a group of flies because it has been shown that these crosses are more successful than crosses with single pairs (REED; MARKOW, 2004). After 72 hours, males from all crosses were discarded, and females (in pairs) were transferred to new fresh vials to lay eggs. This process was repeated five times every 48 h, so the females laid eggs for 10 days (Figure 2). Immediately after the transfers, the eggs laid were recorded under a stereomicroscope, and after eclosion (~19 days after the crosses), the number of imagoes was verified once a week for four weeks. From the number of eggs and

adults, the average fecundity of the females (number of eggs/female) and offspring viability (adults/eggs x 100) were calculated.

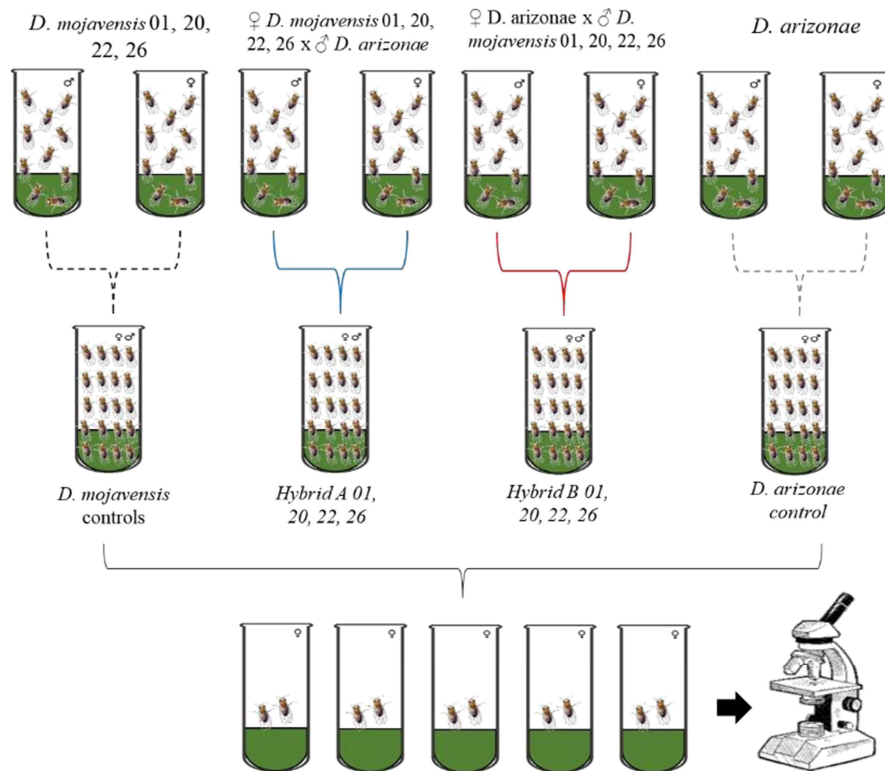


Figure 2. Scheme representing intra- and interspecific crosses of four subspecies of *D. mojavensis* and *D. arizonae*. Source: prepared by the author.

Productivity analyses

New interspecific and intraspecific crosses were carried out in five replicates under the same conditions previously used. In each cross, 10 couples were mated for 7 days, and then the males were discarded. The females were counted (to verify possible deaths of females and to avoid bias in the index of average productivity per female) and transferred to fresh yeasted cactus-banana vials. In all replicates, the females were transferred every four days for a total of five transfers. The adult number in each vial was recorded once a week for four weeks. The individual productivity in each tube was estimated as the ratio between the number of imagoes and the number of females from each vial.

Sterility analyses

Two traits that can result in sterility were studied: gonadal dysgenesis and sperm motility. For these analyses, new interspecific and control crosses were performed in yeasted cactus-banana vials. To obtain as many hybrids as possible, three-day-old virgin flies were used because in previous tests, we noticed an increased production of hybrids when the two species were kept together before they reached sexual maturity. All crosses were performed in five replicates under the same temperature and light/dark conditions for 12 days. After that, the parents were discarded, and the imagoes were separated by sex daily. The descendants were maintained in yeasted food vials until they reached 10 days of age (sexually mature). Then, they were used in gonadal dysgenesis and sperm motility experiments.

Gonadal dysgenesis, a morphological component of hybrid dysgenesis, is characterized by several degrees of abnormalities in the reproductive organs (ALMEIDA; CARARETO, 2002). To verify this component, we analysed 10 F1 males and females from each reciprocal cross, as well as the control flies. The reproductive organs were dissected in phosphate-buffered saline (PBS) and then checked and photographed under a stereomicroscope. Statistical analyses were not performed because all flies presented normal testes and ovaries.

Sperm motility analyses were carried out in 20 F1 male testes and seminal vesicles of each control and interspecific cross, according to Reed *et al.* (2008). No statistical analyses were performed because for each cross, all males presented the same phenotype or presented motility or immobility, which differs from the results verified by Reed *et al.* (2004) and Reed *et al.* (2008). However, following the method described in Reed *et al.* (2008), we noticed differences among the degrees of motility in hybrids from some specific crosses.

Fertility analyses

Three-day-old female and male hybrids from reciprocal interspecific crosses were backcrossed with their respective parents, *D. arizonae* and *D. mojavenensis* (from their respective

subspecies). Crosses were performed with five couples per replicate in five replicates by cross direction. To ensure that the absence of offspring was due to possible prezygotic, postmating prezygotic or postzygotic isolation mechanisms, we increased the crossing time and allowed the couples to mate for 15 days. After that, all parents were discarded, and fertility was evaluated based on the presence or absence of offspring, as reported by Canelossi *et al.* (2014). F1 x F1 crosses were also performed using offspring of each interspecific cross under the same conditions as the backcrosses. To certify that tubes containing only eggs would not produce offspring, they were maintained for 20 days after parent removal and then discarded.

Hybrid Status

To test the hybrid status in offspring of each interspecific cross, amplification of the ITS-1 (internal transcribed spacer 1) region was performed. For this analysis, DNA from five random individuals of each replicate of the interspecific crosses was extracted, and the ITS-1 sequence was amplified using a forward primer that hybridizes to the 3'-end of the 18S rDNA gene and a reverse primer that hybridizes at the beginning of the 5.8S rDNA gene [NCBI Reference Sequence: EU306666.1] (BAFFI; CERON, 2002). It was expected that male and female hybrids would present two fragments of 500 and 550 bp corresponding to the amplified ITS-1 region of the rDNA of their parents *D. arizonae* and *D. mojavenensis*, respectively.

Statistical analyses

Statistical analyses were performed for average fecundity by female, average productivity by female and viability for each replicate of intraspecific and interspecific crosses by using R software R v. 3.6.1 (The R Core Team). Normality and variance tests (Shapiro-Wilk and Levene's test, respectively) were carried out, and when we obtained significant p-values (non-normal distribution), a nonparametric Kruskal-Wallis test was performed. Then, a post hoc Wilcoxon test was performed to determine significant differences between the treatments. For

results with no significant p-values for normality and variance tests, one-way ANOVA was performed using Tukey's post hoc test.

RESULTS

Fecundity and viability analyses

Fecundity is a phenotypic parameter intrinsic to each species and can be related to postmating prezygotic isolation mechanisms. Table 1 shows the percentage of females able to lay eggs from homogamic and heterogamic matings. Notably, *D. m. mojavenensis* and *D. m. sonorensis* control crosses presented the lowest number of eggs compared to their interspecific crosses (Table 1). However, all of the other interspecific crosses presented few egg-laying females compared to their respective intraspecific crosses, independent of cross direction. Accordingly, it was verified that *D. m. mojavenensis* and *D. m. sonorensis* presented the lowest average fecundity (Figure 3A).

Table 1. Percentage of females that laid eggs in intraspecific and interspecific crosses for the 50 females analysed.

Percentage of females which laid eggs					
Crossings	♂ <i>D. arizonae</i>	♂ <i>D. m. mojavenensis</i>	♂ <i>D. m. baja</i>	♂ <i>D. m. wrigleyi</i>	♂ <i>D. m. sonorensis</i>
♀ <i>D. m. mojavenensis</i>	80	36	-	-	-
♀ <i>D. m. baja</i>	76	-	96	-	-
♀ <i>D. m. wrigleyi</i>	48	-	-	96	-
♀ <i>D. m. sonorensis</i>	0	-	-	-	28
♀ <i>D. arizonae</i>	92	80	32	84	52

Source: prepared by the author.

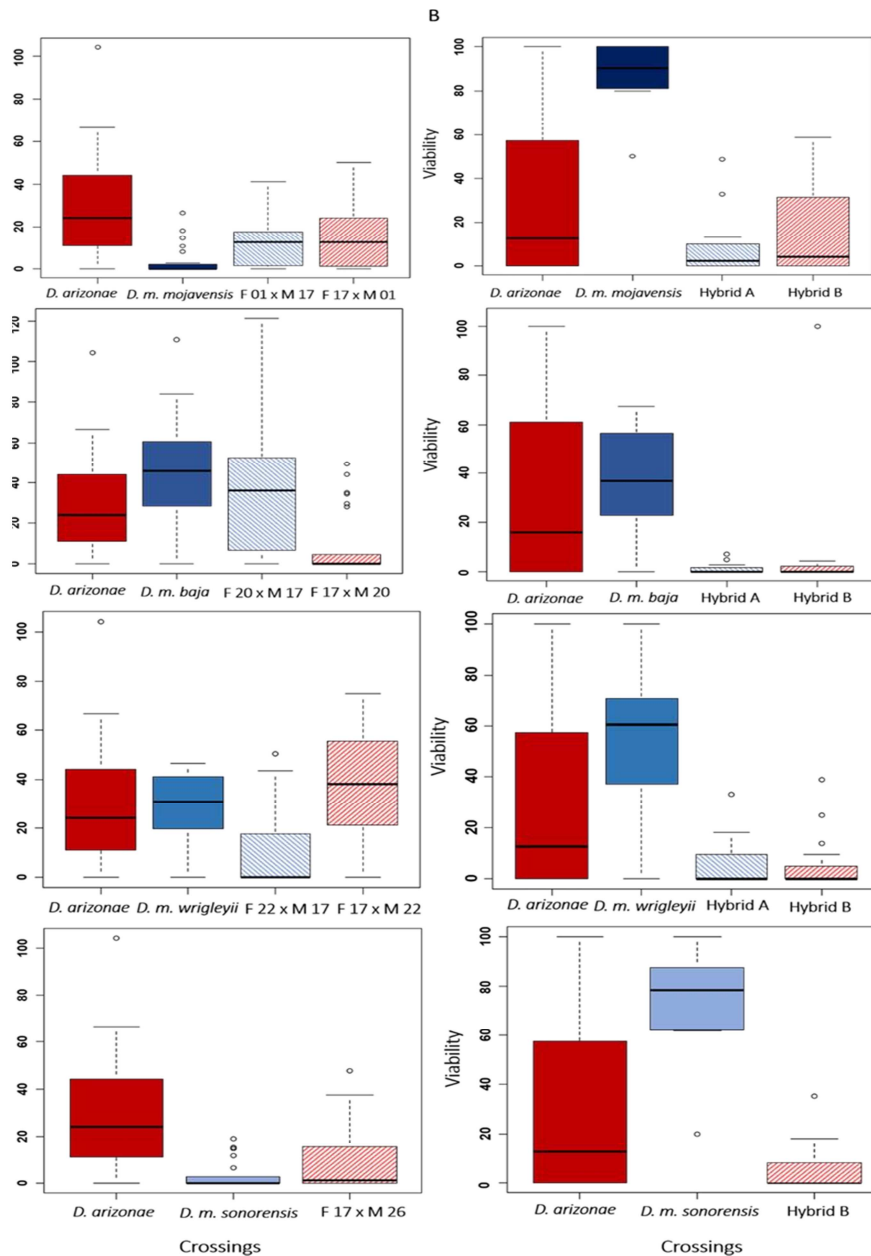


Figure 3. Fecundity and viability of control and interspecific crosses. A) Boxplot showing the average fecundity by females of *D. m. mojavenensis*, *D. m. baja*, *D. m. wrightleyi*, *D. m. sonorensis*, *D. arizonae* and their reciprocal hybrids. B) Boxplot representing the viability of descendants from *D. m. mojavenensis*, *D. m. baja*, *D. m. wrightleyi*, *D. m. sonorensis*, and *D. arizonae* control crosses and their reciprocal hybrids. * outliers. Source: prepared by the author.

Figure 3 shows that among the interspecific crosses, the highest average fecundity was seen in *D. m. baja* females mated with *D. arizonae* males (48.5 eggs/female each 48 h). The fecundity was widely variable between the subspecies of *D. mojavenensis* and *D. arizonae*. The average differences of interspecific compared to control crosses were significant in all groups (Supplementary Table S1). The paired comparisons showed that the fecundity of F01 x M17; F17 x M01; F17 x M20 and F22 x M17 compared with their respective control crosses, as well as F17 x M26 in relation to the *D. arizonae* control cross, were significantly different (Supplementary Table S2). Interestingly, the fecundity in the interspecific crosses did not seem to be related to the fecundity of their respective controls. In other words, we expected that

interspecific crosses using *D. mojavensis* mothers would show a similar fecundity as their control, with the same expectation for interspecific crosses with *D. arizonae* mothers and control crosses, because fecundity is an intrinsic characteristic of the specific strains; therefore, we used the same lines to perform the crosses. However, this was not observed in our experiments, evidencing that other factors may be behind this characteristic.

The averages of viability showed significant differences in all groups of comparisons (Supplementary Table S3). Interestingly, although *D. m. mojavensis* and *D. m. sonorensis* exhibited the lowest fecundity, they had the highest viability (86.8% and 70.9%, respectively). The other control lines, *D. m. baja*, *D. m. wrigleyi* and *D. arizonae*, showed large variations in the average viability of 38.67%, 51.93% and 29.58%, respectively. In all interspecific crosses, a decrease in viability compared to the intraspecific crosses was verified (Supplementary Table S4). The average hybrid viability ranged from 1.16 to 14.2%. The average of viability in hybrids was, at least, seven times as small as the average viability of control crosses. Among the interspecific crosses, Hybrid A 20 presented the lowest viability (Figure 3B, Table 2). This is an interesting observation, since the heterospecific crosses between *D. m. baja* females and *D. arizonae* males presented the highest average fecundity. On the other hand, Hybrid B 01 presented the highest viability compared to other interspecific crosses (Figure 3B, Table 2).

In the paired comparisons, not significant differences were found in all reciprocal hybrids from the crosses between the four subspecies of *D. mojavensis* and *D. arizonae*. This result indicates there are similar deleterious effects in the hybrids regardless of cross direction. We were not able to analyse the fecundity and viability of crosses between *D. m. sonorensis* (26) females from the Sonoran Desert mated with *D. arizonae* males because there was no egg production.

Table 2. Avarage fecundity in intraspecific and interspecific crosses and avarage viability of purebread and hybrid offspring.

Crosses	Offspring	Avarage fecundity (egg/female)	Avarage viability (%)
♀ <i>D. arizonae</i> x ♂ <i>D. arizonae</i> (F17xM17)	<i>D. arizonae</i>	29.56	29.58
♀ <i>D. m. mojavenensis</i> x ♂ <i>D. m. mojavenensis</i> (F01xM01)	<i>D. m. mojavenensis</i>	3.36	86.8
♀ <i>D. m. wrigleyi</i> x ♂ <i>D. m. wrigleyi</i> (F22xM22)	<i>D. m. wrigleyi</i>	28.84	51.93
♀ <i>D. m. baja</i> x ♂ <i>D. m. baja</i> (F20xM20)	<i>D. m. baja</i>	45.84	38.67
♀ <i>D. m. sonorensis</i> x ♂ <i>D. m. sonorensis</i> (F26xM26)	<i>D. m. sonorensis</i>	3.34	70.9
♀ <i>D. m. mojavenensis</i> x ♂ <i>D. arizonae</i> (F01xM17)	Hybrid A 01	12.7	7.5
♀ <i>D. arizonae</i> x ♂ <i>D. m. mojavenensis</i> (F17xM01)	Hybrid B 01	14.7	14.2
♀ <i>D. m. wrigleyi</i> x ♂ <i>D. arizonae</i> (F22xM17)	Hybrid A 22	10.64	3.85
♀ <i>D. arizonae</i> x ♂ <i>D. m. wrigleyi</i> (F17xM22)	Hybrid B 22	35.42	4.74
♀ <i>D. m. baja</i> x ♂ <i>D. arizonae</i> (F20xM17)	Hybrid A 20	36.98	1.16
♀ <i>D. arizonae</i> x ♂ <i>D. m. baja</i> (F17xM20)	Hybrid B 20	9.04	13.02
♀ <i>D. m. sonorensis</i> x ♂ <i>D. arizonae</i> (F26xM17)	Hybrid A 26	No egg production	-
♀ <i>D. arizonae</i> x ♂ <i>D. m. sonorensis</i> (F17xM26)	Hybrid B 26	10.14	5.9

Source: prepared by the author.

Productivity analyses

All interspecific crosses produced fewer offspring than parental crosses (Figure 4). Among the control crosses, *D. m. mojavenensis* and *D. arizonae* presented the highest productivity average (Figure 4A), while *D. m. baja* presented the lowest productivity (Figure 4B). Among interspecific crosses, the highest observed productivity was in Hybrid A 26, from F26 x M17 crosse (Figure 4D). On the other hand, the lowest productivity was seen in Hybrid B 20 (F17 x M20) (Figure 4B). In agreement, statistical tests showed significant differences between the productivity of almost all interespecific crosses compared with intraspecific crosses (Table S6).

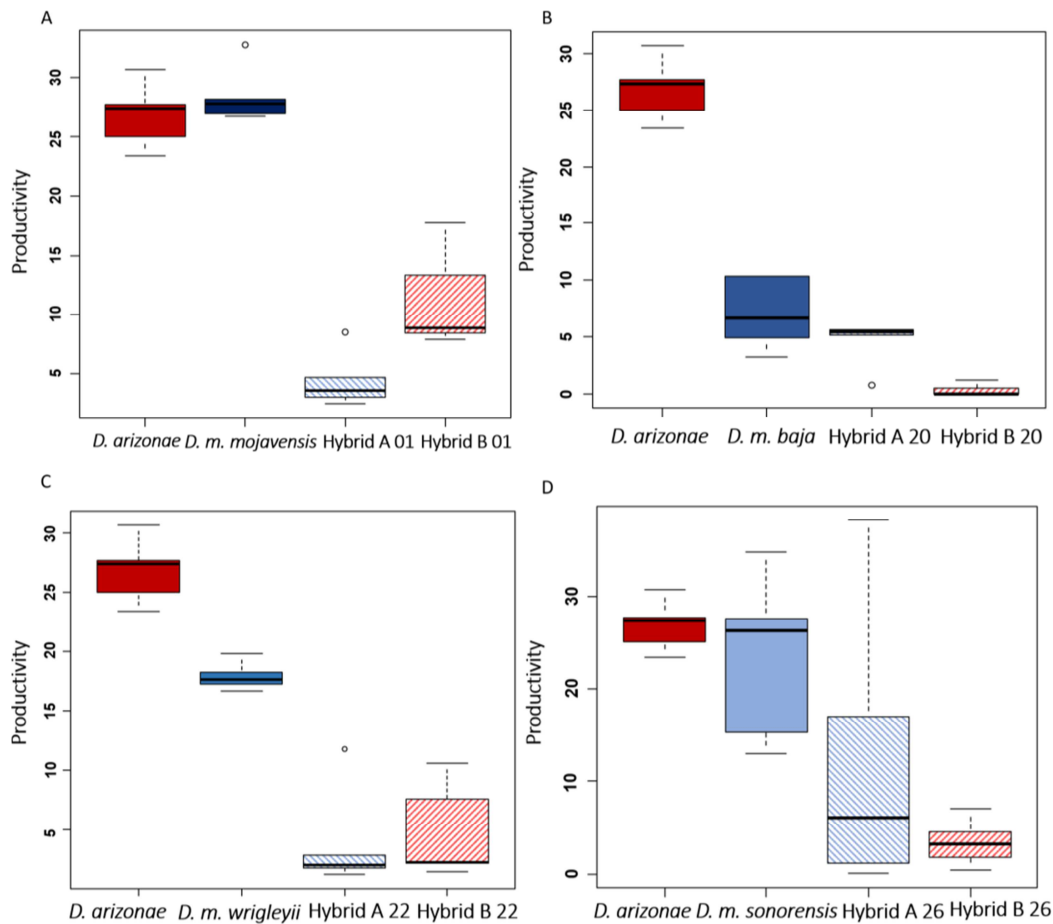


Figure 4. Boxplots showing the average productivity of control and interspecific crosses. A) productivity in *D. arizonae* and *D. m. mojavenensis* control crosses and their reciprocal hybrids; B) productivity in *D. arizonae* and *D. m. baja* control crosses and their reciprocal hybrids; C) productivity in *D. arizonae* and *D. m. wrigleyi* control crosses and their reciprocal hybrids; D) productivity in *D. arizonae* and *D. m. sonorensis* control crosses and their reciprocal hybrids. * outliers. Source: prepared by the author.

Sterility analyses

For sterility analyses new crosses were performed using 3-days-old flies in order to increase hybrid production. With this methodology we were able to recovery hybrids from crosses between the four subspecies of *D. mojavenensis* and *D. arizonae*, in both directions.

No evidence of gonadal dysgenesis was found in males and females. All ovaries and ovarioles, as well as the testes and seminal vesicles, were completely formed in all offspring of *D. m. sonorensis* (26), *D. m. mojavenensis* (01), *D. m. baja* (20), *D. m. wrigleyi* (22) and *D. arizonae* (17) intraspecific crosses, as well as in their reciprocal interspecific crosses (Figure 5). Differences in the size of the gonads were verified and may be related to specific strain polymorphisms. In each cross direction analysed, all males presented the same phenotype; in other words, we did not find phenotypic differences within each cross.

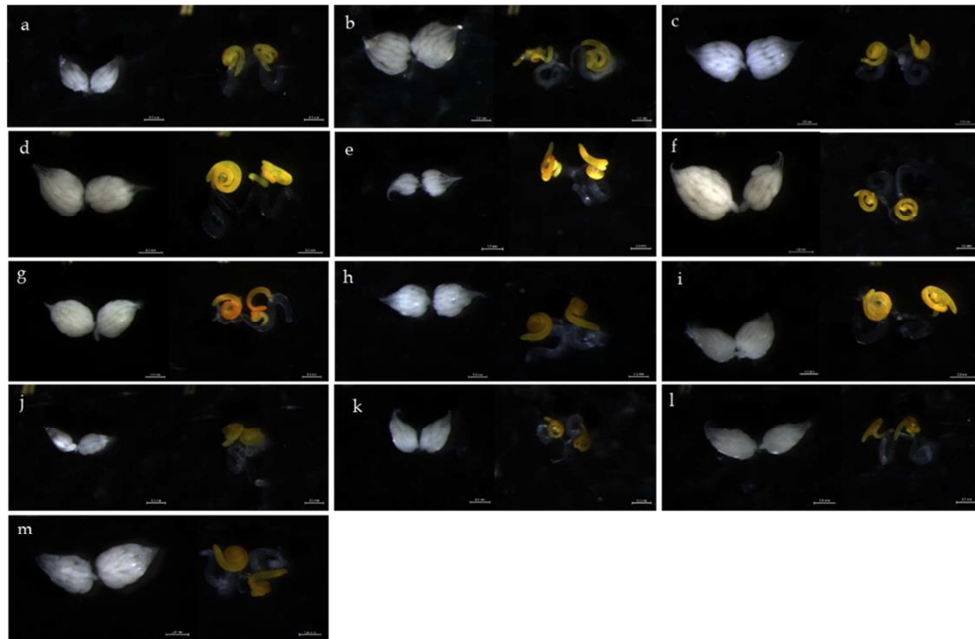


Figure 5. Morphology of ovaries and testes in control and interspecific crosses a) Ovaries and testes of *D. m. sonorensis*; b) ovaries and testes of Hybrid A 26; c) ovaries and testes of Hybrid B 26; d) ovaries and testes of *D. m. mojavenensis*; e) normal ovaries and testes of Hybrid A 01; f) normal ovaries and testes of F Hybrid B 01; g) ovaries and testes of *D. m. baja*; h) ovaries and testes of Hybrid A 20; i) ovaries and testes of Hybrid AB20; j) ovaries and testes of *D. m. wrigleyi*; k) ovaries and testes of F Hybrid A 22; l) normal ovaries and testes of F Hybrid B 22; m) normal ovaries and testes of *D. arizonae*. Bar: 5 mm. Source: prepared by the author.

Sperm motility analyses have shown that all males from control crosses presented the maximum level of motility, constituting the wild phenotype, following the zero-to-six scale (REED *et al.*, 2008). On the other hand, hybrids from *D. mojavenensis* females presented lower sperm motility levels. In general, in Hybrid B from *D. arizonae* mothers, the sperm were immobile (Table 3).

Table 3. Sperm motility index based on the zero-to-six scale of sperm motility in *Drosophila* from Reed *et al.* (2008).

Control or interspecific crosses	Mean of sperm motility				
	♂ <i>D. arizonae</i>	♂ <i>D. m. mojavenensis</i>	♂ <i>D. m. baja</i>	♂ <i>D. m. wrigleyi</i>	♂ <i>D. m. sonorensis</i>
♀ <i>D. m. Mojavenensis</i>	4.2	6	-	-	-
♀ <i>D. m. Baja</i>	3.7	-	5.6	-	-
♀ <i>D. m. Wrigleyi</i>	3.2	-	-	6	-
♀ <i>D. m. Sonorensis</i>	3.8	-	-	-	6
♀ <i>D. arizonae</i>	6	0	0	0	5.3

Source: prepared by the author.

The only exception was Hybrid B 26, from crosses between ♂ *D. m. sonorensis* (26) x ♀ *D. arizonae* (17). They presented high sperm motility similar to the wild phenotype. In addition, their motility level was higher than that of their reciprocal hybrids (Hybrid A 26). It is important to highlight that *D. m. sonorensis* is a sympatric population. In addition, among the hybrids presenting mobile sperm, we can found great variation in the motility levels. This evidence indicates the presence of some specific polymorphisms that may contribute to the evolution of postzygotic isolation mechanisms in each line of *D. mojavensis*.

Fertility analyses

Hybrid sterility was analysed by backcrosses and F1 x F1 crosses for all male and female offspring from interspecific crosses. The results show that all hybrid females were fertile, regardless of the cross direction.

In contrast, we observed that males from crosses between *D. m. mojavensis* (01) and *D.m. wrigleyi* with *D. arizonae* were sterile in both directions (Hybrids A 01 and 22; Hybrids B 01 and 22), although they presented mobile sperm (Table 2). On the other hand, Hybrid A 20, from crosses between *D. m. baja* females and *D. arizonae* males, were fertile despite low sperm motility since their F1 x F1 crosses produced offspring; however, in backcrosses, no offspring were verified. Nevertheless, Hybrid B 20 (from ♀ *D. arizonae* x ♂ *D. m. baja*) were sterile, which was expected, as they presented no sperm motility (Table 3). Interspecific crosses between *D.m. sonorensis* and *D. arizonae* were the only crosses that presented male and female hybrids, regardless of cross direction (Supplementary table S7).

DISCUSSION

To better understand the speciation process, it is important to analyse and identify the reproductive barriers that contribute to reproductive isolation. Among them, postzygotic reproductive barriers can be responsible for reducing hybrid fitness compared to the parental

lines. According to Coyne and Orr (2004), postzygotic isolation is split into two categories: intrinsic and extrinsic. Intrinsic barriers are those that can cause inviability or sterility, while extrinsic barriers are those that allow normal hybrid development but can present problems for attracting mates (behavioural sterility). According to Orr (2009), differences in fitness caused by stress conditions, such as hybridization, can arise in any stage of the life cycle and affect the fitness components (e.g., viability, mating success, and fecundity) contributing to differences in total fitness among individuals.

To identify some factors acting on postzygotic mechanisms, we characterized the life-history traits of offspring from interspecific crosses between *D. arizonae* and four subspecies of *D. mojavensis*, such as fecundity, productivity, viability, hybrid dysgenesis, sperm motility and fertility.

Fecundity and viability analyses

The low egg production observed in the *D. m. mojavensis* and *D. m. sonorensis* crosses was an unexpected result, as they were control crosses. This low egg production may have occurred because *D. mojavensis* needs to mate to lay eggs, a necessity possibly related to male investment in egg production (KELLEHER; MARKOW, 2007). It has been proposed that because *D. mojavensis* females live in a harsh environment often with limited resources, they need to incorporate male-derived molecules into somatic tissues and oocytes (MARKOW; ANKNEY, 1984). Therefore, in this specific case, *D. m. mojavensis* and *D. m. sonorensis* control crosses may not have mated, since no eggs were seen. Regarding interspecific crosses, the small number of females laying eggs might have been related to the strong prezygotic isolation between *D. mojavensis* and *D. arizonae*, which prevented mating for most females. Accordingly, it has been shown that 50% of the females from interspecific crosses between three subspecies of *D. mojavensis* and *D. arizonae* did not lay eggs (KELLEHER; MARKOW, 2007).

According to Massie and Markow (2005), *D. arizonae* females rarely can cross with *D.*

mojavensis males, regardless of the origin of the population, while *D. mojavensis* females from sympatric populations (Sonoran Desert) exhibit stronger prezygotic isolation related to *D. arizonae* males than *D. mojavensis* females from allopatric regions. This pattern of strong prezygotic isolation influencing fecundity was verified, mainly in crosses with female *D. m. sonorensis* and *D. arizonae* males derived from sympatric populations, corroborating the Wasserman and Koepfer (1977) observations and the reinforcement of prezygotic reproductive isolation. In addition, Jennings and Etges (2009) found that factors related to the maintenance of flies in the laboratory, such as the experimental crossing conditions, can increase the prezygotic isolation between *D. arizonae* and *D. mojavensis*, and this may be a factor interfering in the frequency of mating in our crosses.

However, prezygotic isolation cannot be the only factor influencing oviposition in interspecific crosses. Postmating prezygotic isolation (PZPM) has been extensively studied, and evidence shows that it can influence oviposition levels, mainly in heterospecific matings (PATTERSON, 1946; MARKOW; HOCUTT, 1998; KNOWLES; MARKOW, 2001; BONO *et al.*, 2011). According to Markow and Hocutt (1998), females from the *repleta* group, such as *D. arizonae* and *D. mojavensis*, present an insemination reaction (PATTERSON, 1946; ALONSO-PIMENTEL *et al.*, 1994), which consists of the enlargement of the vagina produced by the male ejaculate in intraspecific crossings, forming an opaque mass that prevents remating behaviour temporarily (PATTERSON, 1946). *D. arizonae* and *D. mojavensis* normally present a mating system characterized by frequent female remating relative to *D. melanogaster*. This can be linked to the fact that females use nutrients from the male ejaculate, and males transfer less material at each copulation to take advantage of the increased mating opportunities in their population (MARKOW; ANKEY 1984; ALONSO-PIMENTEL *et al.*, 1994; BONO *et al.*, 2011; BONO *et al.*, 2015). On the other hand, in heterospecific crosses, the insemination reaction can be stronger, and this mass can last days in the uterus of flies, preventing regular remating behaviour and oviposition, and can even sterilize females (KELLEHER *et al.*, 2007; BONO *et*

al., 2011, BONO *et al.*, 2015).

According to Bono *et al.* (2011), heterospecifically mated *D. mojavenensis* females exhibit perturbations in a number of processes occurring within their reproductive tract that result in a high incidence of failed fertilization, a reduced rate of oviposition, and ultimately the production of few hybrid offspring, as verified in our crosses. These problems are associated with deficiencies in the heterospecifically mated female's storage and retention of sperm and in the degradation of the insemination reaction (BONO *et al.*, 2011; PATTERSON 1946). Additionally, Bono *et al.* (2011) found that PMPZ isolation was strong in all crosses involving *D. mojavenensis* females, suggesting that this barrier was the earliest to evolve. However, in our results, the average fecundity in interspecific crosses did not seem to be stronger in *D. mojavenensis* females than in *D. arizonae* females.

The reduction in hybrid viability found in this study agrees with the results of Kelleher and Markow (2007), who found that viability decreased from 60-70% in homospecific crosses to 1-4% in heterospecific crosses. Additionally, in interspecific crosses between *D. virilis* and *D. lummei*, species from the *repleta* group, it was verified that the low viability of hybrids was due to early embryo lethality, which differed with cross direction, since in *D. virilis* female x *D. lummei* male crosses, progeny production was reduced by 62%, while in the reciprocal cross, progeny production was reduced by 50% (CASTILLO; MOYLE, 2019). Therefore, the low hybrid survival in our interspecific hybrids indicates that there are several levels of isolation between the parental species. The results reinforce that despite the evident prezygotic isolation between *D. mojavenensis* and *D. arizonae*, there are several barriers to postzygotic isolation, decreasing the fitness of reciprocal hybrids and influencing the initial steps of the speciation process.

Productivity analyses

Our results showed a decrease in productivity in all interspecific crosses, in agreement

with Kelleher and Markow (2007) and Ruiz *et al.* (1990). This fitness component can be influenced by prezygotic, postmating-prezygotic and postzygotic isolation, such as fecundity and viability. Regarding prezygotic isolation, the different subspecies of *D. mojavensis* present different degrees of isolation related to *D. arizonae*. In general, *D. m. sonorensis* is almost completely isolated from *D. arizonae*, followed by *D. m. baja* and by the least isolated subspecies *D. m. mojavensis* (WASSERMAN; KOEPFER, 1977; MASSIE; MARKOW, 2005). In addition, postmating prezygotic isolation can influence productivity; however, it does not seem to be related to maternal origin. Therefore, we consider postzygotic isolation to be the reproductive barrier that has the greatest influence on the average productivity.

Sterility analyses

Hybrid dysgenesis corresponds to abnormal phenotypes in F1 hybrid gonads, which can influence the speciation process (FONTDEVILA, 2005; MICHALAK, 2009). Gonadal dysgenesis is a rare phenomenon in purebred lines under optimal developmental conditions, such as constant temperature and nutritional availability. However, this phenomenon can occur due to germline development problems, heat shock, transposable element mobilization, and genomic shock as hybridization. In the analysis of male and female gonads from four subspecies of *D. mojavensis*, *D. arizonae* and their reciprocal hybrids, no evidence of gonadal dysgenesis was observed, consistent with the results of Reed and Markow (2004) for hybrids of *D. m. baja*, *D. m. sonorensis* and *D. m. wrigleyi* mated with *D. arizonae*. However, our results showed different ovary sizes, which may be related to the strain characteristics because the number of ovarioles can vary within and between species (MARKOW; O'GRADY, 2007). Because each ovariole produces eggs autonomously, the number of ovarioles is an important determinant of fecundity, thereby influencing evolutionary fitness (ORR, 2009).

In species of the *Drosophila* genus, where the male is the heterogametic sex, sterile hybrid males have mostly atrophied seminal vesicles with normal testes morphology, although a

small proportion of hybrids might also show whole or partial testes atrophy (Ferguson *et al.*, 2013). It was shown the testes and seminal vesicles of *D. mojavenensis* males (11 days old) were longer than those of *D. arizonae*, which was expected, since the sperm of the former are slightly longer than those of the latter (HARDY *et al.*, 2011), and their hybrids presented different testes and seminal vesicle sizes compared to both parents. In our study, all males from intraspecific and interspecific crosses presented normal testes and seminal vesicles, even males that presented sterility confirmed by sperm motility or fertility analyses. However, we could not affirm that the parents and hybrids presented no differences in gonad size, as no measures were performed.

In our analyses of sperm motility and fertility, all female hybrids (homogametic sex) were fertile, regardless of the cross direction. On the other hand, almost all male hybrids were sterile, depending on the cross direction and the *D. mojavenensis* subspecies, in agreement with Haldane's (1922) rule and findings of Turissini *et al.* (2018), who showed that in interspecific crosses of recently diverged *Drosophila* species, the hybrid males usually are sterile while the females remain fertile. We also observed that all hybrids from *D. mojavenensis* females and *D. arizonae* males had mobile sperm, although the sperm motility level was lower in hybrids than in their respective controls and was variable among the hybrids from different *D. mojavenensis* subspecies. Different levels of motility in hybrids might be related to intraspecific polymorphisms in the different populations of *D. mojavenensis*, as also observed by Reed and Markow (2004). On the other hand, almost all males from intercrosses of *D. arizonae* females presented immobile sperm. Although the factors responsible for sperm immobility in these hybrids are not completely known, some studies have reported that it could be related to incompatibilities between the *D. mojavenensis* Y chromosome and *D. arizonae* IV chromosome (ZOUROS *et al.*, 1981, 1988; PANTAZIDIS, 1988).

Among hybrids from *D. arizonae* mothers, we found one exception regarding sperm immotility. Hybrids from *D. arizonae* females and *D. m. sonorensis* males presented high sperm motility, which was approximately equal to the motility of males of pure lineages. These hybrids

are from crosses between *D. arizonae* females and males from the Sonora Desert, one of the sympatric populations analysed. From this result, we can verify that although the prezygotic isolation between these species is very strong (MASSIE; MARKOW, 2005), when this barrier is overcome, several offspring are produced, and they present lower postzygotic isolation than interspecific offspring from allopatric populations.

Fertility analyses

Sterility is a complex trait that can have multiple influences and be controlled genetically at different phenotypic levels (REED; MARKOW, 2004). In general, *Drosophila* sterility is measured by the presence or absence of sperm motility (COYNE; ORR, 1997). However, this may not necessarily always be correct because males presenting motile sperm may be unable to produce offspring. Due to this factor, the fertility of the hybrids with mobile sperm was tested by backcrosses with both parental lines and F1 x F1 crosses. The results verified that the hybrid males from the crosses between *D. arizonae* males and *D. m. baja* females were fertile, since they produced offspring. In addition, hybrids from *D. arizonae* females and *D. m. sonorensis* males, the only males that presented sperm motility in this cross direction, were also fertile, evidencing weak postzygotic isolation in hybrids from sympatric populations. On the other hand, male hybrids from *D. m. mojavenensis* and *D. m. wrigleyi* females crosses with *D. arizonae* males, which showed sperm motility, were unable to produce offspring, in agreement with Reed and Markow (2004). The authors found that some males displaying sperm motility were able to produce offspring, while others were sterile. Therefore, motility is not a factor that always indicates fertility, which suggests that this phenotypic trait has a complex genetic base, which needs to be better understood.

CONCLUSION

Here, we verified that all hybrids presented reduced fitness compared to their parental

lines, regardless of the cross direction and the source of the parental population. Moreover, male sterility was shown in almost all hybrids, while all female hybrids were fertile, corroborating Haldane's rule. Regarding male sterility, it was observed that some hybrids presented mobile sperm, and when this occurred, the motility exhibited different levels compared to the parental males and between the hybrids. This feature suggests the presence of polymorphic factors dependent on the *D. mojavensis* subspecies that can influence the fitness of F1 hybrids. In addition, an interesting result was observed: *D. arizonae* and *D. m. sonorensis*, which are sympatric populations, present strong prezygotic isolation, however, when this barrier is overcome, they are able to produce a large number of fertile male hybrids, in both cross directions, differently from the male hybrid offspring of allopatric populations.

Therefore, these results suggest the presence of several genetic mechanisms influencing reproductive isolation between these sibling species, which are species-specific. Considering this, the next steps will be to analyse the expression of some candidate genes involved in the reproductive isolation process between *D. arizonae* and all subspecies of *D. mojavensis*, as well as the possible influence of transposable elements in the first steps of speciation.

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SUPPLEMENTARY MATERIAL

Table S1. Statistical analyses performed for fecundity data. Group A: comparisons between *D. m. mojavenensis*, *D. arizonae* and their hybrids. Group B: comparisons between *D. m. baja*, *D. arizonae* and their hybrids. Group C: comparisons between *D. m. wrigleyi*, *D. arizonae* and their hybrids. Group D: comparisons between *D. m. sonorensis*, *D. arizonae* and their hybrids. Significant p-value < 0.05.

Fecundity	Shapiro-Wilk (W)/p-value	Levene (F)/p-value	Kruskal-Wallis/p-value
Group A	0.79415/1.644e-10*	7.9439/0.00008713*	30.469/ 1.099e-06*
Group B	0.89526/0.0000008523*	3.6122/0.01603 *	27.881/ 3.846e-06*
Group C	0.93113/0.00005773*	2.5666/0.059	20.892/0.0001109*
Group D	0.74646/4.645e-10*	7.8078/0.0008532 *	27.429/ 1.106e-06*

Source: prepared by the author.

Table S2. Average fecundity of paired comparisons between interspecific and intraspecific crosses of *D. mojavenensis* and *D. arizonae*. P-value of the Wilcoxon test performed in the R software.

	<i>D. m. mojavenensis</i>	<i>D. arizonae</i>	(A)
<i>D. arizonae</i>	8.3e-07*		
(A) F 01 x M 17	0.0005*	0.004*	
(B) F 17 x M 01	0.0004*	0.021*	0.654 n/s
	<i>D. m. baja</i>	<i>D. arizonae</i>	(A)
<i>D. arizonae</i>	0.0156*		
(A) F 20 x M 17	0.173 n/s	0.4541 n/s	
(B) F 17 x M 20	1.2e-06*	0.0002*	0.0004*
	<i>D. m. wrigleyi</i>	<i>D. arizonae</i>	(A)
<i>D. arizonae</i>	0.627 n/s		
(A) F 22 x M 17	0.0001*	0.0008*	
(B) F 17 x M 22	0.268 n/s	0.281 n/s	0.0001*
	<i>D. m. sonorensis</i>	<i>D. arizonae</i>	
<i>D. arizonae</i>	5.1e-07*		
(B) F 17 x M 26	0.058 n/s	0.0007*	

n/s: non-significant, * p < 0.05

(A) interspecific crosses between *D. mojavenensis* females and *D. arizonae* males

(B) interspecific crosses between *D. arizonae* females and *D. mojavenensis* males

Source: prepared by the author.

Table S3. Statistical analyses performed for viability data. Group A: comparisons between *D. m. mojavenensis*, *D. arizonae* and their hybrids. Group B: comparisons between *D. m. baja*, *D. arizonae* and their hybrids. Group C: comparisons between *D. m. wrigleyi*, *D. arizonae* and their hybrids. Group D: comparisons between *D. m. sonorensis*, *D. arizonae* and their hybrids. Significant p-value < 0.05.

Viability	Shapiro-Wilk (W)/p-value	Levene (F)/p-value	Kruskal-Wallis/p-value
Group A	0.78221/ 0.000000006968*	4.0436/0.01054 *	24.973/1.565e-05*
Group B	0.80625/0.00000002158*	7.2651/0.0002639 *	28.499/2.854e-06*
Group C	0.82275/0.00000002189*	7.5465/0.0001744 *	37.488/3.628e-08
Group D	0.7982 0.000002669*	4.2251/0.02146 *	15.413/0.0004*

Source: prepared by the author.

Table S4. Viability of paired comparisons between interspecific and intraspecific crosses of *D. mojavenensis* and *D. arizonae*. P-value of the Wilcoxon test performed in the R software.

	<i>D. m. mojavenensis</i>	<i>D. arizonae</i>	(A)
<i>D. arizonae</i>	0.0004*		
(A) Hybrid A 01	1.6e-05*	0.03*	
(B) Hybrid B 01	3.6e-05*	0.216 n/s	0.295 n/s
	<i>D. m. baja</i>	<i>D. arizonae</i>	(A)
<i>D. arizonae</i>	0.1586 n/s		
(A) Hybrid A 20	2.1e-07*	0.0014*	
(B) Hybrid B 20	0.0036*	0.0496*	0.6904 n/s
	<i>D. m. wrigleyi</i>	<i>D. arizonae</i>	(A)
<i>D. arizonae</i>	0.0049*		
(A) Hybrid A 22	1.1e-05 *	0.0237*	
(B) Hybrid B 22	8.0e-08 n/s	0.0042*	0.9112 n/s
	<i>D. m. sonorensis</i>	<i>D. arizonae</i>	
<i>D. arizonae</i>	0.00889*		
(B) Hybrid B 26	0.00027*	0.02868*	

n/s: non-significant, * p < 0.05; (A) hybrids from interspecific crosses between *D. mojavenensis* females (01, 20, 22, 26) and *D. arizonae* males; (B) hybrids from interspecific crosses between *D. arizonae* females and *D. mojavenensis* males (01, 20, 22, 26).

Source: prepared by the author.

Table S5. Statistical analyses of productivity data. Group A: comparisons between *D. m. mojavenensis*, *D. arizonae* and their hybrids. Group B: comparisons between *D. m. baja*, *D. arizonae* and their hybrids. Group C: comparisons between *D. m. wrigleyi*, *D. arizonae* and their hybrids. Group D: comparisons between *D. m. sonorensis*, *D. arizonae* and their hybrids. Significant p-value < 0.05.

Productivity	Shapiro-Wilk (W)/p-value	Levene (F)/p-value	Anova (F)/p-value
Group A	0.8381/0.1597	0.3729/0.7737	74.23/0.00000000132 *
Group B	0.75825/0.03543*	1.9088/0.1689	120.7/3.37e-11 *
Group C	0.82674/ 0.1315	0.4407/0.7271	53.87/0.0000000139*
Group D	0.98419/0.9557	1.8553/0.1779	6.512/0.00436*

Source: prepared by the author.

Table S6. Paired comparisons of mean productivity between interspecific and intraspecific crosses of *D. mojavenensis* and *D. arizonae*. P-value of the Tukey's test performed in the R software.

	<i>D. m. mojavenensis</i>	<i>D. arizonae</i>	(A)
<i>D. arizonae</i>	0.8308 n/s		
(A) Hybrid A 01	<0.001*	<0.001*	
(B) Hybrid B 01	<0.001*	<0.001*	0.0143*
	<i>D. m. baja</i>	<i>D. arizonae</i>	(A)
<i>D. arizonae</i>	<0.001*		
(A) Hybrid A 20	0.347 n/s	<0.001*	
(B) Hybrid B 20	0.0018*	<0.001*	0.060 n/s
	<i>D. m. wrigleyi</i>	<i>D. arizonae</i>	(A)
<i>D. arizonae</i>	0.00355*		
(A) Hybrid A 22	<0.001*	<0.001*	
Hybrid B 22	<0.001*	<0.001*	0.976 n/s
	<i>D. m. sonorensis</i>	<i>D. arizonae</i>	
<i>D. arizonae</i>	0.936 n/s		
(A) Hybrid A 26	0.294 n/s	0,113 n/s	
(B) Hybrid B 26	0.017*	0.005*	0.434 n/s

n/s: non-significant, * p < 0.05; (A) interspecific crosses between *D. mojavenensis* females and *D. arizonae* males; (B) interspecific crosses between *D. arizonae* females and *D. mojavenensis* males.

Source: prepared by the author.

Table S7. Fertility of reciprocal hybrids from F1 x F1 crosses and backcrosses with their respective parental lines.

Crossings	Egg production	Offspring production
♀ Hybrid A 01 x ♂ Hybrid A 01	Yes	No
♀ <i>D. arizonae</i> x ♂ Hybrid A 01	Yes	No
♀ Hybrid A 01 x ♂ <i>D. arizonae</i>	Yes	Yes
♀ <i>D. m. mojavenensis</i> x ♂ Hybrid A 01	Yes	No
♀ Hybrid A 01 x ♂ <i>D. m. mojavenensis</i>	Yes	Yes
♀ Hybrid B 01 x ♂ Hybrid B 01	Yes	No
♀ <i>D. arizonae</i> x ♂ Hybrid B 01	Yes	No
♀ Hybrid B 01 x ♂ <i>D. arizonae</i>	Yes	Yes
♀ <i>D. m. mojavenensis</i> x ♂ Hybrid B 01	Yes	No
♀ Hybrid B 01 x ♂ <i>D. m. mojavenensis</i>	Yes	Yes
♀ Hybrid A 20 x ♂ Hybrid A 20	Yes	Yes
♀ <i>D. arizonae</i> x ♂ Hybrid A 20	Yes	No
♀ Hybrid A 20 x ♂ <i>D. arizonae</i>	No	No
♀ Hybrid A 20 x ♂ <i>D. m. baja</i>	Yes	Yes
♀ <i>D. m. baja</i> x ♂ Hybrid A 20	No	No
♀ Hybrid B 20 x ♂ Hybrid B 20	Yes	No
♀ <i>D. arizonae</i> x ♂ Hybrid B 20	Yes	No
♀ Hybrid B 20 x ♂ <i>D. arizonae</i>	Yes	Yes
♀ <i>D. m. baja</i> x ♂ Hybrid B 20	No	No
♀ Hybrid B 20 x ♂ <i>D. m. baja</i>	No	No
♀ Hybrid A 22 x ♂ Hybrid A 22	Yes	No
♀ Hybrid A 22 x ♂ <i>D. arizonae</i>	Yes	Yes
♀ <i>D. arizonae</i> x ♂ Hybrid A 22	Yes	No
♀ Hybrid A 22 x ♂ <i>D. m. wrigleyi</i>	Yes	Yes
♀ <i>D. m. wrigleyi</i> x ♂ Hybrid A 22	Yes	No
♀ Hybrid B 22 x ♂ Hybrid B 22	Yes	No
♀ Hybrid B 22 x ♂ <i>D. arizonae</i>	Yes	Yes
♀ <i>D. arizonae</i> x ♂ Hybrid B 22	Yes	No
♀ Hybrid B 22 x ♂ <i>D. m. wrigleyi</i>	Yes	Yes
♀ <i>D. m. wrigleyi</i> x ♂ Hybrid B22	Yes	No
♀ Hybrid A 26 x ♂ Hybrid A 26	Yes	Yes
♀ <i>D. arizonae</i> x ♂ Hybrid A 26	Yes	Yes
♀ Hybrid A 26 x ♂ <i>D. arizonae</i>	Yes	Yes
♀ <i>D. m. sonorensis</i> x ♂ Hybrid A 26	No	No
♀ Hybrid A 26 x ♂ <i>D. m. sonorensis</i>	No	No
♀ Hybrid B 26 x ♂ Hybrid B 26	Yes	Yes
♀ <i>D. arizonae</i> x ♂ Hybrid B 26	Yes	No
♀ Hybrid B 26 x ♂ <i>D. arizonae</i>	Yes	Yes
♀ <i>D. m. sonorensis</i> x ♂ Hybrid B 26	Yes	No
♀ Hybrid B x ♂ <i>D. m. sonorensis</i>	Yes	Yes

Source: prepared by the author.

4 CAPÍTULO 2

Article - Discoveries

Comparative transcriptomics between *Drosophila mojavensis* and *D. arizonae* reveal underexpression of spermatogenesis-related genes in male hybrids

Cecilia A. Banho,^{1,2} Vincent Mérel,² Thiago Y. K. Oliveira,³ Claudia M. A. Carareto,¹ and Cristina Vieira^{2*}

¹Department of Biology, UNESP - São Paulo State University, São José do Rio Preto, São Paulo State (SP), Brazil.

² Laboratoire de Biométrie et Biologie Evolutive, Université Lyon 1, CNRS, UMR 5558, Villeurbanne F-69622, France.

³Laboratory of Molecular Immunology, The Rockefeller University, New York, NY, USA.

*Author for Correspondence: Cristina Vieira, Laboratoire de Biométrie et Biologie Evolutive, Université Lyon 1, CNRS, UMR 5558, Villeurbanne F-69622, France, telephone number +33 6 08 91 58 15, e-mail: cristina.heddi@univ-lyon1.fr.

Keywords: *repleta* group, gene expression, hybrid male sterility

Abstract

Interspecific hybridization is a stressful condition that can lead to sterility and/or inviability through improper gene regulation in *Drosophila* species with a high divergence time. However, the extent of these abnormalities in hybrids of recently diverging species is not well known. Some studies have shown that in *Drosophila*, the mechanisms of postzygotic isolation may evolve more rapidly in males than in females, and that the degree of viability and sterility is associated with the genetic distance between species. Here, by testing the hypothesis that the severity of the hybrid phenotype is associated with the degree of gene misregulation, we observed that hybrids with different phenotypes (female fertility and male sterility with and without sperm motility) showed different degrees of gene misregulation. Through the use of gene expression analyses in hybrids between *Drosophila mojavensis* subspecies and *D. arizonae* (repleta group, *Drosophila*), we have shown that the degree of gene differential expression is greater in the testes than in ovaries. Moreover, the degree of gene misregulation was higher and presented a bias for underexpression in hybrids without motile sperm. In addition, for these hybrids, we identified candidate genes that were mostly associated with spermatogenesis dysfunction. We suggest that the disruption in gene expression of *D. arizonae*-*D. mojavensis* male hybrids is probably related to connected incompatibilities in regulatory sequences, due to adaptative divergence of alleles between the parental species, and is the main factor responsible for the male sterile phenotype.

Introduction

Speciation is a complex process resulting from the divergence of two populations from an ancestral lineage by reproductive barriers capable of preventing gene flow (Orr and Coyne 1989; Coyne and Orr 1997). Among these barriers, postzygotic isolation mechanisms contribute to hybrid incompatibility, and their consequences can be observed by the presence of two main traits, hybrid sterility and/or hybrid inviability, which can evolve at different rates. Overall,

hybrid sterility evolves faster than hybrid inviability, mainly when considering the different sexes, since these barriers evolve faster in males than in females (Turissini et al. 2018). Indeed, several studies considering intraspecific and interspecific hybridization have shown that the germline is primarily affected, and sterility is often detected (Ruiz et al. 1990; Ranz et al. 2004; Moerhring et al. 2007; Llopart 2012; Brill et al. 2016; Hill et al. 2016; Li et al. 2016). Although the process of speciation has been widely studied, the causes of postzygotic incompatibility in hybrids are not fully understood. According to the model of Dobzhansky–Muller (1936, 1942), genetic variation could lead to deleterious epistatic interactions, disrupting regulatory networks and leading to serious consequences in the hybrids (Orr and Coyne 1989; McManus et al. 2010; Kelleher et al. 2012; Romero-Soriano et al. 2017). In addition, according to Haldane’s rule (Haldane 1922), sterility in hybrids is more likely to affect the heterogametic sex, and the degree and extent of these genetic incompatibilities are related to the time of divergence and are likely the result of divergent regulatory sequences (McManus et al. 2010; Kelleher et al. 2012; Ryazansky et al. 2014; Llopart et al. 2018). Many studies have searched for genes involved with postzygotic isolation, particularly in *Drosophila*. So far, few genes that may play a role in hybrid incompatibility have been identified. Among these genes, *Odiseus* (*OdsH*), which is located on the X-chromosome and encodes a transcription factor, has been found to be one of the responsible factors contributing to hybrid sterility between *Drosophila simulans* and *D. mauritiana* (Ting et al. 1998). In addition, the genes *Hybrid male-rescue* (*Hmr*) and *Lethal male-rescue* (*Lmr*) are known to be related to hybrid unviability in *D. melanogaster*–*D. simulans*. These genes encode centromeric proteins, and the interaction of the alleles of *D. melanogaster Hmr* with *D. simulans Lmr* leads to hybrid breakdown (Barbash et al. 2004; Barbash and Lorigan 2007; Satyaki et al. 2014). *Nup96*, a gene located on the X chromosome that encodes a nuclear pore protein, also seems to contribute to *D. melanogaster*–*D. simulans* hybrid unviability, likely due the deleterious interaction between the alleles *D. simulans Nup96* and *D. melanogaster Nup153*, located on the X chromosome (Presgraves et al. 2003; Tang and Presgraves 2015).

Overdrive (*Ovd*) is another gene responsible for male sterility and segregation distortion in F_1 hybrids of *D. p. pseudoobscura* and *D. p. bogotana* by epistatic X-autosomal interactions (Phadnis and Orr, 2009; Phadnis 2011). Moreover, multiple studies have shown that in sterile hybrids, several differentially expressed genes (DEG) have a bias for underexpression (Michalak and Noor 2003; Haerty and Singh 2006) and function in spermatogenesis, such as *Acyp*, *spermatocyte arrest*, *bag of marbles*, *always early*, *twine*, *cookie monster*, *meiosis I arrest*, *janusB*, *gonadal* and *don juan* (White-Cooper et al. 1998; Jiang and White-Cooper 2003; Michalak and Noor 2003, 2004; Ma and Michalak 2011; Gomes and Civetta 2014).

Most of the hybrid incompatibility genes identified so far seems to be species-specific, indicating that hybrid incompatibility is a complex polygenic trait that has different epistatic effects related to the rapid accumulation of genetic changes over time. Studies of recently diverged species presenting different phenotypes regarding the postzygotic isolation mechanisms can help to clarify early disruptions in gene regulation and expression that may influence the speciation process. *Drosophila mojavensis* and *D. arizonae* are appropriate for such a study. They are sibling species, recently diverged (~1.5 mya, Sanches-Flores et al. 2016), and widely used in speciation studies due to their ability to produce hybrids in the laboratory; however, introgression evidence has not yet been found in nature despite of the favourable ecological conditions for hybridization, mainly in sympatric populations (Jennings and Etges 2009). *D. mojavensis* is composed of four ecologically distinct subspecies, which are distributed in four different geographic regions, and each one uses a specific host cactus as a feeding and breeding site, with no evidence of gene flow, constituting well-structured populations (Knowles and Markow 2001; Reed and Markow 2004; Massie and Markow 2005; Reed et al. 2007; Etges et al. 2010). In crosses between *D. mojavensis* subspecies and *D. arizonae*, hybrids are produced in both directions. Nevertheless, they present incomplete and asymmetric postzygotic isolation, since in crosses with *D. mojavensis* females the sterility of the male hybrids is variable, whereas in crosses with *D. arizonae* females the male hybrids are always sterile because they do not have

motile sperm (Ruiz et al. 1990; Reed and Markow 2004; Carnelossi et al. 2014).

With this in mind, we sought to assess the degree of genetic incompatibility by analysing gene expression in male and female offspring from *D. arizonae* $\times$ *D. mojavensis* crosses. We have shown that in fertile hybrid females, very few DEG are identified. However, in male hybrids, the degree of deregulation of gene expression was related to the severity of the sterile phenotype, because males without motile spermatozoa had several DEG, with a bias for underexpression and spermatogenesis related-functions. By contrast, in sterile hybrids with motile spermatozoa, the degree of deregulation was lower, had a bias for overexpression, and the gene functions it was not directly related with spermatogenesis.

Results

The transcriptomes from the ovaries and testes of three parental allopatric strains (*D. m. mojavensis*, *D. m. wrigleyi* and *D. arizonae*) and their reciprocal hybrids (Figure 1) were sequenced. We were able to recover 11,654 coding genes from *D. mojavensis* r1.04 transcripts. From those, approximately 9,321 (80%) genes were expressed in *D. arizonae*, *D. m. mojavensis* and HB_m ovaries, while 9,714 (83.3%) were expressed in *D. arizonae*, *D. m. wrigleyi*, HA_w and HB_w female gonads. In testes, we found a larger number of expressed genes; 11,146 (95.6%) were expressed in *D. arizonae*, *D. m. mojavensis* and HB_m, and 11,242 (96.4%) were expressed in *D. arizonae*, *D. m. wrigleyi*, HA_w and HB_w. The read alignment rate ranged from 81.7 to 86% in ovaries and from 76.4 to 80.8% in testes (Supplementary Table S1). A similar alignment rate for *D. arizonae*, *D. mojavensis* subspecies and their reciprocal hybrids indicated that the use of the *D. mojavensis* r1.04 reference can be used in analyses involving *D. arizonae* and the hybrids. Principal component analysis (PCA) was performed to verify the variance of the biological replicates. In ovaries and testes, the replicates were grouped together (Supplementary Fig. S1), indicating low variance between replicates; this allowed us to proceed with the differential expression analyses.

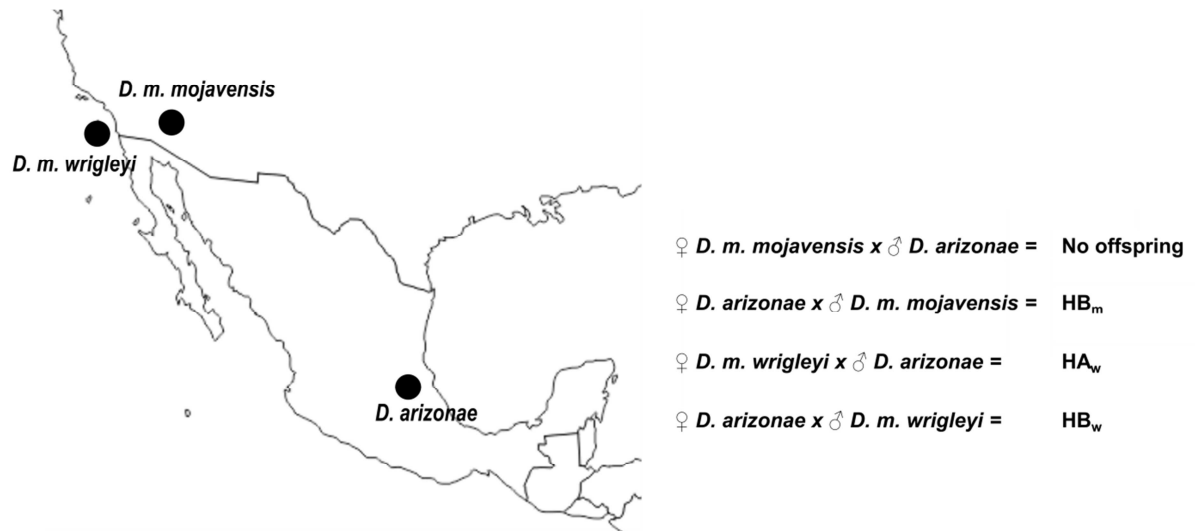


Fig. 1. Crosses performed between *D. arizonae* and *D. mojaveensis* subspecies and differential gene expression between parental lines and hybrids. Geographic distribution of *D. m. wrigleyi*, *D. m. mojaveensis* and *D. arizonae* lines used in this study and their respective cross direction with offspring identification. HA_w and HB_w are offspring from crosses between *D. arizonae* and *D. m. wrigleyi*, HB_m are hybrids from crosses between *D. arizonae* and *D. m. mojaveensis*. A and B represent the cross direction (A = crosses with *D. mojaveensis* mothers; B = crosses with *D. arizonae* mothers). Source: prepared by the author.

Differential expression of genes in *D. arizonae* and *D. mojaveensis* gonads

We compared the transcriptomes of *D. arizonae* and *D. m. mojaveensis* and *D. arizonae* and *D. m. wrigleyi* and we identified 501 (5.3%) and 621 (6.3%) DEG in ovaries, respectively, with the majority of the genes overexpressed in *D. arizonae* (Figure 2A). In testes, there was a higher number of DEG, which reached 16% when comparing *D. arizonae* with each *D. mojaveensis* subspecies; however, there was a similar distribution between the over- and underexpressed genes (Fig. 2E). Gene ontology (GO) analyses of DEG between parental lines showed enrichment in the ovaries of genes related to response to stimulus, multicellular organismal process, multicellular organismal development, developmental process, cellular

process, biological process, biological regulation, anatomical structure development, among others (Fig. 3, supplementary tables S2 and S3). For the testes, enriched DEG were related to similar functions as in females, but also with behaviour, sensory perception, regulation of cellular process, regulation of biological process, among others (Fig. 3, supplementary tables S4 and S5).

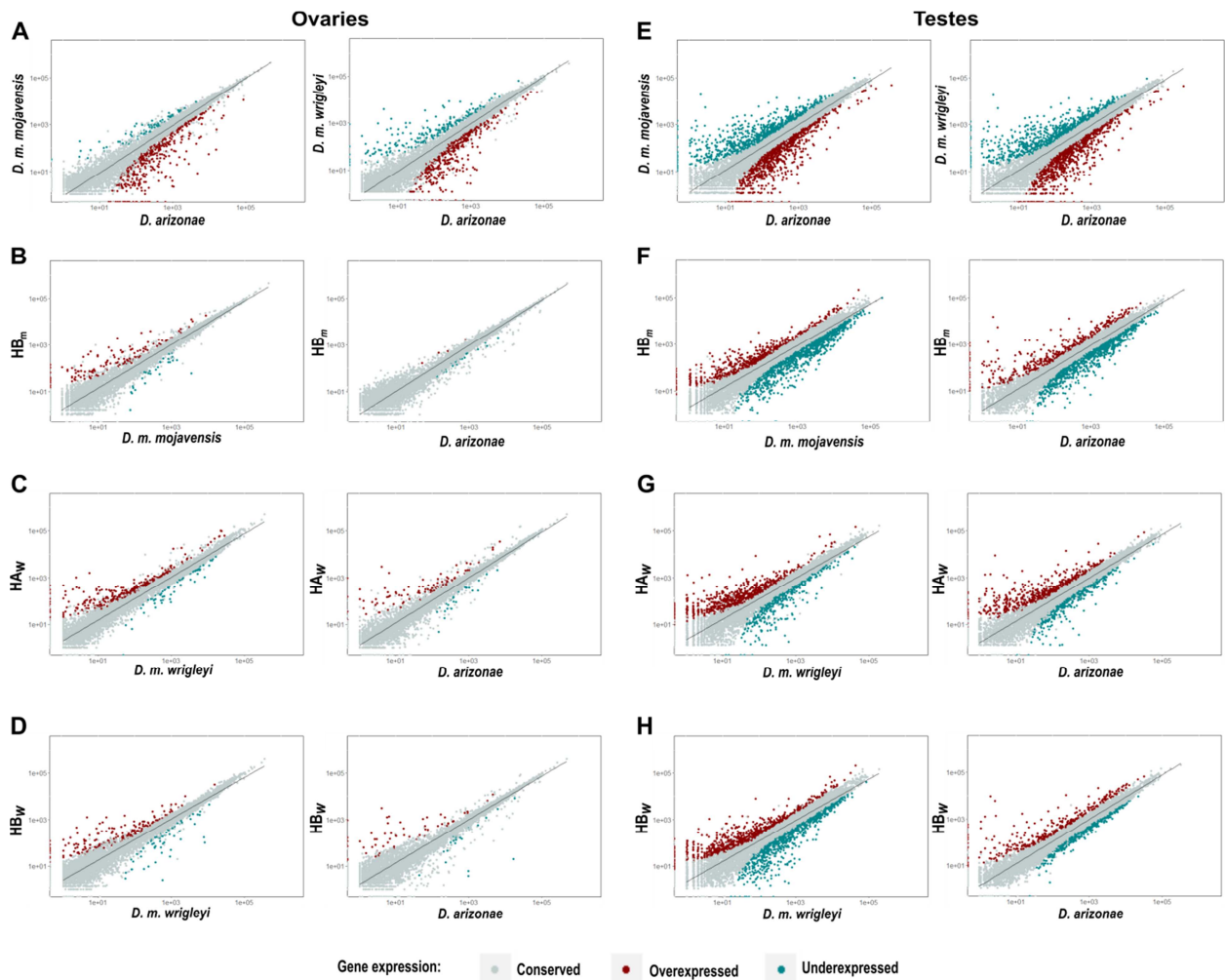


Fig. 2. Differential expression between *D. arizonae* and *D. mojaveensis* subspecies and between hybrids and their respective parental lines. Scatter plots representing differential gene expression in ovaries (left) and testes (right) between parental lines as well as between hybrids and their respective parental lines. Genes were considered as differentially expressed when they presented 2-fold of differences and p-value corrected by FDR < 0.01. Red dots = overexpressed genes; blue dots = underexpressed genes; grey dots = not differentially expressed.

A) Differential expression in ovaries between *D. m. mojaveensis* x *D. arizonae* and *D. m. wrigleyi*

x *D. arizonae*. B) Differential expression in ovaries between HB_m x *D. m. mojavenensis* and *D. arizonae*. C) Differential expression in ovaries between HA_w x *D. m. wrigleyi* and *D. arizonae*. D) Differential expression in ovaries between HB_w x *D. m. wrigleyi* and *D. arizonae*. E) Differential expression in testes between *D. m. mojavenensis* x *D. arizonae* and *D. m. wrigleyi* x *D. arizonae*. F) Differential expression in testes between HB_m x *D. m. mojavenensis* and *D. arizonae*. G) Differential expression in testes between HA_w x *D. m. wrigleyi* and *D. arizonae*. H) Differential expression in testes between HB_w x *D. m. wrigleyi* and *D. arizonae*. Source: prepared by the author.

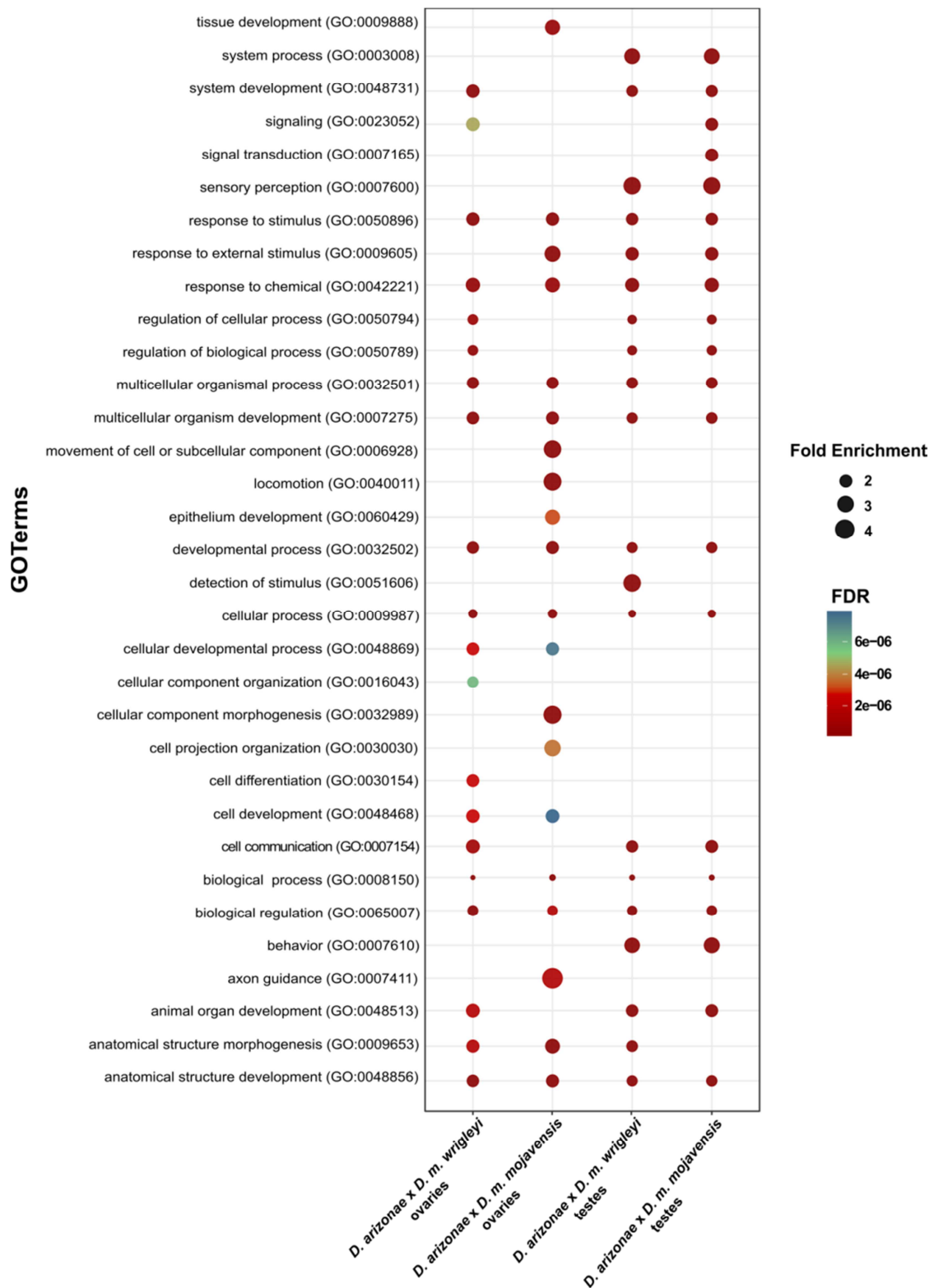


Fig. 3. Functions of DEG between *D. arizonae* and *D. mojavensis* subspecies. Dot plot representing GO enrichment (biological process, FDR < 0.05) for DEG in ovaries and testes between *D. arizonae* and *D. mojavensis* subspecies (FDR < 0.01, FC > 2). Source: prepared by the author.

Gene expression in the hybrid female gonads is very similar to the parental lines

We compared the expression of hybrids to their parental lines. The expression profile of the HB_m ovaries revealed that 1.93% of DEG between the hybrid and *D. m. mojavensis*, in contrast to the comparison with *D. arizonae*, which showed almost no differences (0.26%) (Fig. 2B, supplementary table S6). Gene expression in HA_w and HB_w ovaries showed a larger number of DEG, mostly overexpressed in the hybrids when compared to *D. m. wrigleyi* (4.2% and 2.7%, respectively), regardless of the direction of the crossing (Fig. 2C and D, supplementary table S7).

To identify the genes that were over- or underexpressed relative to both parental lines in all crosses, we filtered those that presented a greater than 2-fold difference and significant adjusted p-values (< 0.01) in HB_m, HA_w and HB_w ovaries. This reduced greatly the number of genes. The HB_m ovaries presented no overexpressed genes and only three underexpressed genes. These included FBgn014602, which corresponds to *alpha-Est5* and has carboxylic ester hydrolase activity, FBgn028050, corresponding to *Maverick* gene, which has a role in signalling pathways, the regulation of neuromuscular junctions, dendrite development and imaginal disc-derived wing size, and FBgn0146651, which has an unknown function (From Flybase database, available at: <http://flybase.org>). By contrast, HA_w ovaries showed 16 overexpressed genes (supplementary table S8), however no significant enrichment was found due to the few number of DEG, and none underexpressed genes was found in relation to both parental lines. The HB_w ovaries showed 13 overexpressed genes (supplementary table S9) and only one underexpressed gene (FBgn0135298, with unknown function) when compared with both parental lines. Additionally, HA_w and HB_w ovaries did not show any common overexpressed genes.

Gene expression in hybrid male germline is divergent from the parental lines

Compared to ovaries, hybrid testes presented a larger number of DEG in comparison with the parental lines. HB_m, the offspring from crosses with *D. arizonae* females, displayed 11.9%

and 16% of DEG compared with *D. arizonae* and *D. m. mojavenensis*, respectively, and most of them were underexpressed (Fig. 2F, supplementary table S6). In HA_w testes (offspring from *D. mojavenensis* females), 7.9% and 9.7% DEG compared with *D. arizonae* and *D. m. wrigleyi* were found, respectively, and presented a bias for overexpression (Fig. 2G, supplementary table S7). Similarly, hybrids from reciprocal cross (HB_w) also exhibited a larger number of DEG compared with *D. m. wrigleyi* than when compared with *D. arizonae*. However, in these hybrids, as was observed for HB_m, most them were underexpressed (Fig. 2H, supplementary table S7).

Regarding the deregulated genes in HA_w testes, 130 were overexpressed and 60 underexpressed compared with both parental species (supplementary tables S10 and S11), while in HB_w testes 79 and 172 genes were commonly over- and underexpressed, respectively, compared with *D. arizonae* and *D. m. wrigleyi* (supplementary tables S12 and S13). As with HB_w, the common deregulated genes in the testes of HB_m compared to the parental species also showed an underexpression bias, as 57 genes were overexpressed and 519 were underexpressed (supplementary tables S14 and S15).

Spermatogenesis-related functions of DEG in testes

In the male gonads, the deregulated gene expression pattern differed depending on the direction of crossing. In HA_w testes, from the 190 deregulated genes, 160 were orthologous to *D. melanogaster*, allowing us to find their functions. Most of the DEG were significantly enriched for functions associated with plasma membrane (Fig. 4). On the other hand, in HB_w testes, 251 genes were deregulated, and the *D. melanogaster* orthologues were recovered for 185 of them. From enrichment analysis we detected that, unlike HA_w, the function of most of these genes are related with axoneme, cilium, dynein and microtubule (Fig. 4). In HB_m testes, 598 genes were identified as differentially expressed, and 462 had a *D. melanogaster* orthologue. In these hybrids, most of the over- and underexpressed genes were enriched for some functions similar to HA_w and HB_w (Fig. 4).

Globally, hybrid testes presented a larger number of deregulated genes. However, the over- and underexpressed gene ratio depended on the direction of crossing. HA_w testes showed a larger number of overexpressed genes, while HB_w and HB_m exhibited a larger number of underexpressed genes. We searched for shared deregulated genes in the different hybrids in both gonad tissues and found 31 overexpressed and 8 underexpressed genes shared in the HA_w and HB_w males (supplementary table S16).

Comparisons of HB_m and HB_w male gonads, corresponding to hybrids from crosses of different *D. mojavensis* subspecies, but in the same direction, showed 31 shared overexpressed genes (supplementary table S17) and 146 underexpressed genes (supplementary table S18). Interestingly, many of these genes exhibited enrichment for spermatogenesis-related functions, as cilium movement (Fig. 3, Table 1), which are linked to the hybrid sterile phenotype. Looking specifically these genes it is remarkable that some of them might have a direct role in reproduction, since they participate in male meiosis, male courtship, mating behaviour, as well as playing a role in gene silencing and pre-miRNA processing (Table 1). Additionally, for many of these shared DEG genes, the mutant phenotype already described for other *Drosophila* corresponds to male sterility (Table 1). To test our hypothesis that genes with specific expression in testes would show signatures of positive selection, as already reported in some studies (Meiklejohn et al. 2003; Artieri and Haerty 2007, Llopart 2012) we calculated Ka/Ks for a set of DEG testis-specific. However, no signatures of positive selection were observed in these genes, although several of them present evidence of having undergone relaxed selection.

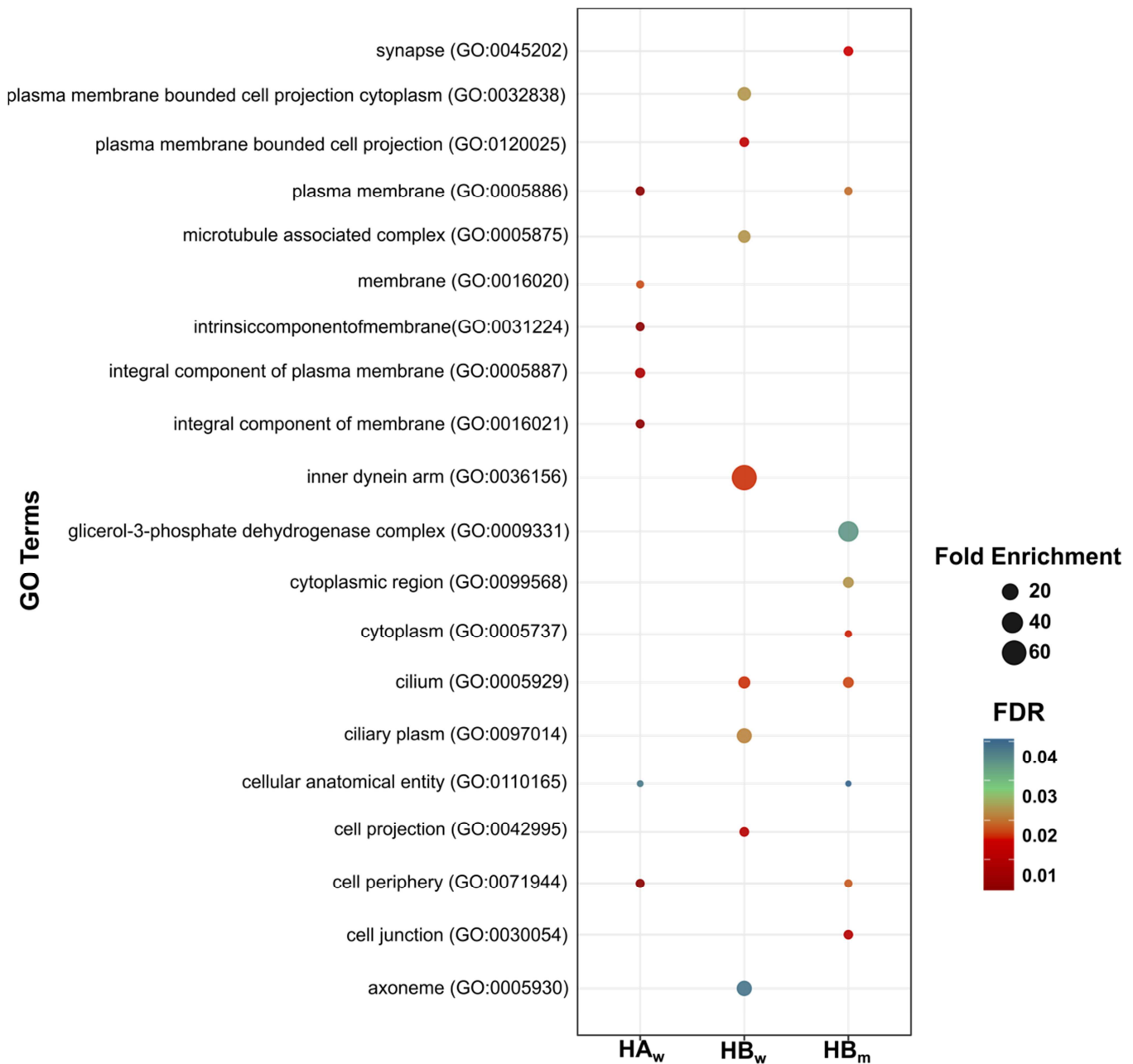


Fig. 4. Functions of over and underexpressed genes in male hybrids. Dot plot representing GO enrichment (cellular component, FDF < 0.05) for all DEG between hybrid testes and their parental lines (FDR < 0.01, FC > 2). Source: prepared by the author.

Table 1. DEG in male hybrids gonads with reproductive functions. Genes commonly misexpressed in male hybrid gonads presenting spermatogenesis-related functions and their mutant phenotype in other *Drosophila* species associated to them.

<i>D. mojavensis</i> ID	<i>D. melanogaster</i> ID	Gene name	Biological process	Mutant phenotype	Reference	Expression		
						HA _w	HB _w	HB _m
FBgn0140112	FBgn0028858	<i>CG10839</i>	GO:0007018 - microtubule-based movement	-	-	Not DF*	Underexpressed	Underexpressed
FBgn0145039	FBgn0039812	<i>CG15548</i>	GO:0000022 - mitotic spindle elongation	-	-	Not DF*	Underexpressed	Underexpressed
FBgn0135509	FBgn0035581	<i>Dnah3</i>	GO:0007018 - microtubule-based movement,	Immotile sperm	Karak et al. 2015	Not DF*	Underexpressed	Underexpressed
FBgn0140104	FBgn0028901	<i>CG18109</i>	GO:0007020 - microtubule nucleation	-	-	Not DF*	Underexpressed	Underexpressed
FBgn0147429	FBgn0063261	<i>CG31275</i>	GO:0007018 - microtubule-based movement	-	-	Not DF*	Underexpressed	Underexpressed
FBgn0142040	FBgn0027066	<i>Eb1</i>	GO:0000226: microtubule cytoskeleton organization.	Defects in spindle elongation and orientation; reduction in astral microtubules.	Rogers et al. 2002	Not DF*	Underexpressed	Underexpressed
FBgn0143126	FBgn0032225	<i>CG5022</i>	GO:0031032 - actomyosin structure organization	-	-	Not DF*	Underexpressed	Underexpressed
FBgn0137431	FBgn0283476	<i>Dhc16F</i>	GO:0001539 - cilium or flagellum-dependent cell motility	Male sterility.	Lage et al. 2019	Not DF*	Underexpressed	Underexpressed
FBgn0136866	FBgn0039925	<i>Kif3C</i>	GO:0007018 - microtubule-based movement	Roles in flagellar/ciliary motilities.	Henson et al. 1997Gumy et al. 2013	Not DF*	Underexpressed	Underexpressed
FBgn0140824	FBgn0031952	<i>cdc14</i>	GO:0071850 - mitotic cell cycle arrest	-	-	Not DF*	Underexpressed	Underexpressed
FBgn0140999	FBgn0262123	<i>l(2)41Ab</i>	GO:0070286 - axonemal dynein complex assembly	-	-	Not DF*	Underexpressed	Underexpressed
FBgn0146436	FBgn0038565	<i>CG7794</i>	GO:0007017 - microtubule-based process	-	-	Not DF*	Underexpressed	Underexpressed
FBgn0280294	FBgn0023090	<i>dtr</i>	GO:0060271 – cilium morphogenesis	Male sterility.	Lage et al. 2019	Not DF*	Underexpressed	Underexpressed
FBgn0140392	FBgn0002673	<i>twe</i>	GO:0007140 - male meiosis, GO:0007283 - spermatogenesis	Male sterility. Absence of meiotic divisions in male germline and no motile sperm.	Courtot et al. 1992; Lin et al. 1996; Maines and Wasserman 1999	Not DF*	Underexpressed	Underexpressed
FBgn0144612	FBgn0267326	<i>Ntl</i>	GO:0030317 - sperm motility	Male sterility. Immotile sperm and fail to be transferred to the seminal vesicle.	Chatterjee et al. 2011	Not DF*	Underexpressed	Underexpressed
FBgn0281134	FBgn0001313	<i>kl2</i>	GO:0003341: cilium movement	Male sterility. Defects in sperm individualization.	Zhang et al. 2020	Not DF*	Underexpressed	Not DF*
FBgn0142949	FBgn0265512	<i>mlt</i>	GO:0007291 - sperm individualization	Male sterility. Defects in sperm individualization.	Fabrizio et al. 2012	Not DF*	Not DF*	Underexpressed
FBgn0142705	FBgn0002865/ FBgn0004171	<i>Mst98Ca/ Mst98Cb</i>	GO:0007286 - Spermatid development	Structural proteins in the sperm tail.	Schafer et al. 1993	Not DF*	Not DF*	Underexpressed
FBgn0146037	FBgn0260942	<i>bond</i>	GO:0007112 - male meiosis cytokinesis	Failure of cytokinesis in dividing spermatocytes.	Szafer-Glusman et al. 2008	Not DF*	Not DF*	Underexpressed

Table 1 Cont.

FBgn0138561	FBgn0052529	<i>Hers</i>	GO:0006342: chromatin silencing	Affect the regulation process of cell proliferation/differentiation	Ito et al. 2012	Not DF*	Overexpressed	Overexpressed
FBgn0145802	FBgn0037470	<i>Tailor</i>	GO:0031054 - pre-miRNA processing	Subfertility. Reduction in sperm production.	Lin et al. 2017	Overexpressed	Overexpressed	Not DF*
FBgn0141892	FBgn0010052	<i>Jhe</i>	GO:0060179 - male mating behavior	Reduced courtship mutant males.	Ellis and Carney 2010; Wijesekera et al. 2016	Overexpressed	Not DF*	Overexpressed
FBgn0140780	FBgn0086681/ FBgn0261349	<i>mst36F/ mst36Fb</i>	GO:0007018: microtubule-based movement	Reduction of male fertility.	Di Cara et al. 2010	Not DF*	Not DF*	Underexpressed

*DF= Differentially Expressed

Source: prepared by the author.

Inheritance of gene expression

We compared the level of gene expression of HB_m, HA_w and HB_w with each parental line, following the six inheritance categories of McManus et al. (2010). Most of the genes in the ovaries and testes of the interspecific hybrids showed conserved expression (Fig. 5; supplementary tables S19 and S20).

HB_m showed conserved expression for 97.8% of the genes in ovaries and for 78.5% in testes (Fig. 5; supplementary table S19). Few genes were classified having additive expression in the female and male gonads (0.05 and 1.45%, respectively). In the dominant categories, HB_m exhibited an overrepresentation of genes with *D. arizonae*-like expression (1.8%, in ovaries and 9.5% in testes), and few genes were *D. mojavenensis*-dominant (Fig. 5; supplementary table S19). In the over-dominant and under-dominant categories, no genes were found for ovaries (0.03% of DEG). However, the testes showed several genes in these categories, with 0.5% over-dominant and 4.6% under-dominant (Fig. 5; supplementary table S19).

Similar to HB_m, most of the expressed genes in HA_w and HB_w female and male gonads had conserved expression, but in testes the conserved inheritance was lower than that in ovaries (Fig. 5; supplementary table S20). Additionally, very few genes in ovaries of HA_w and HB_w (0.3 and 0.22%) and testes (1.1 and 1.2%) displayed additive inheritance expression (Fig. 5; supplementary table S20), as it was observed for HB_m. An interesting finding is that most of the genes classified in dominant categories displayed a *D. arizonae*-like pattern of expression, regardless of crossing direction or gonad tissue (Fig. 5; supplementary table S20). Considering the over-dominant and under-dominant categories, few genes were in these categories in ovaries (0.16% in HA_w and 0.13% in HB_w) (Fig. 5; supplementary table S20). In hybrid testes, however, a large number of genes were classified as over- (1.1% in HA_w and 0.7% in HB_w) or under-dominant (0.53% in HA_w and 1.5% in HB_w) (Fig. 5; supplementary table S20).

Comparing all inheritance categories of HA_w and HB_w, significant differences in the expression profile of reciprocal hybrids were observed in ovaries ($X^2 = 146.67$, $P < 0.001$), which

was mainly influenced by the paternal effects, since 201 HA_w genes and 23 HB_w genes showed paternally dominant inheritance. In the testes, significant differences in the expression profile were also found between the reciprocal hybrids ($X^2 = 191.70$, $P < 0.001$), however, unlike in the ovaries, these differences were mainly influenced by maternal and subdominant inheritance. More specifically, 452 and 811 genes were found exclusively with maternal inheritance and 60 and 172 genes with subdominant expression in the HA_w and HB_w, respectively.

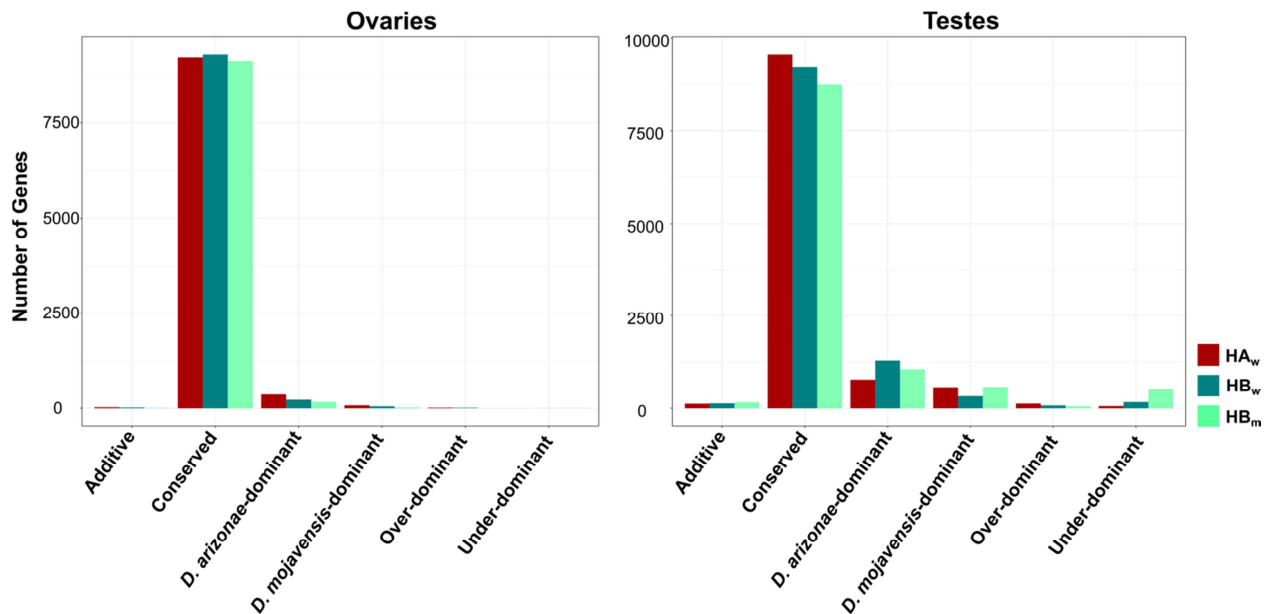


Fig. 5. Inheritance gene expression in ovaries and testes of HA_w, HB_w and HB_m. Genes were classified in six categories of inheritance expression depending on the significance of the differential expression measured by doing pairwise comparisons in all conditions, according McManus et al. (2010). These categories are Additive, when the expression levels are different between the two parental lines, but the hybrid expression is intermediate. Conserved, when the level of gene expression in hybrids is similar to both parental lines. *D. arizonae*-dominant or *D. mojaveensis*-dominant, when the hybrid expression is similar to only one parental line. Over-dominant, when the expression in the hybrids are significantly higher than in both parental lines and Under-dominant, when the hybrid expression is significantly lower than in both parental lines. Source: prepared by the author.

Discussion

In our study, the target species *D. mojavensis* and *D. arizonae* showed approximately 6% DEG in the ovaries and approximately 16% in the testes, agreeing with our previous findings of hybrid female transcriptomes (Lopez-Maestre et al. 2017) and with results obtained for male hybrid gonads (Llopart 2012). The divergence time of the studied species appeared to influence the rate of DEG, since *D. arizonae* and *D. mojavensis* presented levels of differential expression in the range of what was previously shown for other *Drosophila* species. It was reported that the pairs *D. yakuba* and *D. melanogaster* (~ 6.1 mya divergence time, Russo et al. 1995), *D. melanogaster* and *D. simulans* (~1.2 to ~5 mya divergence time, Russo et al. 1995; Kliman et al. 2000; Tamura et al. 2004; Cutter 2008), and *D. melanogaster* and *D. sechellia* (~1.2 to ~5 mya divergence time, Russo et al. 1995; Kliman et al. 2000; Tamura et al. 2004; Cutter 2008) exhibited 29-2%, 18-24 (Rifkin et al. 2003) and 78% DEG (McManus et al. 2010), respectively. In more recently diverged species, fewer DEG are observed, since in males of *D. yakuba* and *D. santomea* (~0.4 mya, Caricou et al. 2001) and *D. p. bogotana* and *D. p. pseudoobscura* (~0.25 mya, Wang et al. 1997), 19% (Llopart 2012) and 14.6% (Gomes and Civetta 2015) DEG were found, respectively.

The differences in the number of DEG between ovaries and testes could be explained by two main factors. First, there is a larger number of genes expressed in the male germline than that in the ovaries. Second, there is faster evolution for male-specific genes, such as male-biased genes, which has been previously reported in other *Drosophila* species. A previous study found that 83% of DEG in hybrids of *D. melanogaster* and *D. simulans* showed a sex-specific pattern, with a greater differential expression in males, indicating that some specific genes can evolve faster than others (Ranz et al. 2003). Moreover, other studies have shown that during the divergence process, male-biased genes display higher evolutionary rates, driven by positive selection (Artieri and Haerty 2007), and most of them are preferentially expressed in gonad tissues (Meiklejohn et al. 2003; Civetta et al. 2006; Haerty and Singh et al. 2006). Additionally,

gene ontology analyses showed that in testes, several DEG were related to mating and courtship behaviours, indicating that prezygotic reproductive barriers evolve faster among species, as was observed by Turissini et al. (2018).

Therefore, as was observed for other *Drosophila* species, the number of differentially expressed genes found in both gonad tissues of *D. arizonae* and *D. mojavensis* could be related to the divergence time, and hence, might be driven by regulatory differences, as was previously reported in drosophilids (McManus et al. 2010). The higher deregulation observed in the testes of *D. arizonae* and *D. mojavensis* subspecies can indicate that male-biased genes evolve faster than female-biased genes or non-sexual genes, agreeing with the faster-male hypothesis (Wu and Davis 1993) and with other results previously reported for *Drosophila* (Meiklejohn et al. 2003; Artieri and Haerty 2007; Llopart et al. 2018).

Differential expression in hybrid ovaries

Female hybrid gonads exhibited few DEG when compared with both parental lines, and most of them presented overexpression. Previous studies in hybrids of *D. arizonae*-*D. m. mojavensis* (Lopez-Maestre et al. 2017) and *D. melanogaster*-*D. simulans* (Kelleher et al. 2012) showed similar patterns. However, this result was not observed in *D. buzzatii* and *D. koepferae* (~4.49 mya) female hybrids, since no bias for over- or underexpression was observed (Romero-Soriano et al. 2017). We suggest that in hybrid ovaries from recently diverged species, the DEG tend to be overexpressed, but over time the global deregulation will increase due to the accumulation of genetic changes, and the number of over- and underexpressed genes will be symmetric.

Overall, no massive deregulation was observed in hybrid ovaries, since few genes were over- or underexpressed compared with the parental lines. One explanation for this finding is the higher stability of the female genome, which shows a slower evolution of gene expression, mainly for female-biased genes, as was reported in other *Drosophila* species (Meiklejohn et al.

2003; Llopart et al. 2018). This could have an important role in the fertile phenotype observed in these hybrids.

Differential expression in hybrid testes

Overall, male gonads showed a higher proportion of DEG compared with ovaries in all hybrids analysed. Moreover, hybrids from different crossing directions presented very different numbers of DEG compared with one parental line, agreeing with results obtained with hybrid males of *D. simulans*–*D. mauritiana* (Mohering et al. 2007) and of fire ants (Ommeto et al. (2012). Thus, in *D. mojaveensis* x *D. arizonae* male hybrids, one specific parental line could display a greater dominance of genetic elements, leading to higher divergence of the expression profile.

In general, hybrids from different crossing directions exhibited distinct proportions of over- or underexpressed genes. HA_w showed twice as many overexpressed genes than underexpressed genes in comparison to parental lines, while HB_w exhibited twice as many underexpressed genes than overexpressed genes. Similarly, HB_m exhibited nine times more underexpressed genes than overexpressed genes. Two main factors could influence these differences in the over- and underexpressed genes regarding the crossing direction. These hybrids differ only in a few genetic characteristics, such as the origin of the X chromosome and the genetic background; thus, it is possible that some specific epistatic factors among sexual chromosomes and autosomes play a role in the differential expression profile, as was found by Reed et al. (2008). Moreover, there is evidence that in hybrids of *D. arizonae* (females) x *D. mojaveensis* (males) crosses, type HB_m in this study, the immotile sperm observed can be an outcome from interactions between the *D. mojaveensis* Y chromosome and the 3rd autosome or X chromosome from *D. arizonae* (Vigneault and Zouros 1986). Regarding the reciprocal cross, it was previously shown that incompatibilities leading to hybrid sterility were related to interactions between the Y chromosome of *D. arizonae* and the 4th autosome of *D. mojaveensis*

(Pantazidis and Zouros 1988). In addition, in previous analyses of sperm motility and fertility that we have performed (data not shown), we found that HB_w and HB_m have no motile sperm and are therefore sterile. On the other hand, the sperm of HA_w was motile, although no progeny were produced in F₁×F₁ crosses or backcrosses; therefore, they were also sterile indicating that the disruption of spermatogenesis was weaker than in HB_w and HB_m males. These findings are in agreement with Hardy et al. (2011), who observed that *D. arizonae*-*D. m. mojavenensis* reciprocal hybrids display abnormal spermatid development. However, the disruption of spermatid formation is lower in hybrids carrying *D. mojavenensis* X chromosomes, as they exhibit sperm with normal tails; in contrast, hybrids with *D. arizonae* X chromosomes do not have individualized spermatids and their tails are severely disrupted. Considering these results, it can be suggested that the level of gene over- or underexpression can be related to the degree of spermatogenesis disturbance in recently diverged species. Consistent with these findings, it was reported that in male hybrids between *D. santomea* × *D. yakuba* presented less severe sterility (Moehring et al. 2006) and exhibited more overexpression of male-biased genes, than those presenting greater severity in sterility (Moehring et al. 2006) which showed more deregulated genes that were underexpressed (Llopart 2012), as it was observed for HB_m and HB_w.

Although a large number of misexpressed genes is probably related to the degree of the sterile phenotype, other factors can also influence this feature. Many studies have shown that genes involved in spermatogenesis are usually underexpressed in hybrids and display rapid sequence divergence driven by positive selection (Ranz et al. 2003; Michalak and Noor 2003; Haerty and Singh 2006; Moehring et al. 2007; Llopart 2012; Llopart et al. 2018). In agreement with these findings, one study observed that the substitution rate in male-biased DEG of hybrids was higher than that in non-sexual DEG and that the substitution rate of underexpressed genes was greater than that of overexpressed genes (Artieri and Haerty 2007). Therefore, we suggest that the higher level of differential expression in *D. arizonae*-*D. mojavenensis* hybrids, displaying stronger sterility mechanisms and exhibiting a higher number of underexpressed genes, can be

linked to the divergence of regulatory elements between the species, causing a breakdown in gene regulation during spermatogenesis, as was previously reported for hybrids of other *Drosophila* species (Wu and Davis 1993; Meiklejohn et al. 2003; Civetta et al. 2006; Haerty and Singh 2006; DuMont et al. 2007; Sundararajan and Civetta 2011).

The majority of DEG in testes of hybrids have functions related to spermatogenesis.

In male hybrids, the functionally enriched categories of DEG in HA_w testes were mainly related to components of plasma membrane, including cell adhesion functions. In agreement with these results, Gomes and Civetta (2015) also reported enrichment in overexpressed genes of *D. p. pseudoobscura*-*D. p. bogotana* male hybrids for similar categories. Cell adhesion genes are thought to play a role in the hybrid male sterility phenotype, since fertilization requires cell–cell recognition (Go et al. 2019). In fact, studies in other organisms, such as mammals and sea urchins, have shown that cell-adhesion proteins are fundamental for sperm-egg binding and that they can rapidly evolve to be species-specific (Palombi et al. 1992; Gao and Garbers 1998). Thus, the genes classified in these categories can have a direct role in the reproduction of HA_w males. However, we cannot discard the influence of DEG with other functions, such as those involved in regulatory processes and silencing, which can also have an impact on reproduction.

Unlike, in HB_w and HB_m testes most of DEG (over- and underexpressed) present enrichment for spermatogenesis and sperm movement function. Indeed, several studies have reported that in male hybrids most of the DEG are underexpressed and their functions are associated with spermatogenesis (Moehring et al. 2007; Brill et al. 2016; Li et al. 2016). A previous work that investigated DEG in male hybrids of *D. simulans* and *D. mauritiana* showed that the underexpressed genes presented functions related to the pre-meiotic stages of spermatogenesis, contributing to their sterile phenotype (Michalak and Noor 2003). Our findings of underexpressed genes in HB_w and HB_m primarily showed enrichment for sperm movement categories, as cilium, corroborating Hardy et al. (2011), and showed an association between

DEG and the immotile sperm phenotype.

Among the DEG shared by HB_m and HB_w males, several were directly linked to reproductive functions such as meiosis, spindle formation and sperm tail movement. This finding confirms the specific sterile phenotype of hybrids from crosses with *D. arizonae* females (Ruiz et al. 1990; Reed et al. 2008). Additionally, several studies have reported that males presenting the underexpression of specific genes, such as those involved in meiotic arrest and male-specific genes related to spermatogenesis and spermiogenesis, are often observed in sterile hybrids (White-Cooper et al. 1998; Maines and Wasseman 1999; Fossela et al. 2000; Jiang and White-Cooper 2003; Moehring et al. 2006; Fabian and Brill 2012; Lindsley et al. 2013).

Inheritance of expression

Our analyses showed that most of the genes had conserved inheritance of expression in hybrid ovaries and testes. This result is similar to previous findings for *D. arizonae*-*D. m. mojaveensis* female hybrids (Lopez-Maestre et al. 2017) but is quite different from that found in *D. sechellia*-*D. melanogaster* and *D. simulans*-*D. melanogaster* hybrids, since only 6% and 11% of their genes had conserved expression, respectively (Ranz et al. 2004; McManus et al. 2010). Furthermore, in *D. sechellia*-*D. melanogaster* (Cutter 2008) and *D. arizonae*-*D. mojaveensis* (Sanches-Flores et al. 2016), which present similar divergence times, the amount of genes classified as conserved is quite different, indicating that the interactions of different genetic changes accumulated in distinct species might have stronger or weaker effects on hybrids, causing disturbances in gene expression to variable degrees.

Among the DEG in the dominant categories in ovaries and testes of all hybrids, most showed *D. arizonae*-like expression, regardless of the crossing direction. These findings could indicate that in hybrid gonads of these species, the effect of *D. arizonae* genome is more important than the maternal effects. Therefore, we could speculate that for hybrid ovaries, *D. arizonae* has a stronger effect on the hybrid gene expression, likely due to regulatory sequences.

This result corroborates the findings of (Lopez-Maestre et. 2017), who verified that among the genes classified in the dominant category, for both crossing directions, *D. arizonae*-dominant inheritance was stronger. Likewise, the expression profile in ovaries of hybrids between *D. melanogaster* females x *D. sechellia* males showed that 49% of the genes were classified as dominant, and among these, 84% showed *D. sechellia*-like expression (McManus et al. 2010), indicating that the maternal species does not influence the expression profile of the hybrids. In the target species of the current study, the hybrid female gonads showed very few genes classified as over- or under-dominant, agreeing with previously reported results (Lopez-Maestre et al. 2017) and demonstrating the greater stability of their gene expression. In testes, on the other hand, the number of differentially expressed genes classified in these categories was higher, with a higher number of under-dominant genes for one specific cross direction. In male hybrid gonads, the underexpression of genes, mainly related to reproduction, has often been observed (Gomes and Civetta 2014; Haerty and Singh 2006; Li et al. 2016; McManus et al. 2010; Ometto et al. 2012), indicating that this can be related to disruptions in regulatory networks driven by the rapid divergence of male-biased genes leading to sterility (Haerty and Singh 2006; Llopart 2012).

Conclusion

Here, we showed that in hybrids of recently diverged species, as *D. arizonae* and *D. mojavensis*, the degree of gene misexpression is related with the severity of the sterile phenotype. Female hybrids, which are fertile, displayed few DEG, which probably have no influence on reproductive features. In contrast, in males hybrids, the degree of gene misexpression was dependent on the subspecies of *D. mojavensis* and, most importantly, the cross direction. Hybrids carrying the X chromosome of *D. mojavensis*, which are sterile but with motile sperm, displayed a smaller number of misexpressed genes, with a bias to overexpression, which are not directly related to sterile phenotype, since few genes acting on reproduction were

found. Nevertheless, in hybrids carrying the X chromosome of *D. arizonae*, which are sterile with immotile sperm, the disruption of gene expression was higher and presented a bias towards underexpression. Surprisingly, most of these genes were directly related with spermatogenesis-related functions, as well as, sperm movement. Therefore, our data suggest that the disruption in gene expression in hybrid males, from recently diverged species, could be connected to incompatibilities in regulatory sequences, due to adaptive divergence of alleles between the parental species, leading to misregulation of gene expression in hybrids, which could be the main factor responsible for the male sterile phenotype. However, more analyses must be undertaken to clarify the regulatory differences between this pair of species. These include investigating the divergence of male-biased gene sequences, regulatory studies (cis-trans regulatory changes and the impact of micro-RNAs in gene expression), and functional analyses of the genes identified in the current study. Thus, we can obtain a better understanding of their relationship with the sterile phenotype in the initial steps of hybrid incompatibility.

Materials and Methods

***Drosophila* strains and RNA sequencing**

Intraspecific and interspecific reciprocal crosses were performed between *D. arizonae*, from Metztitlan, Hidalgo, México (stock number: 15081-1271.17), and two subspecies of *D. mojavensis*: *D. m. mojavensis*, from Anza Borrego Desert, California, USA (stock number: 15081-1352.01) and *D. m. wrigleyi*, from Catalina Island, California, USA (stock number: 15081-1352.22). Crosses were performed with 3-day-old flies, ten males and ten females, in 2.3 × 9.5 cm vials containing a standard *Drosophila* medium supplemented with yeast under the same temperature (23°C) and humidity conditions. One-day-old virgin female and male offspring (control and F₁ hybrids) were collected after hatching and were isolated until they reached sexual maturity. The male and female reproductive tracts of 9-12-day-old flies were dissected in PBS (phosphate-buffered saline) and stored at -80°C until used for RNA extraction.

To verify the hybrid status of the F₁ offspring of interspecific crosses, 10 individuals of each cross were randomly collected for DNA extraction, and PCR for the ribosomal ITS-1 (*internal transcribed spacer 1*) from the 18S gene region (NCBI Reference Sequence: EU306666.1) (Baffi and Ceron 2002) was performed. The oligonucleotide primer for ITS-1 amplified 500 bp and 550 bp amplicons, respectively, in *D. arizonae* and in *D. mojavensis*. Therefore, in hybrids, two different fragments corresponding to *D. arizonae* and *D. mojavensis* alleles were expected.

After confirming the hybrid status, 30 pairs of ovaries and 50 pairs of testes were used to perform total RNA extraction, in two biological replicates, using the RNeasy kit (Qiagen). The samples were treated with DNase (DNA-free Kit, Ambion) and stored at -80°C. The samples were quantified by fluorescence in a Bioanalyzer 2100 (Agilent). Sequencing was performed using the GenomEast platform by a member of the France Génomique consortium (ANR-10-INBS-0009) with an Illumina HiSeq 4000. The samples were sequenced in 2 × 100 paired-end reads, and the average size of the inserts was 300 base pairs.

Twelve transcriptomes were sequenced with two biological replicates, corresponding to *D. arizonae*, *D. m. mojavensis*, *D. m. wrigleyi* (controls, ovaries and testes) and hybrids from crosses between *D. arizonae* and both *D. mojavensis* subspecies (ovaries and testes). The hybrid transcriptomes from *D. m. mojavensis* female and *D. arizonae* male crosses were not sequenced because the hybrid incompatibility in this direction of the cross is very high and it was not possible to obtain enough material to perform RNA extraction.

Mapping and quantification of expression

The sequenced transcriptomes were trimmed using UrQt (Modolo and Lerat 2015) to remove polyA tails (from RNA-Seq reads) and low-quality nucleotides. The sequence quality was then checked with FastQC software. The transcriptomes were aligned against all annotated coding sequences (CDS) of the *D. mojavensis* r1.04 public genome (Clark et al. 2007) (available

at <http://flybase.org/>). Overall, 21,915 RefSeq sequences were downloaded from <https://www.ncbi.nlm.nih.gov/refseq/>. From those sequences, 20,110 corresponding to mRNA were used as a reference to perform the alignments. This approach was used because the public genome of *D. mojavensis* presents the best quality of sequences and, because *D. mojavensis* and *D. arizonae* are recently diverged species, a large divergence in their coding protein genes was not expected. Kallisto (Bray et al. 2016) was used to map the reads from parental and hybrid transcriptomes against the *D. mojavensis* r1.04 reference transcripts. Kallisto is able to perform rapid pseudoalignment to quickly determine the compatibility of the reads with their respective targets. The pseudoalignment of reads preserves the key information needed for quantification and is robust against error, presenting a similar accuracy as other alignment tools (Bray et al. 2016). After the mapping procedure, biomaRt (Durinck et al. 2005), an R (3.6.1) (R Core Team 2018) Bioconductor package, was used to recover the gene names corresponding to each transcript from the reference. This was possible because the biomaRt database is maintained by Ensembl, providing direct access to a diverse set of data and enabling a wide range of powerful online queries, from gene annotation to database mining. Subsequently, due to several genes displaying different isoforms, the package tximport (Soneson et al. 2015) was used to summarize the transcript level estimation for the gene level analysis, allowing us to use these data for the differential expression of gene-level counts.

Differential expression analyses

Differential expression analyses were performed using DESeq2 (Love et al. 2014), an R (3.6.1) package [85], using raw read counts to identify differential gene expression in the hybrids compared to the parental species (controls lines) for each gonad tissue. This package normalizes counts using size factors that are estimated according to the median counts taken for all genes. Additionally, DESeq2 estimates the means and variances of raw read counts and tests for differential expression based on a model using a negative binomial distribution and uses

Benjamini–Hochberg multiple test correction (FDR level of 0.1) (Benjamini and Hochberg 1995). Genes were classified as significantly differentially expressed when the p-value adjusted, by FDR level, was below 0.01 and an at least 2-fold change in differential expression was identified. Transcripts that presented less than ten mapped counts, in all conditions tested, were excluded from the analyses.

Gene ontology enrichment analyses

GO terms (Ashburner et al. 2000) were investigated using a list of DEG in ovaries and testes of hybrids. For this, an orthologous gene table for *Drosophila* species was downloaded from <http://flybase.org/>. The *D. melanogaster* ortholog genes corresponding to DEG in hybrids were identified and submitted to the PANTHER Classification System platform (Mi et al. 2013; Thomas et al. 2003) available at <http://geneontology.org/>, in order to verify the gene function by biological processes and cellular components. In this analysis, a set of genes (target gene list) were compared with a reference list, which corresponds to all the genes in the PANTHER database for a selected organism, using as statistical test Fisher's exact test with FDR correction. After that, we selected all the GO terms, from our target gene list, present in FDR < 0.05 and submitted them to REVIGO web server (Supek et al. 2011). By using REVIGO we were able to summarize and remove redundant GO terms.

Inheritance classifications

The R (3.6.1) package (R Core Team 2018) was used to sort genes in terms of differences in their expression levels between each parental line and their respective reciprocal hybrids, according to McManus et al. (2010). The expression data was transformed into log percentage, and a threshold of 2-fold change and adjusted p-value < 0.01 was set to determine the significance of differential gene expression. Genes not differentially expressed were considered to have the same expression level as the parental lines, being conserved. Genes considered

differentially expressed were classified as additive, conservative, dominant, under-dominant or over-dominant. Chi-square statistical tests were performed in R (3.6.1) package.

Data Availability Statement:

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have no competing interests.

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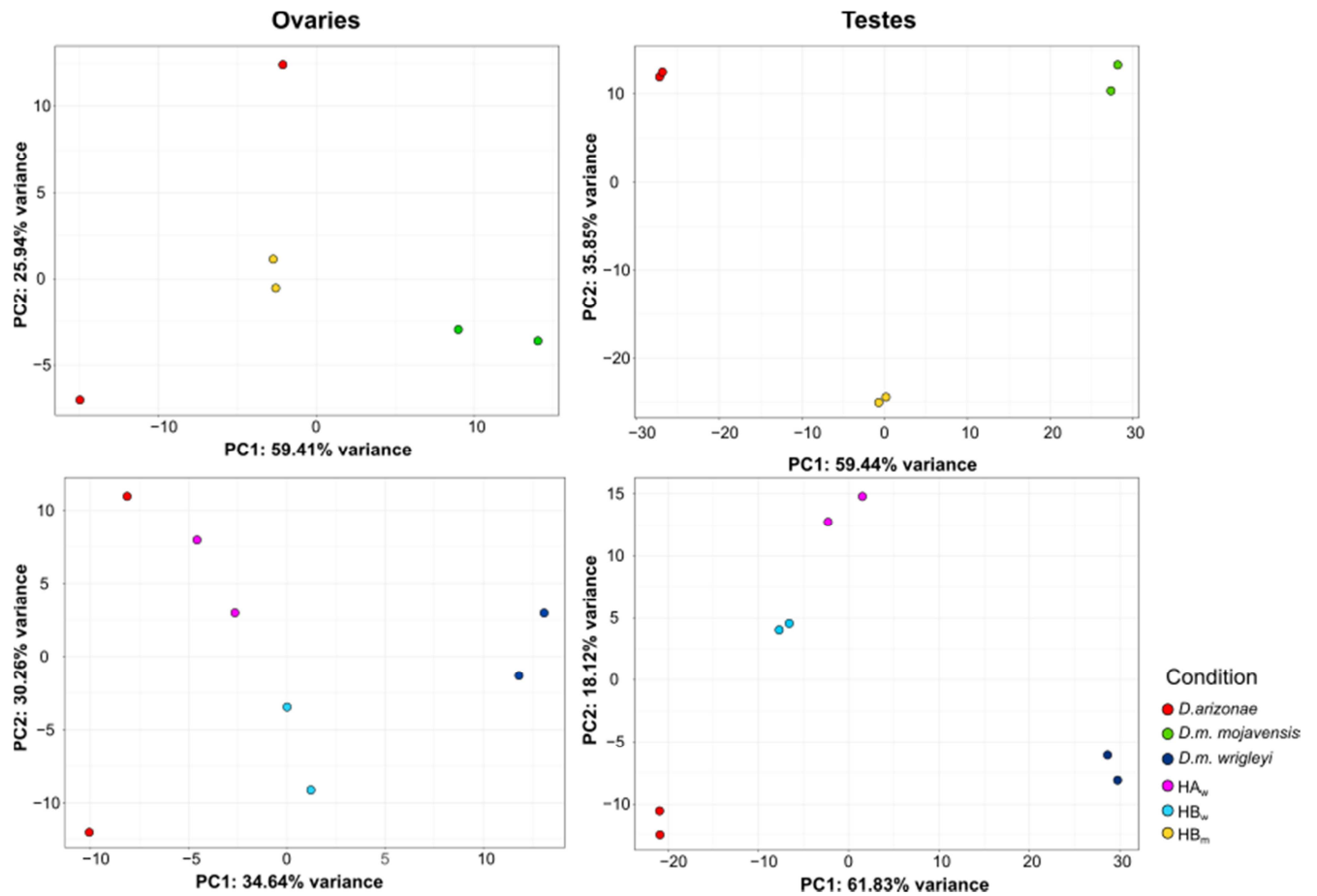
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Supplementary material

Tables S2, S3, S4, S5, S8, S9, S10, S11, S12, S13, S14, S15, S16, S17, S18, S19 and S20:

Available from the author on reasonable request.



Supplementary Fig. S1. Principal component analysis (PCA). Transcriptome replicates of

ovaries (on the left) and testes (on the right) for *D. arizonae*, *D. m. mojavenensis*, *D. m. wrigleyi*,

HA_w, HB_w and HB_m. Source: prepared by the author.

Supplementary table S1. Number of sequenced and aligned reads of *D. arizonae*, *D. mojavensis* and their reciprocal hybrids. The number of reads is showed by replicate and the percentage values are the alignment rate for each transcriptome.

Alignment rate by replicate				
Samples	Ovaries		Testes	
	Number of sequenced reads	Number of uniquely mapped reads	Number of sequenced reads	Number of uniquely mapped reads
<i>D. arizonae</i> replicate 1	40,110,913	34,139,096 (85.1%)	28,086,191	21,559,308 (76.7%)
<i>D. arizonae</i> replicate 2	44,205,411	37,795,821 (85.5%)	34,237,590	26,573,274 (77.6%)
<i>D. m. mojavensis</i> replicate 1	35,696,266	31,236,825 (87.5%)	29,643,424	23,686,597 (79.9%)
<i>D. m. mojavensis</i> replicate 2	42,995,065	36,736,531 (85.4%)	28,912,494	23,289,405 (80.5%)
<i>D. m. wrigleyi</i> replicate 1	28,401,359	24,114,079 (85%)	29,865,407	23,830,369 (79.7%)
<i>D. m. wrigleyi</i> replicate 2	39,477,665	34,288,043 (86.8%)	33,417,722	27,025,306 (80.8%)
HA _w replicate 1	41,216,806	34,383,790 (83.4%)	30,456,531	24,176,528 (79.3%)
HA _w replicate 2	35,564,989	29,404,539 (82.6%)	34,858,245	27,328,461 (78.4%)
HB _w replicate 1	35,696,107	30,726,812 (86%)	34,971,680	26,731,095 (76.4%)
HB _w replicate 2	40,326,965	33,984,764 (84.2 %)	26,878,152	20,859,798 (77.6%)
HB _m replicate 1	31,426,297	26,590,922 (84.6%)	40,654,656	31,636,630 (77.8%)
HB _m replicate 2	34,951,127	29,528,040 (84.4%)	36,636,279	28,206,652 (80%)

Source: prepared by the author.

Supplementary Table S6. Number of DEG between HB_m and their respective parental lines. O: overexpressed genes; U: underexpressed genes.

Comparisons	<i>D. arizonae</i> x <i>D. m. mojavensis</i>	
	Ovaries	Testes
HB _m x <i>D. arizonae</i>	25 (0.26%)	1327(11.9%)
	O: 4 (%)	O: 431 (32.5%)
	U: 21 (%)	U: 895 (67.5%)
HB _m x <i>D. m. mojavensis</i>	180 (1.93%)	1801 (16%)
	O: 145 (%)	O: 597(33.1%)
	U: 35 (%)	U: 1204 (66.9%)

Source: prepared by the author.

Supplementary Table S7. Number of DEG between HA_w and HB_w versus their respective parental lines and between the reciprocal hybrids. O: overexpressed genes; U: underexpressed.

Comparisons	<i>D. arizonae</i> x <i>D. m. wrigleyi</i>	
	Ovaries	Testes
HA _w x <i>D. arizonae</i>	124 (1.27%) O: 99 (79.8%) U: 25 (20.2%)	895 (7.9%) O: 532 (59.5%) U: 363 (40.5%)
HA _w x <i>D. m. wrigleyi</i>	415 (4.2%) O: 345 (83.1%) U: 70 (16.9%)	1101 (9.7%) O: 694 (63%) U: 407 (37%)
HB _w x <i>D. arizonae</i>	89 (0.91%) O: 65 (73%) U: 24 (24%)	719 (6.3%) O: 325 (45.2%) U: 394 (54.8%)
HB _w x <i>D. m. wrigleyi</i>	266 (2.7%) O: 201 (75.5%) U: 65 (24.5%)	1684 (14.9%) O: 767 (45.5%) U: 917 (54.5%)
HA _w x HB _w	40 (0.4%) O: 25 (62.5%) U: 15 (37.5%)	728 (6.4%) O: 494 (67.8%) U: 234 (32.2%)

Source: prepared by the author.

Supplementary Table S19. Inheritance expression in hybrids from *D. arizonae* and *D. m. mojaveensis*. Number of genes classified in each category of inheritance for ovaries and testes hybrids.

Category	<i>D. arizonae</i> x <i>D. m. mojaveensis</i>	
	HB _m ovaries	HB _m testes
Additive	5 (0.05%)	162 (1.45%)
Conservative	9124 (97.8%)	8757 (78.5%)
<i>D. arizonae</i>-dominant	172 (1.8%)	1063 (9.5%)
<i>D. mojaveensis</i>-dominant	17 (0.18%)	588 (5.2%)
Over-dominant	0	57 (0.5%)
Under-dominant	3 (0.03 %)	519 (4.6%)

Source: prepared by the author.

Supplementary Table S20. Inheritance expression in hybrids from *D. arizonae* and *D. m. wrigleyi*. Number of genes classified in each category of inheritance for ovaries and testes hybrids.

Category	<i>D. arizonae</i> x <i>D. m. wrigleyi</i>			
	Ovaries		Testes	
	HA _w	HB _w	HA _w	HB _w
Additive	29 (0.3%)	22 (0.22%)	126 (1.1%)	135 (1.2%)
Conservative	9220 (94.9%)	9295 (95.6%)	9562 (85%)	9225 (82%)
<i>D. arizonae</i>-dominant	370 (3.8%)	230 (2.3%)	785 (6.9%)	1298 (11.5%)
<i>D. mojavensis</i>-dominant	79 (0.81%)	53 (0.54%)	579 (5.1%)	333 (2.9%)
Over-dominant	16 (0.16%)	13 (0.13%)	130 (1.1%)	79 (0.7%)
Under-dominant	0	1 (0.01%)	60 (0.53%)	172 (1.5%)

Source: prepared by the author.

5. CAPÍTULO 3

Misregulation of transposable elements in *Drosophila mojavensis* and *Drosophila arizonae* hybrids

Cecilia Artico Banho^{1,2}, Vincent Mérel², Edoardo Estevam de Oliveira Lobl¹, Thiago Yukio Kikuchi Oliveira³, Annabelle Haudry², Cristina Vieira², Claudia Marcia Aparecida Carareto¹

¹UNESP - São Paulo State University, Department of Biology, São José do Rio Preto, São Paulo State (SP), Brazil

² Université de Lyon, Université Lyon 1, CNRS, Laboratoire de Biométrie et Biologie Evolutive UMR 5558, F-69622 Villeurbanne, France

³Laboratory of Molecular Immunology, The Rockefeller University, New York, NY, USA

Corresponding author: ceci.abanho@gmail.com, carareto@ibilce.unesp.br

ABSTRACT

Interspecific hybridization may lead to sterility and/or inviability through the misexpression of genes and transposable elements (TEs). In *Drosophila*, many studies have reported the mobilization of few TEs at the intraspecies level, which are potentially responsible for gonadal dysgenesis, while at the interspecies level, massive TE mobilization has been observed in species with large divergence times. However, few studies have examined the consequences of interspecies hybridization in species with recent divergence times. Therefore, we chose *Drosophila mojavensis* and *D. arizonae* to investigate this question. These species present a recent divergence (~1.5 million years (m.y.)) and are able to produce hybrids in the laboratory. We have sequenced their transcriptomes by performing of RNA-Seq from the ovaries and testes of *D. arizonae*, two *D. mojavensis* subspecies, and their reciprocal hybrids to analyse the effect of hybridization on post-zygotic reproductive isolation. In hybrid ovaries, most of the TE

families are not deregulated (98-99.6%). However, in testes, TE deregulation was observed at a rate of 11.4% and presented a bias towards overexpression. We have sequenced the piRNAs from the ovaries and testes of hybrids and parental lines to obtain a better understanding of the causes of TE upregulation. Here, several deregulated TEs in male and female gonads have had complementary piRNAs mapped onto them; however, the control of their expression was not observed. In males, some genes, such as chromatin genes and piRNA pathway genes, showed divergent expression in hybrids compared with that in the parental lines, potentially indicating that TE deregulation is associated with the divergence of regulatory genes that play a role in modulating transcriptional and post-transcriptional mechanisms.

Keywords: TEs, differential expression, piRNA, *repleta* group, hybridization.

INTRODUCTION

Speciation is a complex process resulting from the split of one ancestral lineage in two reproductively isolated populations (ORR; COYNE, 1989; COYNE; ORR, 1997). However, under specific conditions, species present incomplete reproductive isolation and are able to cross; nevertheless, their hybrids might exhibit a decrease in fitness that is often due to inviability and sterility. According to the Dobzhansky–Muller model (1936, 1942), the presence of allele-specific genetic variation in different species or populations may lead to deleterious epistatic interactions, disrupting regulatory networks and causing severe consequences in hybrids (ORR; COYNE, 1997; KELLEHER *et al.*, 2012, ROMERO-SORIANO *et al.*, 2017). Although numerous efforts have focused on obtaining a better understanding of the mechanisms underlying reproductive isolation, few studies have specifically analysed deregulated genes or other factors directly associated with reproductive barriers (TING *et al.*, 1998; MICHALAK; NOOR, 2003; RANZ *et al.*, 2004; HAERTY; SINGH, 2006; MA; MICHALAK, 2011; GOMES; CIVETTA, 2014; LI *et al.*, 2016; HILL *et al.*, 2016). Moreover, the deregulation of gene expression may not be the only or the main cause of hybrid breakdown, but a consequence of

other factors, such as divergence in regulatory sequences, mobilization of transposable elements or epigenetic changes (MCMANNUS *et al.*, 2010; REBOLLO *et al.*, 2012; AKKOUCHE *et al.*, 2013; IWASAKI *et al.*, 2016; FABLET *et al.*, 2019).

Transposable elements (TEs), which are repetitive DNA sequences capable of moving from one place to another in the genome and between genomes, are important sources of genetic variability and genomic evolution (KIDWELL; LISCH, 2001; VAN de LAGEMAAT *et al.*, 2003; WONG; CHOO, 2004). Several studies have reported the effect of TEs on reproductive isolation, due to their ability to modify regulatory networks and gene expression and lead to structural rearrangements (KIDWELL; LISCH, 2001; SERRATO-CAPUCHINA; MAMUTE, 2018). TE activation is induced by environmental stress, such as temperature and pathogens (CAPY *et al.*, 2000), and, as proposed by Barbara McClintock (1980), by genomic shock, such as interspecific hybridization (O'NEILL *et al.*, 1998; LABRADOR *et al.*, 1999; FONTDEVILA, 2005; GUERREIRO, 2012). The disruption of genome stability resulting from hybridization is attributed to the divergence of regulatory sequences and/or the content of TEs (SESSEGOLO *et al.*, 2016). The effects of TEs on interspecific hybrids may be beneficial or deleterious, depending on the species. In successful hybrids of *Helianthus annuus* and *Helianthus petiolaris* (BAACK *et al.*, 2005; KAWAKAMI *et al.*, 2011), a burst of transposition was identified involving two main TE families (*Ty1/Copia*-like and *Ty3/Gypsy*-like), which contributed to increase the size of the hybrid genome by ~50%. In animals, on the other hand, the consequences of hybridization are potentially more severe. Hybrids of marsupials in the *Macropodidae* family have shown centromeric expansion and chromosomal instability, which was associated with the amplification of *KerV-1* transposable elements and satellite repeats, decreasing the fitness of the hybrids (O'NEILL *et al.*, 1998).

Several studies have described the consequences of TE mobilization in *Drosophila* species at different levels of hybridization. In intraspecies hybrids, some TEs are responsible for hybrid dysgenesis syndrome, such as the I/R system (PICARD, 1976) and *hobo* element

(BLACKMAN *et al.*, 1987; YANNOPOULOS *et al.*, 1987) in *D. melanogaster*, the P/M system (KIDWELL *et al.*, 1977; HILL *et al.*, 2016) in *D. melanogaster* and *D. simulans*, and the gonadal dysgenesis caused by the mobilization of some specific TEs in *D. virilis*, such as the *Penelope* element (PETROV *et al.*, 1995). This syndrome is characterized by gonadal atrophy and generally affects offspring obtained from crosses in only one direction. The deregulation of some TEs in one specific direction of the cross is associated with differences in the TE content between the genomes of the parental populations and their regulatory system, in which the piRNAs (a class of small RNAs named piwi-interacting RNAs) play the main role (ARAVIN *et al.*, 2007; MALONE *et al.*, 2009; SENTI; BRENNECKE, 2010). These small RNAs function in the *Drosophila* germline at post-transcriptional and transcriptional levels together with a complex of *Argonaute* proteins, which recognize homologous TEs and induce their degradation (KLATTENHOFF; THEURKAUF, 2008; SENTI; BRENNECKE, 2010; ROZHKOV *et al.*, 2013; LUO; LU, 2017). In intraspecies or interspecies crosses, females lacking one or a few TE families are not able to produce specific piRNAs, which are maternally deposited, and hence, silence TE expression (LUO; LU, 2017). As an example, in interspecies hybrids obtained from *D. virilis* and *D. lummei* crosses, the magnitude of prezygotic and postzygotic isolation depends on the presence of the transposon *Penelope*. The lack of this element in the female parent is responsible for hybrid inviability, likely because of parental mismatch between the number or identity of TEs and the machinery responsible for *Penelope* repression (CASTILLO; MOYLE, 2019). This potential to cause negative genetic interactions via TE derepression in hybrids places TEs within the classical Dobzhansky-Muller model (1936, 1942) for the evolution of incompatibilities. Overall, the role of repetitive elements, such as TEs and satellite sequences, in postzygotic reproductive isolation has been suggested by some authors that correlate differences in their content between species with increased transcription and dysfunction in hybrids (SATYAKI *et al.*, 2014; ROMERO-SORIANO *et al.*, 2017; KOTOV *et al.*, 2019). Moreover, in interspecies *Drosophila* hybrids obtained from highly divergent species, high levels of TE

expression have been observed (SATYAKI *et al.*, 2014). In hybrids of *D. melanogaster* x *D. simulans*, which diverged between 1.2 to 5 m.y. (RUSSO *et al.*, 1995; KLIMAN *et al.*, 2000; TAMURA *et al.*, 2004; CUTTER, 2008), and *D. buzzatii* x *D. koepferae*, which diverged between 4.02 to 4.63 mya (GOMEZ; HASSON 2003; LAAYOUNI *et al.*, 2003; OLIVEIRA *et al.*, 2012), bursts of transposition were observed. This massive TE mobilization was associated with the adaptive divergence of piRNA pathway genes, leading to the disruption of the system regulating TEs and contributing to hybrid sterility to some extent (LABRADOR *et al.*, 1999; KELLEHER *et al.*, 2012; VELA *et al.*, 2014; GUERREIRO, 2015; ROMERO-SORIANO *et al.*, 2017).

In addition, some studies have reported the causes and consequences of mobilization of TEs at the intra- and interspecies level. The focus of the present study is the role of TEs in the early stages of hybrid incompatibility. Thus, we chose *D. arizonae* and *D. mojavensis* (*repleta* group, *Drosophila* genus) to investigate this topic because they are sibling species with a shorter divergence time (1.5 m.y., SANCHES-FLORES *et al.*, 2016). They produce hybrids in the laboratory with asymmetrical sterility, because the females are fertile and the males, most of which are sterile, have different phenotypes, with the sperm of the sterile males being mobile or immobile, depending on the direction of crossing (REED; MARKOW, 2004; REED *et al.*, 2008; JENNINGS; ETGES, 2009). Moreover, the TE contents of these species have been characterized, enabling a study of differential expression in hybrids and both parental lines. In the first publication of the *D. mojavensis* genome (r 1.04), the annotation of TEs showed that 8.92% of the genome is composed of TEs, 45% of which are LTRs, ~28% are LINEs and 13% are DNA transposons (CLARK *et al.*, 2007). However, using a new approach to annotate TEs (the pipeline TEdenovo present in the suite of applications REPET, FLUTRE *et al.*, 2011), we showed that the *D. mojavensis* r 1.04 public genome is composed of 16.6% TEs (data not shown), the same content reported by Rius *et al.* (2016) and twice as many TE families that were previously identified by Clark *et al.* (2007). However, fewer TEs were annotated in the genomes

of two *D. arizonae* lines, 10% (SANCHEZ-FLORES *et al.*, 2016) and 6.4% (data not shown), which is explained by the underestimation of TE sequences in genomes obtained using NGS compared to genomes obtained using Sanger sequencing. In the latter species, 3.5% of TEs are Class I elements, known as retrotransposons, and 2.8% are Class II elements, known as DNA transposons (data not shown).

Considering the characteristics mentioned above, this pair of species is interesting as a model to study differences in the expression of TEs in the reproductive organs of hybrids, as well as their regulation in different gonad tissues by analysing the piRNA pathway. At the same time, they allow us to test the hypothesis that the rate of TE deregulation in hybrids depends on the time of divergence of the parental species and may exert a direct effect on the male phenotype. Indeed, two previous studies have analysed the expression of TEs in female and male hybrids of *D. arizonae* and *D. mojavensis* (CARNELOSSI *et al.*, 2014; LOPEZ-MAESTRE *et al.*, 2017). According to the findings reported by Lopez-Maestre *et al.* (2017), no global burst of transposition is detected in the female germline of hybrids, but the transposition of specific TE families is detected in male and female gonad tissues (CARNELOSSI *et al.*, 2014). In addition, studies of the expression profile of TEs in male hybrids of these species, as well as their associations with the TE regulatory system in the testes are lacking.

Therefore, in this study, we aimed to investigate the profile of TE expression in the ovaries and testes of hybrids obtained from crosses between *D. arizonae* and two subspecies of *D. mojavensis* (*D. m. mojavensis* and *D. m. wrigleyi*), as well as the association of deregulated TE families with the systems regulating the piRNA and siRNA pathways. A higher level of TE deregulation is observed in the male gonads compared with the ovaries. Moreover, in ovaries and testes, we identified complementary piRNAs that are able to degrade and start the *ping-pong* amplification cycle for most of the deregulated TEs. However, TE expression is not completely controlled by the piRNA pathway, suggesting that other mechanisms might control TEs in reproductive tissues.

MATERIALS AND METHODS

Drosophila strains and crosses

Intraspecies and interspecies reciprocal crosses were performed between *D. arizonae* from Metztitlan, Hidalgo, México (stock number: 15081-1271.17) and two subspecies of *D. mojavensis*: *D. m. mojavensis* from Anza Borrego Desert, California, USA (stock number: 15081-1352.01) and *D. m. wrigleyi* from Catalina Island, California, USA (stock number: 15081-1352.22). Crosses were performed with three-day-old flies, ten males and ten females, in 2.3 × 9.5 cm vials containing a standard *Drosophila* medium supplemented with yeast at the same temperature (23°C) and humidity. One-day-old virgin female and male offspring (control and F1 hybrids) were collected after hatching and isolated until they reached sexual maturity (~9-12-day-old flies).

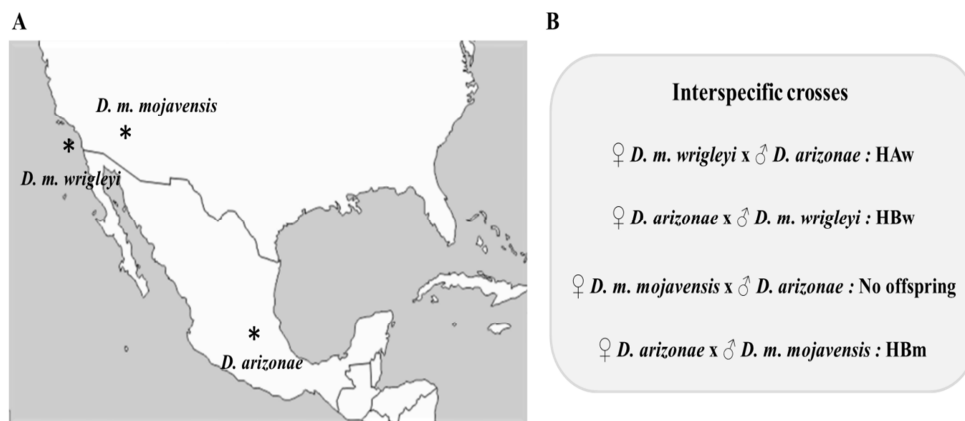


Figure 1. A) Geographic distribution of *D. m. wrigleyi*, *D. m. mojavensis* and *D. arizonae* lines used in this study (http://www.d-maps.com/carte.php?num_car=1404&lang=en). B) Crosses between the subspecies of *D. mojavensis* and *D. arizonae* with their respective hybrids, which were named regarding cross direction. HAw and HBw: hybrids from crosses between *D. arizonae* and *D. m. wrigleyi*, HBm: hybrids from crosses between *D. arizonae* and *D. m. mojavensis*. A and B represent the cross direction (A = crosses with *D. mojavensis* mothers; B = crosses with *D. arizonae* mothers. Source: prepared by the author.

RNA Extraction, library preparation and sequencing

Flies were dissected in PBS (phosphate-buffered saline) 9-12 days after birth, and the male and female gonads were stored at -80°C until used for RNA extraction. We confirmed the hybrid status by randomly collecting 10 F1 individuals from intra- and interspecies crosses for DNA extraction, and PCR was performed to analyse the ribosomal ITS-1 (*internal transcribed*

spacer 1) sequence from the 18S gene region, NCBI Reference Sequence: EU306666.1 (BAFFI; CERON, 2002). The oligonucleotide primers for ITS-1 amplify an amplicon with a size of 500 bp and 550 bp in *D. arizonae* and in *D. mojavensis*, respectively. Therefore, in the hybrids, two different fragments corresponding to the *D. arizonae* and *D. mojavensis* alleles are expected. After confirming the hybrid status, 30 pairs of ovaries and 50 pairs of testes were selected to perform total RNA extraction using the RNeasy kit (Qiagen). The samples were treated with DNase (DNA-free Kit, Ambion) and stored at -80°C. The samples were quantified by measuring the fluorescence in a Bioanalyser 2100 (Agilent). Two Illumina libraries of 2 × 100 paired-end reads containing inserts of 300 bp on average were prepared for each sample (ovaries and testes of *D. arizonae*, *D. m. mojavensis*, *D. m. wrigleyi* and their respective F1 hybrids) using 1 µg of purified RNA. Sequencing was performed using the GenomEast platform, a member of the ‘France Génomique’ consortium (ANR-10-INBS-0009)”, with Illumina technology in an Illumina HiSeq 4000 instrument. The hybrid transcriptomes from *D. m. mojavensis* females and *D. arizonae* males were not sequenced, because the hybrid incompatibility in this direction of the cross is very high and we were unable to obtain a sufficient amount of material to perform RNA extraction.

TE library construction

The new TEs were identified using the TEdenovo pipeline in the REPET v 2.5 package (available at <https://urgi.versailles.inra.fr/Tools/REPET>) (FLUTRE *et al.*, 2011). The consensus sequences obtained were classified up to the family level by performing phylogenetic analyses of the conserved domains of each TE order. The conserved domains of each consensus sequence were aligned with the corresponding domains of reference sequences from *Drosophila* available at Repbase (BAO *et al.*, 2015), along with new reference sequences of LTR elements described by Bargues and Lerat (2017), and *Tc1-Mariner* elements listed in the study by Hernandez-Hernandez *et al.* (2017) using the MAFFT 7 software (KATO; STANDLEY, 2013). The non-

informative locations in each alignment were removed using the trimAL tool v1.3 (CAPELLA-GUTIÉRREZ *et al.*, 2009). The amino acid evolution model for the phylogenetic reconstructions was created using the Smart Model Selection software (LEFORT *et al.*, 2017). The reconstructions of the trees were performed using the maximum likelihood method and PhyML 3.0 software (GUINDON *et al.*, 2010). For the TE annotation, Repbase repeats from *Drosophila* species (BAO *et al.*, 2015) were added to the custom library obtained from the TEdenovo step. The genomes (*D. arizonae*, which was previously sequenced and assembled by our research group, and the *D. mojavensis* r 1.04 public genome) were masked with RepeatMasker (version 4.0.7) (SMIT *et al.*, 2015) and RMBlast engine (version 2.2.27+) using the standard options, except for the cutoff (score 250), no low and no RNA. The “one code to find them all” pipeline (available at <http://doua.prabi.fr/software/one-code-to-find-them-all>) was used to correctly estimate the number of copies of the TEs identified using RepeatMasker.

Small RNA extraction, library Preparation, and sequencing

Small RNAs were extracted from the ovaries (70 pairs) and testes (100 pairs) of *D. arizonae*, *D. m. mojavensis*, *D. m. wrigleyi*, and their interspecific hybrids using HiTrap Q HP anion exchange columns, as described in the study by Greutzinger *et al.* (2014). Two Illumina libraries were prepared for each sample from 1-6 ng of the purified small RNA fraction using the TruSeq Small RNA Library Prep Kit (Illumina, San Diego, CA) and the GenomEast platform, a member of the ‘France Génomique’ consortium (ANR-10-INBS-0009)”, and sequencing was performed using Illumina technology and an Illumina HiSeq 4000 instrument (1x50 bases). Overall, 729 million reads were obtained, and reads with a length of 23–30 nt were retained as piRNAs, whereas reads with a length of 21 nt were considered siRNAs.

TE and gene read mapping and the analysis of differential expression

The sequenced transcriptomes were trimmed using UrQt (MODOLO; LERAT, 2015) to remove polyA tails (from RNA-Seq reads) and low-quality nucleotides, and then the sequence quality was assessed using FastQC software. TE expression analyses were performed with the module TEcount from the TETools pipeline (LERAT *et al.*, 2017; <https://github.com/l-modolo/TETools>) to ensure the accuracy of the map of the RNA-Seq data. RNA-Seq reads were aligned to our homemade TE library containing approximately 894 candidate TE families from the *D. arizonae* and *D. mojavensis* r 1.04 genomes using Bowtie2. The read count step was computed for each TE family by adding all reads mapped on copies of the same family. The parental and hybrid transcriptomes were aligned to all annotated (20,110 mRNAs) Coding Sequence (CDS) of *D. mojavensis* r1.04 public genome (CLARK *et al.*, 2007) available on <http://flybase.org/> using Kallisto, to verify the expression of genes regulating the piRNA pathway (BRAY *et al.*, 2016). After the mapping procedure was complete, biomaRt (DURINCK *et al.*, 2005), an R 3.6.1 (R Core Team, 2018) Bioconductor package, was used to recover the gene names corresponding to each transcript from the reference genome. Afterwards, because several genes are expressed in different isoforms, the package tximport (SONESON *et al.*, 2015) was used to summarize the estimated transcript levels of each gene analysed, allowing us to recover 11,654 coding genes from *D. mojavensis* r 1.04 transcripts. We have used these data in the differential expression analyses. Gene ontology was accessed using an orthologous gene table from *Drosophila melanogaster* downloaded from <http://flybase.org/>, allowing us to identify the genes involved in the piRNA pathway.

Differential expression analyses were performed with the R Bioconductor package DESeq2 (LOVE *et al.*, 2014; <https://www.R-project.org>) using raw read counts to identify differential TE and gene expression in the reproductive tissues from the hybrids compared to the parental species (control lines). This package normalizes counts using size factors that are estimated according to the median counts from all TE families. Additionally, DESeq2 estimates

the means and variances of raw read counts and tests for differential expression based on a model using the negative binomial distribution and the Benjamini-Hochberg correction for multiple tests (FDR level of 0.1, BENJAMINI; HOCHBERG, 1995). TE families and genes were classified as differentially expressed when the adjusted p-value (FDR level) was less than 0.01 and an at least 2-fold difference in expression was identified. TE families and genes that showed ≤ 10 aligned reads mapped under all conditions tested were excluded from the analyses.

Small RNA and *ping-pong* analyses

The small RNA-Seq analyses were performed using the method described by Fablet *et al.* (2019). Thus, the data were initially cleaned using Cutadapt (MARTIN, 2011) to remove the adapter sequences (-a TGGAATTCTCGGGTGCCAAGGAAGTCCAGTCACTTA). PRINSEQ lite version 0.20.4 (SCHMIEDER; EDWARDS, 2011) was used to filter the reads with a size ranging from 23 to 30 nucleotides to ensure that only piRNAs were analysed. The TEcount module from TETools (LERAT *et al.*, 2017) was used to map the sense and antisense piRNA reads to the same TE library that was previously used as a reference in the analysis of differential TE expression. Read counts were normalized to miRNA read counts obtained from the alignment created using Bowtie (with -S and -best arguments to obtain the most sensitive option and retaining a single alignment for reads mapping to multiple positions) (LANGMEAD *et al.*, 2009) of the cleaned small RNA data (before the filtering) against the miRNA sequences of *D. mojavensis* (Flybase: dmoj-all-miRNA-r1.04.fasta.gz). The *ping-pong* signature was analysed using signature.py with the options min_size = 23 and max_size = 30 (ANTONIEWSKI, 2014). SAM alignments of cleaned small RNA-seq reads with complete TE copies that were identified as differentially expressed in ovaries and testes were used as input files when the hybrids were compared with the parental lines.

RESULTS

In ovarian transcriptomes, the expression of 543 TE families was detected in the ovaries of *D. m. mojavenensis* and HB_m, whereas 563 TE families were expressed in *D. m. wrigleyi*, HA_w and HB_w females. In testes transcriptomes of parental lines and hybrids, 586 TE families were expressed of a total of 894 candidate TE families. Moreover, ovaries presented a higher alignment rate than testes for all conditions, except HB_m males (Figure S1).

Differential expression of TEs in the gonads of *D. arizonae* and *D. mojavenensis*

Comparative analyses of *D. arizonae* and *D. m. mojavenensis* transcriptomes showed that 19% (104) of TE families were differentially expressed in ovaries compared with 54.2% (318) of TE families in testes (Tables S1 and S2). Similarly, in comparisons between *D. arizonae* and *D. m. wrigleyi*, fewer differentially expressed TE families were identified in ovaries (28.5% - 153) than in testes (54.7% - 321) (Tables S3 and S4). Among the differentially expressed TE families in both reproductive tissues, LTR (long terminal repeats) elements were overrepresented in all comparisons, followed by TIRs elements and LINE (long interspersed nuclear elements) (Figure 2).

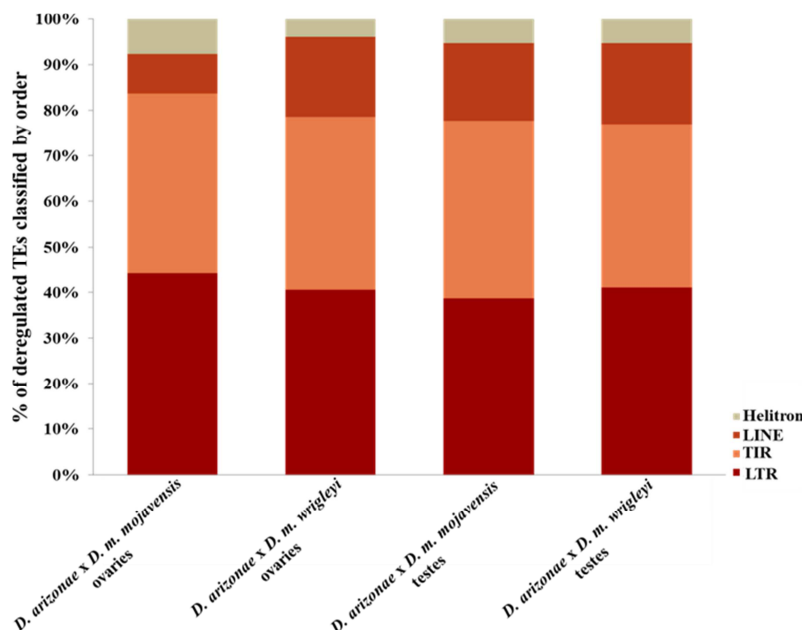


Figure 2. Percentage of differentially expressed TE families among parental lines classified by TE order. The classification was based on the order of each TE family. Transposable elements from TIR and

Helitron orders belong to Class II elements, known as DNA transposons. Transposable elements from LTR (long-terminal repeat) and LINE (long interspersed nuclear element) orders belong to Class I elements, known as retrotransposons. Source: prepared by the author.

The expression profiles in the parental species showed a bias in the distribution of fold changes towards TE overexpression in *D. arizonae* ovaries and testes compared with both *D. mojavensis* subspecies (Figure 3).

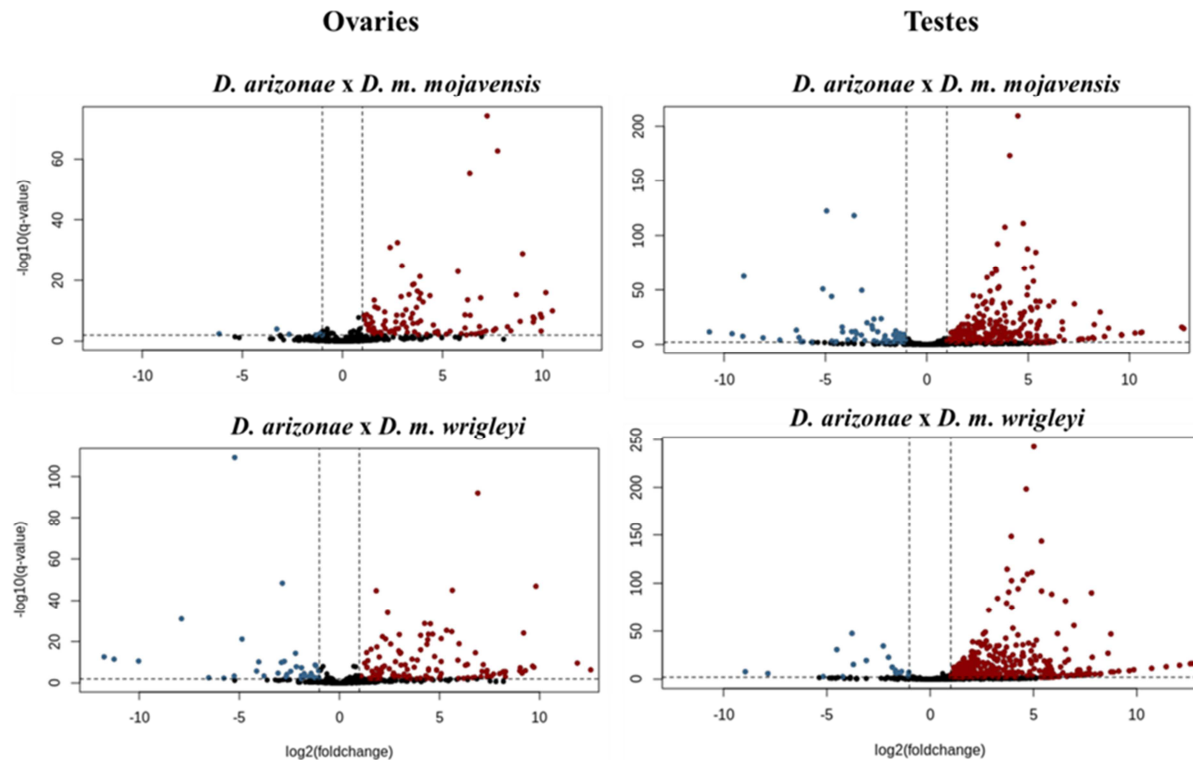


Figure 3. Expression profile of *D. arizonae* compared with *D. mojavensis* subspecies in ovaries (left) and testes (right). TE families were considered as differentially expressed when they presented 2-fold of differences (X-axis) and q-value (p-value corrected by FDR) < 0.01 (Y-axis). Red dots = overexpressed TE families; Blue dots = underexpressed TE families; Black dots = not differentially expressed TE families. Horizontal and vertical dotted lines are the threshold of q-value and $\log_2(\text{foldchange})$, respectively. Source: prepared by the author.

Bias in TE expression in the hybrid female germline compared with the parental lines

Separate comparisons of the expression profile in HB_m ovaries and their parental lines revealed more deregulated TE families in comparisons with *D. m. mojavensis* (69 – 12.7%) than in *D. arizonae* (18 – 3.3%). Likewise, comparisons of HA_w and HB_w females with their parental lines showed higher levels of differential expression in *D. m. wrigleyi* (17.5% - 99 and 16.1% - 91, respectively) than in *D. arizonae* (5.6% - 32 and 5.5% - 31, respectively). Most of the misregulated TEs identified in *D. mojavensis* subspecies were overexpressed (Figure 4).

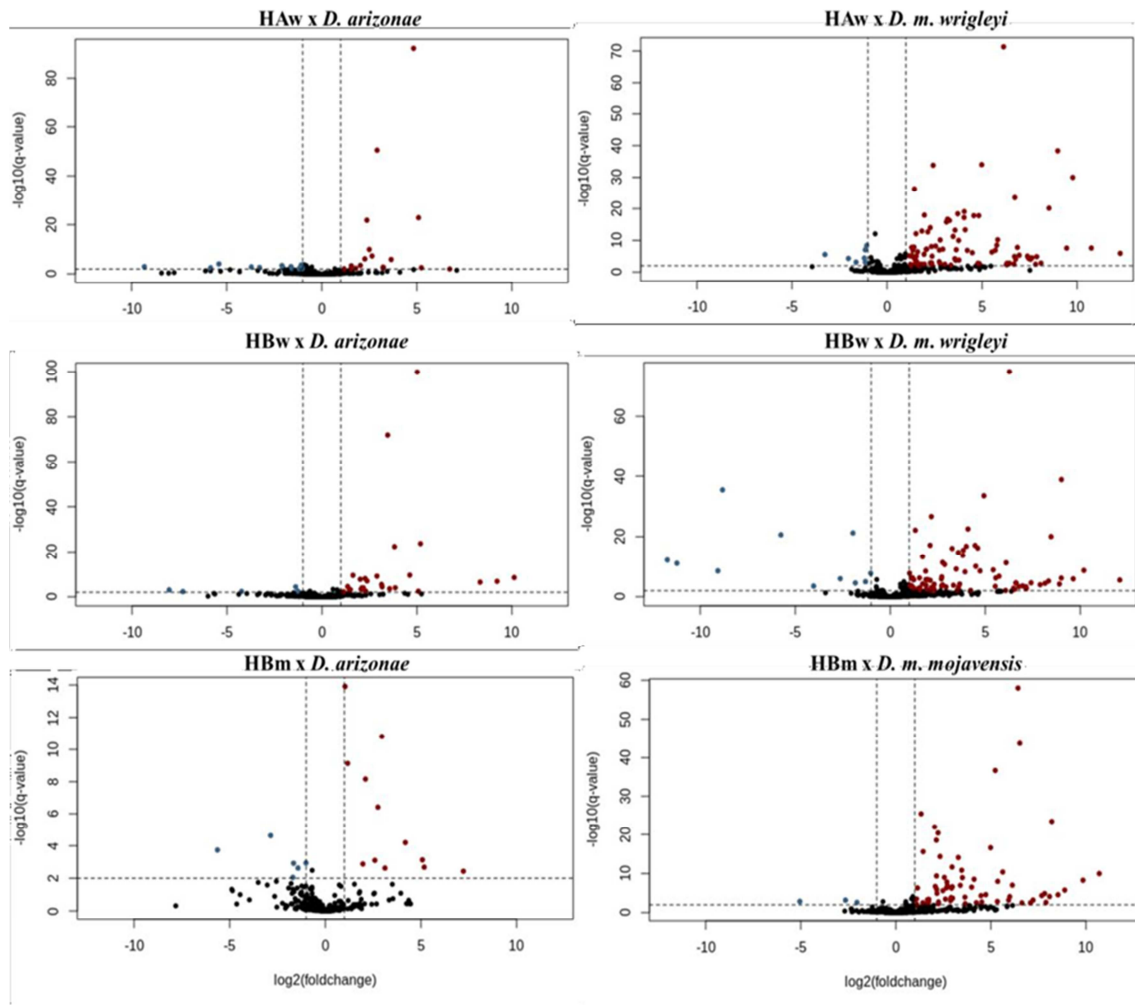


Figure 4. Expression profile in ovaries of interspecific hybrids compared with *D. arizonae* (left) and *D. mojavensis* subspecies (right). TE families were considered as differentially expressed when they presented 2-fold of differences (X-axis) and q-value (p-value corrected by FDR) < 0.01 (Y-axis). Red dots = overexpressed TE families; Blue dots = underexpressed TE families; Black dots = not differentially expressed TE families. Horizontal and vertical dotted lines are the threshold of q-value and $\log_2(\text{foldchange})$, respectively. Source: prepared by the author.

Compared with both respective parental lines, nine TE families were significantly overexpressed in HB_m ovaries, most of which were DNA transposons (Table S5 and Figure 5A). Additionally, the TE presenting the highest fold change (FC) compared with the parental lines belonged to the *Gypsy* superfamily (Figure 5A). On the other hand, HA_w displayed four deregulated TE families compared with both parental lines, two of which were LTR elements, which presented the highest FC (Table S6 and Figures 5A). HB_w ovaries exhibited only two deregulated TE families compared to both parents: one was overexpressed (*Mariner-3_DMo*) and one was underexpressed (*R1-1_DMo*) (Table S7). HA_w and HB_w shared one deregulated TE

family (*Mariner-3_DMo*) compared with both parental lines. These three TEs were identified in the *D. mojavensis* r 1.04 genome with the *de novo* annotation approach used to build the TE library.

The reciprocal hybrids HA_w and HB_w showed 30.1% (17) differentially expressed TEs, most of which were overexpressed in HA_w (Table S8). Among those differentially expressed TEs, an overrepresentation of *Gypsy* superfamily (58.8%) and hence LTR elements (76.4%) was observed (Table S8).

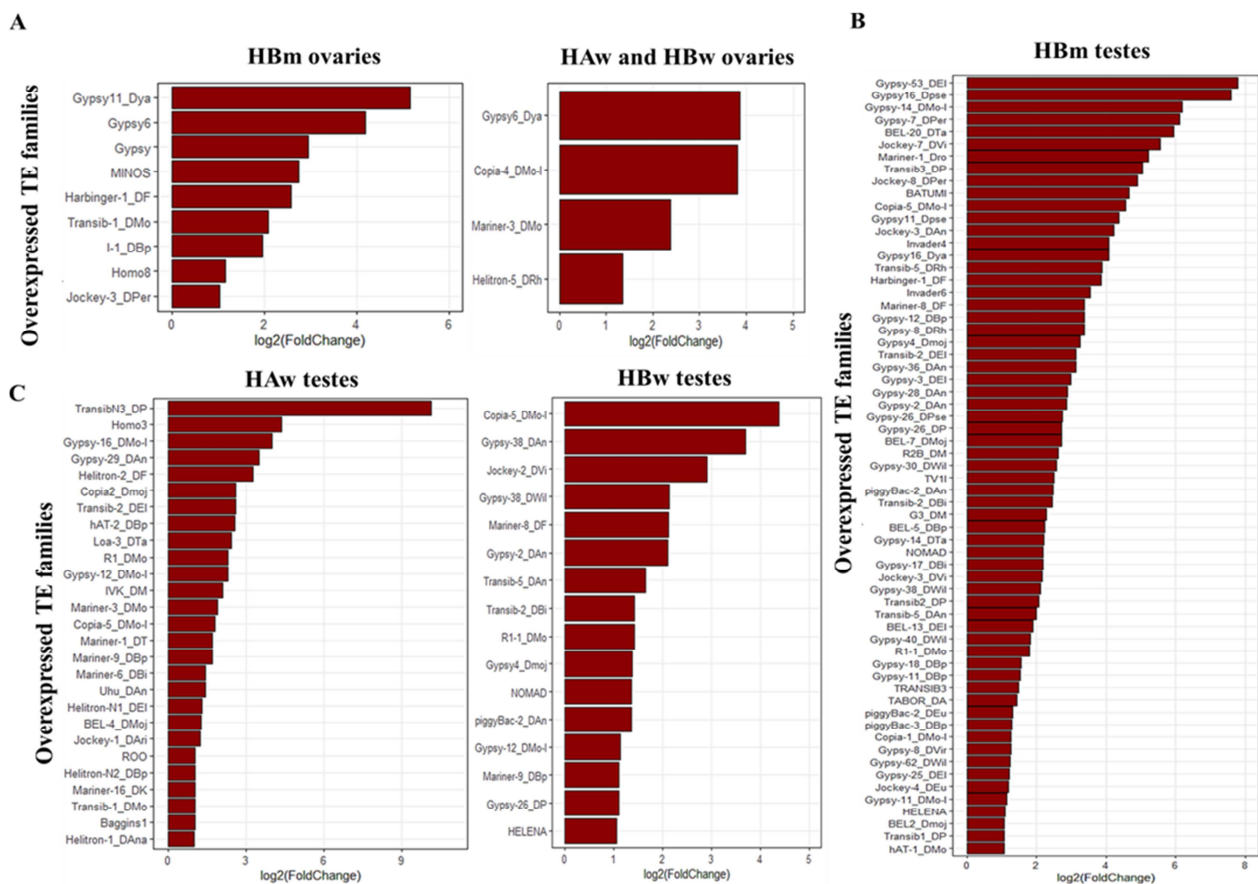


Figure 5. Overexpressed TE families presenting the highest differential of expression ($\log_2(\text{FoldChange})$) in ovaries and testes of hybrids. A) Overexpressed TE families in HB_m (left) and HA_w/HB_w (right) ovaries. B) Overexpressed TE families in HB_m testes. C) Overexpressed TE families in HA_w (left) and HB_w (right) testes. TEs were considered as overexpressed when they presented more than $\log_2(\text{foldchange}) > 1$ and p-value adjusted by FDR < 0.01. Source: prepared by the author.

In testes transcriptomes, TE deregulation was higher than in ovaries (Figure S2). In HB_m testes, similar to female gonads, more TE families were misregulated in comparison with *D. m. mojavensis* than in *D. arizonae* (50.6% - 297 and 28.3% - 166, respectively). Likewise, in

reciprocal hybrids of *D. arizonae* x *D. m. wrigleyi*, more TE families were deregulated compared with *D. mojavensis* subspecies, since 50.5% (296) and 50.1% (294) of TEs were deregulated in HA_w and HB_w, respectively, whereas when their expression profile was compared with *D. arizonae*, 20.4% (120) and 12% (70) of TEs were deregulated. Similar to the hybrid ovaries, most of these misregulated TEs showed a bias towards overexpression in the hybrids compared to their parental lines (Figure 6).

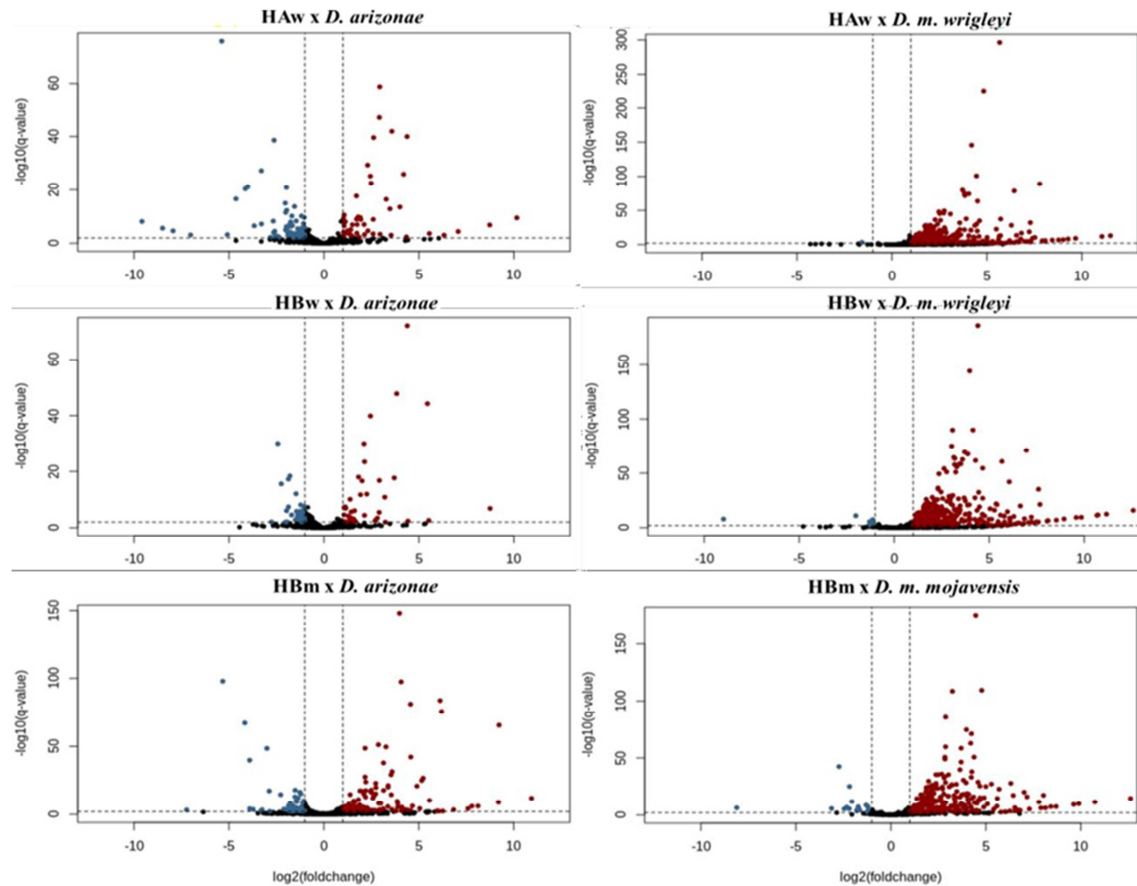


Figure 6. Expression profile in testes of interspecific hybrids compared with *D. arizonae* (left) and *D. mojavensis* subspecies (right). TE families were considered as differentially expressed when they presented 2-fold of differences (X-axis) and q-value (p-value corrected by FDR) < 0.01 (Y-axis). Red dots = overexpressed TE families; Blue dots = underexpressed TE families; Black dots = not differentially expressed TE families. Horizontal and vertical dotted lines are the threshold of q-value and log₂(foldchange), respectively. Source: prepared by the author.

Considering the misregulation in hybrids compared with both parental lines, 67 deregulated TE families (95.5% overexpressed) were identified in HB_m, 58.2% of which are LTR elements (Table S9 and Figure 5B). Among the deregulated TE families in HB_m testes, the highest fold changes were observed for TEs belonging to the *Gypsy* superfamily (Figure 5B). On the other hand, 28 deregulated TE families were identified in HA_w, the majority (39.2%) of

which were DNA transposons (Table S10 and Figure 5C), and the TE family that showed the highest fold change compared with parental lines was a *TransibN3_DP*, a DNA element (Figure 5C). Seventeen misregulated TE families were identified in the reciprocal hybrid HB_W. Forty-seven percent were LTR elements, similar to HB_M (Table S11 and Figure 5C). In this hybrid, the element that presented the highest fold change (>4) was a *Copia-5_DMo-I* (Figure 5C) (an element identified by the *de novo* approach in the *D. mojavensis* r 1.04 genome used to build the TE library).

The reciprocal hybrids HA_W and HB_W shared only two deregulated TE families, a *Mariner-9_DBp* element and *Gypsy-12_DMo-I*, which were identified in the public genome of *D. mojavensis* r 1.04 using the *de novo* annotation approach. Unlike the small number of shared TE deregulated families between the reciprocal hybrids, 92 TEs were differentially expressed and most were the LTR elements (41.3%) (Table S12).

piRNA control in hybrid female gonads

The sequencing and analyses of piRNAs in the transcriptomes of male and female parental lines and hybrids allowed us to identify antisense regulatory piRNA populations for 568 and 478 TE families in the ovaries and testes of *D. m. mojavensis* and HB_M, respectively (Tables S13 and S15). In the female and male gonads of *D. m. wrigleyi*, HA_W and HB_W antisense piRNA populations were observed for 560 and 486 TE families in the ovaries and testes, respectively (Tables S14 and S16).

Differential expression analyses revealed significantly differentially expressed piRNA populations associated with 410 TE families of up to 2-fold in the ovaries of *D. arizonae* and *D. m. mojavensis* (Table S13). In this comparison, ~61.4% of TE families showed lower levels of antisense piRNAs in *D. arizonae*; however, only a few TE families (10) were deregulated in HB_M ovaries (Table S5), where the cross direction with *D. arizonae* are the females. Moreover,

almost all deregulated TE families detected using RNA-Seq in HB_m ovaries displayed lower levels of piRNA expression in *D. arizonae* than in *D. m. mojavensis* and HB_m (Table S13 and Figure 7).

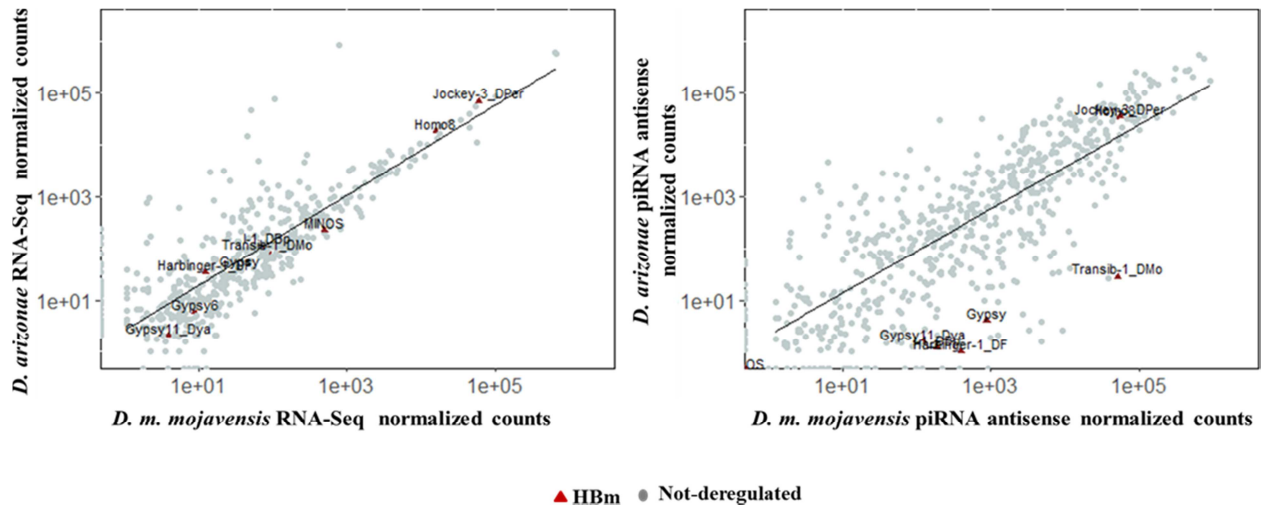


Figure 7. Expression of TE families in ovaries of *D. m. mojavensis* versus *D. arizonae* and the piRNA expression for the correspondent TE families. In red triangles are the TE families overexpressed in HB_m ovaries. TE families were considered as upregulated when they presented $\log_2(2) > 1$ and p-value adjusted < 0.01 in HB_m ovaries compared with both parental lines. Source: prepared by the author.

Similarly, in the comparisons between *D. arizonae* and *D. m. wiggleyi* ovaries, approximately 405 TE families showed antisense piRNA populations with differences in expression greater than 2-fold (Table S14). Among these populations, ~64% were underexpressed in *D. arizonae*; however, in the ovaries of HB_w, only two TE families were deregulated (Table S7). On the other hand, the ovaries of HA_w, where *D. m. wiggleyi* is the maternal source, the three TEs that were uniquely deregulated in this cross showed lower levels of piRNA expression in *D. m. wiggleyi* than in *D. arizonae*, which was also verified by comparing the expression levels of HA_w and *D. arizonae* (Table S14 and Figure 8).

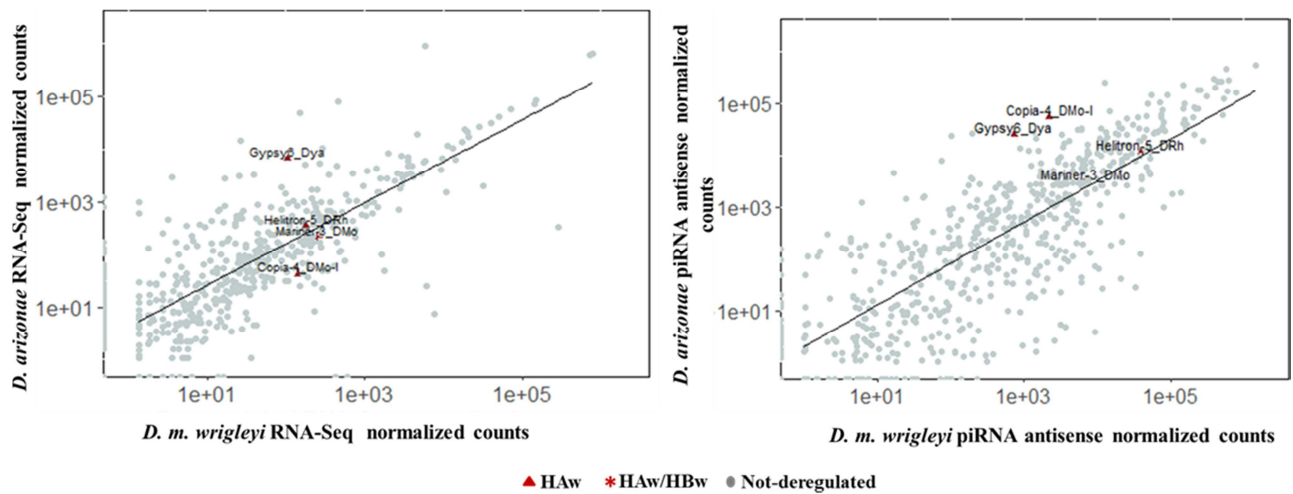


Figure 8. Expression of TE families in ovaries of *D. m. wrigleyi* versus *D. arizonae* and the piRNA expression for the correspondent TE families. In red triangles are the TE families overexpressed in HA_W ovaries, in red stars is the TE family commonly overexpressed in HA_W and HB_W ovaries (*Mariner-3_DM0*). TE families were considered as upregulated when they presented $\log_2(2) > 1$ and p-value adjusted < 0.01 in HA_W and HB_W ovaries compared with both parental lines. Source: prepared by the author.

TE overexpression is not always associated with lower levels of piRNAs in the gonads of hybrid males obtained from the maternal line

Fewer antisense piRNA populations were expressed in the testes than in ovaries. Overall, piRNA expression that was deregulated greater than 2-fold was observed for 279 TE families in the comparison between *D. arizonae* and *D. m. mojavensis* males. Among these piRNAs populations, 63.8% were underexpressed in *D. m. mojavensis* compared with *D. arizonae* (Table S15). Considering the misregulated TEs in HB_m males (Table S9 and Figures 9 and 10A), only 52.2% presented lower piRNA levels in *D. arizonae* (female parent) than in *D. m. mojavensis* (Table S15). Moreover, among the overexpressed TEs in HB_m testes, 70.1% showed lower piRNA levels in the hybrid than in both parental species. Thus, in testes, the maternal piRNAs do not exert a strong regulatory effect on TEs, since even in the presence of a complementary antisense piRNA for some TE families, the TEs were upregulated. Moreover, nine TE families did not have show piRNAs to controll their expression in hybrid and parental male gonads (Table S15).

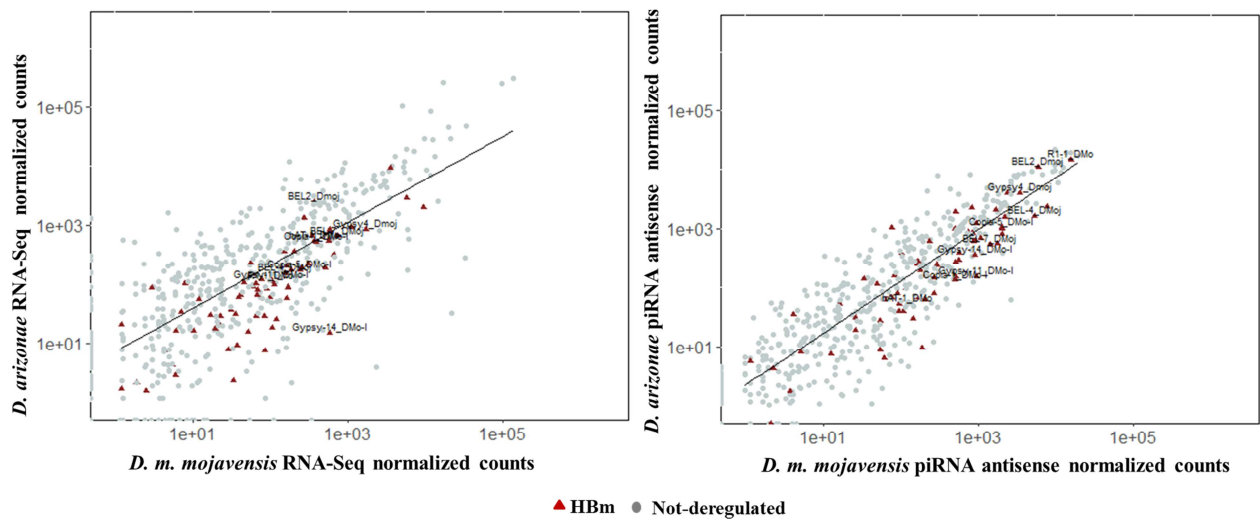


Figure 9. Expression of TE families in testes of *D. m. mojavensis* versus *D. arizonae* and the piRNA expression for the correspondent TE families. In red triangles are the TE families overexpressed in HB_m testes. TE families were considered as upregulated when they presented $\log_2(2) > 1$ and p-value adjusted < 0.01 in HB_m testes compared with both parental lines. Source: prepared by the author.

Similarly, male reproductive tissues from *D. arizonae* and *D. m. wrigleyi* exhibited 317 TE families with differential piRNA expression greater than 2-fold (Table S16). Additionally, 79.4% were underexpressed in *D. m. wrigleyi* compared with *D. arizonae* (Figure 10). Considering separately the misregulated TEs in HAw and HBw, 6 of the 28 TE families that were deregulated in HAw presented higher levels of piRNAs in *D. m. wrigleyi* (the maternal line of HAw) than in *D. arizonae*. Moreover, for eight deregulated TEs (*Baggins1*, *Loa-3_DTa* and *TransibN3_DP*, *ROO*, *Mariner-16_DK*, *Mariner-1_DT*, *IVK_DM* and *Helitron_N1_Del*), no antisense piRNAs were verified. Moreover, the piRNA expression level in HAw was lower than that in both parental lines for only two overexpressed TE families (*Helitron-2_DF* and *Helitron-1_DAna*) (Table S16). Regarding the piRNA expression in HBw testes, of the 17 deregulated TE families identified in this cross direction, the maternal line *D. arizonae* showed a lower amount of antisense piRNAs for three of them (Table S16). In addition, for almost all overexpressed TE families, HBw presented higher piRNA expression levels, indicating that the deregulation may not be linked to the absence of a homologous piRNAs to control TE expression.

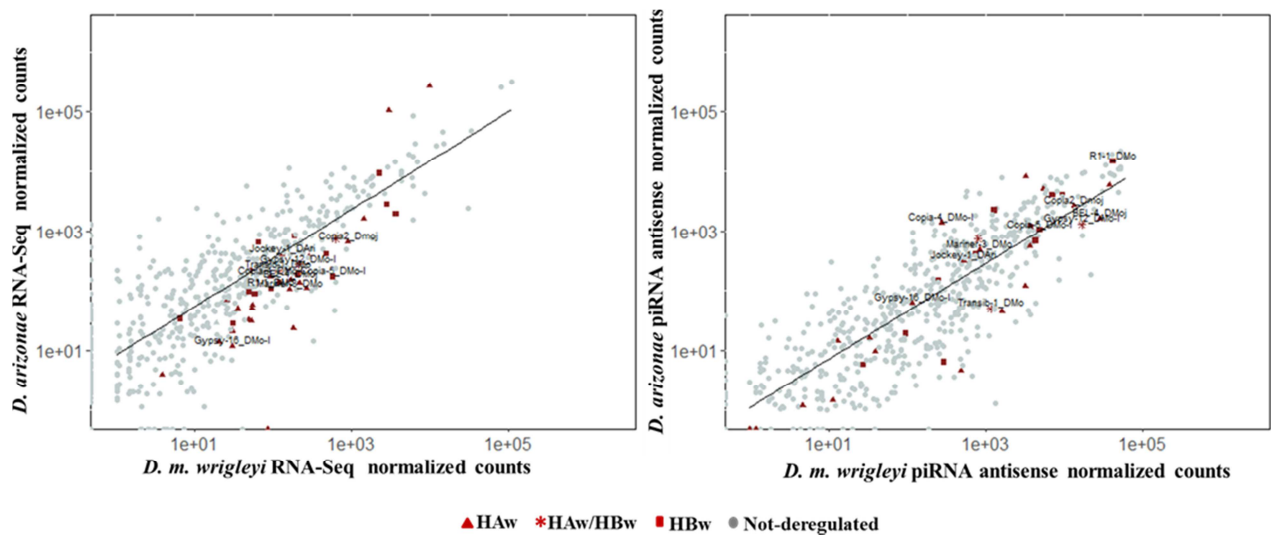


Figure 10. Expression of TE families in testes of *D. m. wrigleyi* versus *D. arizonae* and the piRNA expression for the correspondent TE families. In red triangles are the TE families exclusively overexpressed in HA_w testes, in red stars are the TE families commonly overexpressed in HA_w and HB_w testes, in red squares are the TE families exclusively overexpressed in HB_w testes. TE families were considered as upregulated when they presented $\log_2(2) > 1$ and p-value adjusted < 0.01 in HA_w and HB_w ovaries compared with both parental lines. Source: prepared by the author.

Divergence is detected in piRNA pathway genes

We performed gene expression analyses to verify if the overexpression of specific TEs were associated with divergence of piRNA pathway genes. In female reproductive tissues, no genes participating in the primary and secondary piRNA pathways exhibited differential expression among the parental species (*D. arizonae* x *D. m. mojavensis* and *D. m. wrigleyi*) or among the hybrids and their respective parental species (Tables S17 and S18). Regarding the gene expression in testes, most of the genes involved in the piRNA pathway presented conserved expression, except for *cuff* and *zuc*, which showed differential expression (greater than 2-fold) among *D. arizonae* and both *D. mojavensis* subspecies. Similarly, HB_m also presented differential expression for the same genes compared with its paternal line (*D. m. mojavensis*) (Table S19). Another interesting finding was the underexpression of a heat shock protein (*Hsp70-1*), reaching 18-fold differences, in the testes of HB_m.

Similarly, in HA_w testes, the differential expression of the *cuff* and *aub* genes was verified compared with at least one parental line. On the other hand, HB_w testes displayed

differential expression of *cuff* and *Hsp70-1* (Table S20). Figure 10 shows the levels of expression of piRNA pathway genes in the ovaries and testes of parental lines and hybrids, and the expression of these genes is quite conserved among the hybrids and parental lines. However, the expression of the same genes varied in different gonad tissues (Figure 11).

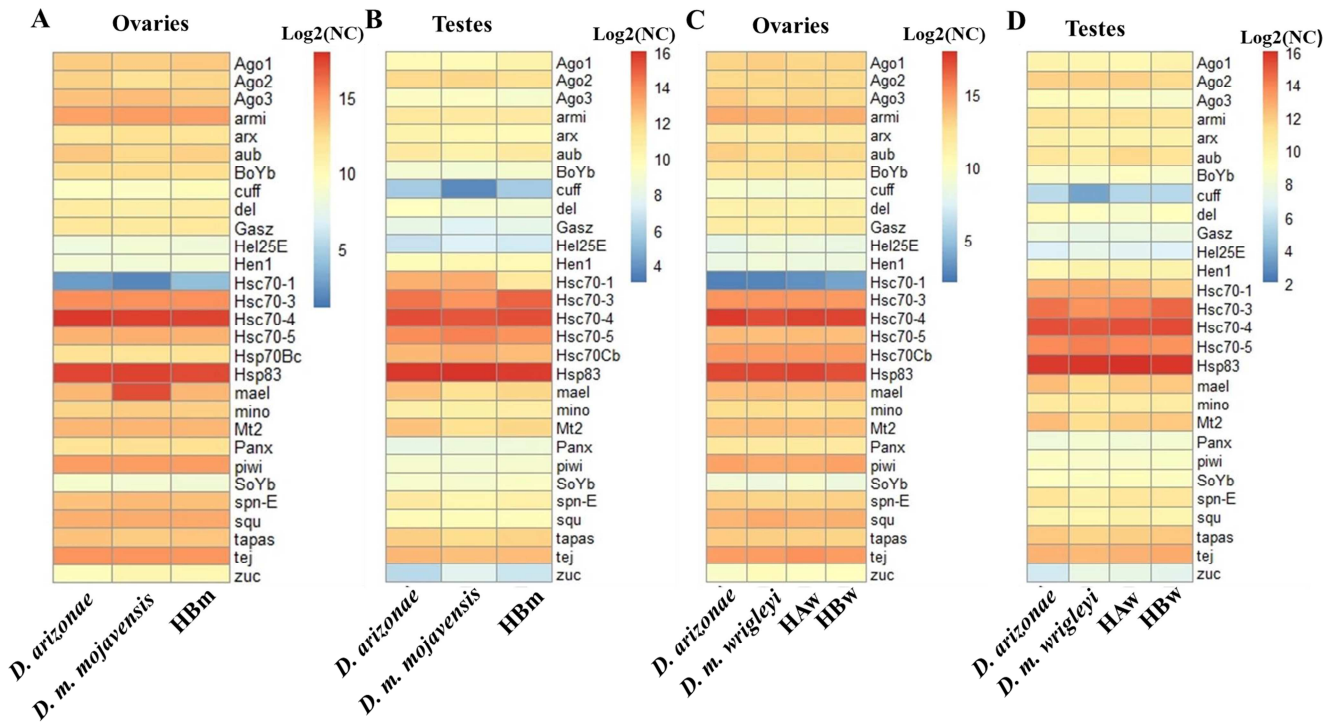


Figure 11. Heatmap of piRNA pathway genes that act in the biogenesis of primary and secondary piRNAs. A) Gene expression in ovaries of *D. arizonae*, *D. m. mojaveensis* and HBm. B) Gene expression in testes of *D. arizonae*, *D. m. mojaveensis* and HBm. C) Gene expression in ovaries of *D. arizonae*, *D. m. wrigleyi*, HAw and HBw. D) Gene expression in testes of *D. arizonae*, *D. m. wrigleyi*, HAw and HBw. Color indicates either overexpression (red) or underexpression (blue) according to log2 of normalized counts. NC: normalized counts. Source: prepared by the author.

The *ping-pong* signature is not completely associated with TE silencing in *D. arizonae* x *D. mojaveensis* hybrids

In male and female germlines, the secondary piRNA pathway plays an important role in regulating TEs. As described above, the genes involved in this pathway are conserved among parental species and hybrids. Therefore, we analysed the *ping-pong* amplification loop for some deregulated TE families in the ovaries and testes to determine whether the biogenesis of secondary piRNAs is active in the hybrids. The deregulated TEs chosen for this analysis were identified from the *de novo* annotation and presented complete copies.

In HBm ovaries, we analysed the piRNA signature for *Transib-1_DMo* and *R1-1_DMo* elements, and in both cases, the hybrids showed the *ping-pong* signature (Figure S3). Regarding the other TEs overexpressed in HBm ovaries, the elements *Gypsy11_Dya*, *Gypsy6*, *Harbinger-1_DF*, *I-1_DBP*, *Minos* showed very few or no antisense and sense piRNAs mapped to them in HBm and in *D. arizonae* (the maternal line) (Table S21). Thus, for those TEs, the *ping-pong* amplification is likely absent in hybrid ovaries and is responsible for their overexpression.

Similarly, in the ovaries of HA_w and HB_w, the three TEs (*Mariner-3_DMo*, *Copia-4_DMo-I* and *R1-1_DMo*) for which the *ping-pong* signature was analysed showed strong *ping-pong* signals (Figure S4). Additionally, for all overexpressed TEs identified using RNA-Seq in HA_w and HB_w ovaries, antisense and sense pools of mapping piRNAs were detected at high levels (Table S22).

In testes, the biogenesis of secondary piRNAs generated by *ping-pong* amplification does not appear to be directly associated with TE regulation. In HBm male gonads, the *ping-pong* signature was analysed for 9 of 67 deregulated TEs. Among these 9 TEs, only three (*hAT-1_DMo*, *Gypsy-11_DMo-I* and *Gypsy-14_DMo-I*) did not show *ping-pong* amplification in the hybrids, which was also absent in *D. arizonae*, but present in *D. m. mojavnensis* testes (Figure S5). Similarly, in ovaries, several of these deregulated TEs that showed the *ping-pong* signature displayed sense and antisense piRNA-mapping TEs, explaining the secondary piRNA biogenesis in the germline (Table S23). Additionally, nine of the 67 misregulated TE families detected in HBm testes did not show homologous antisense or sense piRNAs (Table S23).

In HA_w testes, four of the 28 deregulated TE families were analysed for the *ping-pong* cycle. One showed no *ping-pong* signature for both the parental species and the reciprocal hybrids (*Mariner-3_DMo*) and was also deregulated in HA_w and HB_w ovaries. One showed the *ping-pong* signature only for HA_w testes, in which this TE was overexpressed (*Transib-1_DMo*), and two TE families presented the *ping-pong* signature in the parental lines and the reciprocal hybrids (*Gypsy-12_DMo-I*, also upregulated in HB_w testes, and *Copia-5_DMo-I*) (Figure S6). In

HB_w testes, the *ping-pong* signature was present for the two TEs tested (Figure S7). Consistent with these findings, an analysis of the number of antisense and sense piRNAs mapping to these deregulated TEs revealed sense and antisense piRNAs for most of these deregulated TEs (Table S24). Notably, piRNA expression was not detected for 10 of the TEs overexpressed in HA_w and HB_w testes (Table S24).

Importantly, some overexpressed TE families that do not have complementary antisense or sense piRNAs to regulate their expression are shared among HB_m, HA_w and HB_w testes. More specifically, the TE families that shared a lack of complementary piRNAs in hybrid and parental testes are *Gypsy-38_DWil*, *Helitron-1_DT*, *Helitron-N1_DEl*, *IVK_DM* and *Mariner-16_DK* (Tables S23 and S24).

siRNA control in somatic cells of gonad tissues

We have analysed the siRNA populations for the male and female gonads of the parental lines and hybrids to verify the role of siRNAs in the reproductive tissues. In the ovaries of HB_m, siRNAs associated with 291 TE families were identified. Among these siRNAs, 192 presented differential expression between *D. arizonae* and *D. m. mojavensis*, and most (86%) were expressed at an at least 2-fold lower level in *D. arizonae* (Table S25). Complementary siRNAs were identified for most of the TE families that were overexpressed in HB_m ovaries, and the hybrids generally contained more siRNAs than the parental lines (Table S25). However, four upregulated TE families (*Gypsy11_Dya*, *Gypsy6*, *Harbinger-1_DF* and *I-1_DBP*) identified in the HB_m female ovaries did not have complementary siRNAs. On the other hand, in the ovaries of HA_w and HB_w, siRNAs associated with 267 TE families were detected. Of those TE families, 207 display differential expression of siRNAs between *D. arizonae* and *D. m. wrigleyi*, and most (92.7%) were underexpressed up to 2-fold in *D. arizonae* (Table S26). Considering only the upregulated TE families in HA_w and HB_w ovaries, siRNAs for almost all of these TE families were expressed at higher levels in hybrids, regardless of the cross direction, than in parental

lines. The only exception was verified for the *Gypsy6_Dya* family, for which the levels of siRNAs were lower in HAw ovaries than in both parental lines and in *D. m. wrigleyi* (maternal source) compared with *D. arizonae* (Table S26). However, this difference was less than 2-fold, and hence, not significant.

In testes, siRNA-mediated control is more complex. In HBm male gonads, 213 TE families mapped to siRNAs. Comparing the parental species, 134 presented differential siRNA expression, and, unlike ovaries, most of these siRNAs (79%) were overexpressed in *D. arizonae*. Considering the 67 overexpressed TE families detected in the HBm male reproductive tissue, lower amounts of siRNAs for 15 TE families were detected in hybrids than in both parental lines (Table S27). Interestingly, the majority of these TE families belong to the *Gypsy* superfamily, and *ping-pong* amplification was analysed in three of these 15 TE families. Only *Gypsy-14_DMo-I* did not show the *ping-pong* signature, indicating that this element is either exclusively expressed in the soma or it is absent in *D. arizonae*, since *D. m. mojavenensis* showed *ping-pong* amplification. The elements *Gypsy4_Dmoj* and *R1-1_DMo* exhibited the *ping-pong* signature and lower amounts of siRNAs, suggesting that they are expressed in both the germline and soma, but the overexpression likely occurs in the somatic cells. Another important finding was that 35 of the 67 upregulated TE families showed no associated siRNAs, including eight TE families for which a lack of piRNAs was also detected in this tissue (Table S27). Moreover, some TEs that exhibited *ping-pong* amplification also showed larger amounts of siRNAs in the hybrids than in both parental lines, such as *BEL-7_Dmoj* and *Copia-5_DMo-I*, indicating that these elements may be expressed in somatic cells in the testes, although they are not controlled by siRNA or piRNA pathways.

Similarly, to the HBm testes, 236 TE families showed associated siRNAs in HAw and HBw testes. One hundred sixty-two families displayed differential siRNA expression between *D. arizonae* and *D. m. wrigleyi* testes, and most were expressed at higher levels in *D. arizonae* (79.6%), as it was previously observed in *D. arizonae* x *D. m. mojavenensis* comparisons for the

same tissue. Considering the upregulated TE families in HA_w and HB_w testes, none of TE families with associated siRNAs showed lower expression of siRNAs in the hybrids than in both parental lines (Table S28). However, for some TEs, differences greater than 2-fold were verified in hybrid testes than in one of the parental lines (Table S28). Additionally, 17 deregulated TE families did not present homologous siRNAs, indicating that these elements are not expressed in the soma, since this pattern was observed for hybrids and both parental lines. Moreover, among these 17 TE families without associated siRNAs, 9 TE families lacking piRNAs were identified (*Baggins1*, *Helitron-1_DT*, *Helitron-N1_DEL*, *IVK_DM*, *Loa-3_Dta*, *Mariner-1_DT*, *Mariner-16_DK*, *ROO* and *TransibN3_DP*) (Table S28). Notably, almost all TE families assessed for the *ping-pong* signature in this tissue showed mapping siRNAs, except *Transib-1_DMo*.

DISCUSSION

Differential expression of TE families in the gonads of *D. arizonae* and *D. mojavensis*

We have detected a higher alignment rate of RNA-Seq reads in the TE library in ovary transcriptomes than in testes. However, a greater number of differentially expressed TE families was detected in males, similar to the male and female transcriptomes of *D. buzzatii* and *D. koepferae* (ROMERO-SORIANO *et al.*, 2017). Differences in the alignment rate might be related to difference in the genome size, which is driven by differences in repetitive sequences between sexes. Indeed, males and females presented differences in genome size, such as *D. buzzatii*, *D. koepferae*, *D. mauritiana* and *D. hydei*, in previous studies (GIRARD; HANNON, 2008; ROMERO-SORIANO *et al.*, 2016). Moreover, in males, the dynamic changes in the number of gene and repetitive sequences may be highly variable due to the Y chromosome (CARVALHO *et al.*, 2009), which modulates the differential expression among species.

Based on the number of differentially expressed TEs in each tissue, 19% of TEs were deregulated between *D. arizonae* and *D. mojavensis* ovaries, which was lower than the value reported by Lopez-Maestre *et al.* (2017) (29%) in the comparison *D. arizonae* and *D. m.*

mojavensis transcriptomes sequenced at a different time point. Differences in the number of misregulated TE families between the species of the present study and the study by Lopez-Maestre *et al.* (2017) are likely related to the use of different TE libraries. Here, we have used a library that contains TE sequences derived from the *D. mojavensis* r 1.04 public genome, as well as from *D. arizonae* genome that was recently sequenced, assembled and annotated by our research group using a *de novo* approach.

Furthermore, most of the deregulated TEs detected in the ovaries and testes of *D. arizonae* and the two *D. mojavensis* subspecies were LTR retrotransposons, followed by DNA transposons and LINEs. Interestingly, these proportions of deregulated TE classes for these species are similar to those reported by Clark *et al.* (2007), the 12 Genomes Consortium and by Rius *et al.* (2016) for LTR elements.

Differential expression of TE families and the relationship with post-transcriptional control by piRNA and siRNA pathways in hybrid female gonads

Few TE families showed dysregulated expression in the hybrid female reproductive tissues compared with both parental lines. This result concurs with the findings reported by Lopez-Maestre *et al.* (2017), who analysed ovaries of hybrids from the same parental species. However, it is quite different from the results described by Romero-Soriano *et al.* (2017) for *D. buzzatii* and *D. koepferae* female hybrids (4.96 m.y. of divergence), since ~35% (234 of 658 TE families) of differentially expressed TE families were identified. Similarly, our results differ from those reported for female hybrids of *D. melanogaster* and *D. simulans* (KELLEHER *et al.*, 2012; SATYAKI *et al.*, 2014), where a larger number of deregulated TEs was verified. These results corroborate our hypothesis that a longer divergence time of two genomes results in a greater rate of TE deregulation.

We sequenced and analysed the piRNA pool of parental species and their respective hybrids to obtain a better understanding of the factors that lead to higher levels of TE

transcription in hybrids. In ovaries of HB_m derived from crosses of *D. arizonae* females x *D. m. mojavenensis* males, nine TEs were overexpressed, most belonging to the *Gypsy* superfamily. The maternal source showed fewer sense and antisense piRNAs mapping to TEs, and some of them, such as the *Gypsy11_Dya*, *Gypsy6*, *Harbinger-1_DF*, *I-1_DBp* and MINOS families, showed very few or no antisense and sense mapping piRNAs in *D. arizonae* and HB_m. Thus, these elements may be absent in the maternal species *D. arizonae*, contributing to the upregulation of these TE families in the hybrid genome through a lack of primary piRNAs, maternal deposition or transcription from single-strand piRNA clusters, initiating the *ping-pong* loop. However, analyses of the *ping-pong* signature must be performed to confirm this hypothesis. In fact, the contribution of the maternal cytotype in post-transcriptional silencing mechanisms mediated by piRNAs has been reported in hybrids of *D. arizonae* and *D. m. mojavenensis* by Lopez-Maestre *et al.* (2017) for two LTR retrotransposons. Considering other *Drosophila* species, a similar pattern has been reported in intraspecies hybrids of *D. melanogaster* and *D. simulans* and *D. virilis* intraspecies hybrids (PICARD, 1976; BLACKMAN *et al.*, 1987; YANNOPOULOS *et al.*, 1987; KIDWELL *et al.*, 1977; PETROV *et al.*, 1995; HILL *et al.*, 2016; FUNICOV *et al.*, 2018). Nonetheless, the overexpressed MINOS TE family presented a different pattern, since no antisense and sense piRNAs were detected in hybrids and both parental lines, suggesting that the expression of this element is exclusively somatic.

Additionally, in ovaries of HB_m, HA_w and HB_w hybrids, some upregulated TEs displayed strong *ping-pong* signals and antisense and sense piRNAs in the maternal species, thus failures in silencing the paternally inherited TE family mediated by the secondary biogenesis of piRNAs do not explain their deregulation. Likewise, the element *Frogger* displays a similar pattern in *D. arizonae* x *D. m. mojavenensis* female hybrids (LOPEZ-MAESTRE *et al.*, 2017), since its upregulation was verified in one cross direction and strong *ping-pong* amplification was observed. The authors claimed that the main explanation for this phenomenon was related to the diversity of copies expressed in the parental genome, which limits the capacity to attribute

transcripts and piRNAs to specific insertions (LOPEZ-MAESTRE *et al.*, 2017). Similar results were also observed in female hybrids of *D. melanogaster* $\times$ *D. simulans* and *D. buzzatii* $\times$ *D. koepferae* (KELLEHER *et al.*, 2012; ROMERO-SORIANO *et al.*, 2017), since in both cases, TE derepression in hybrids is not always associated with the disruption of the *ping-pong* cycle for all misregulated TEs, as was verified in the present study. Nevertheless, in hybrids from those more divergent species, the failure to regulate TEs is widely associated with adaptive divergence in piRNA pathway genes (KELLEHER *et al.*, 2012; ROMERO-SORIANO *et al.*, 2017). However, divergence in piRNA pathway genes does not explain the TE dysregulation in hybrids from more recently diverged species, such as *D. arizonae* and *D. mojavensis*, since no differential expression was detected. Similar results were reported by Lopez-Maestre *et al.* (2017), since they observed less than 2% of divergence between orthologous genes of *D. mojavensis* and *D. arizonae* for more than 95% of the genes.

Because some upregulated TE families did not show failures in the secondary biogenesis of piRNAs or divergence of piRNA genes in hybrid ovaries, we speculated that two main reasons may underlie TE deregulation. The first reason is potentially related to the expression of these TEs in somatic cells, which are present in *Drosophila* ovaries and have a specific post-transcriptional regulation system driven by siRNAs (21 nt in length) (reviewed in SATO; SIOMI, 2020). Therefore, failures in this type of silencing might induce TE overexpression in ovaries. We analysed the siRNA population associated with TE families in ovaries of the parental lines and hybrids to confirm this hypothesis. Interestingly, no associated siRNAs were identified for some elements that were upregulated in HB_m ovaries and lacked antisense or sense complementary piRNAs in hybrids and the maternal line (*Gypsy11_Dya*, *Gypsy6*, *Harbinger-1_DF* and *I-1_Dbp*), suggesting that they are exclusively expressed in the germline. Furthermore, their overexpression is likely linked to the lack of primary piRNAs able to start the *ping-pong* amplification cycle. On the other hand, the transposon *Minos*, which was deregulated (up to 2-fold) in HB_m and *D. arizonae* compared with *D. m. mojavensis*, did not display any

antisense or sense complementary piRNAs, but had associated siRNAs. Based on this result, the overexpression of this TE occurs in the somatic cells of the ovary and is related to lower amounts of siRNAs that control its expression in hybrids. Despite the positive correlation between siRNA levels and TE expression, some upregulated TE families identified in the ovaries of HB_m, HA_w and HB_w exhibited larger amounts of associated siRNAs and piRNAs, as well as a strong *ping-pong* signature, suggesting that these TEs are expressed in somatic and germline cells. Indeed, piRNAs and siRNAs have been shown to originate from the same genomic clusters, indicating that they may arise from the same single precursor transcript (GHILDIYAL *et al.*, 2008). Moreover, functional interactions between piRNA and siRNA pathways have been identified in *C. elegans* (BAGIJN *et al.*, 2012), confirming that they may function cooperatively in TE silencing. However, TEs overexpressed in ovaries presented associated piRNA and siRNAs, indicating that other mechanisms might be influencing their deregulation. Importantly, although we postulate that the expression of some specific TEs occurs in somatic and/or germ cells, an analysis designed to locate TE transcript expression in gonad tissues must be performed to confirm these findings.

The second reason underlying TE deregulation might be related to epigenetic changes and failure of the transcriptional regulatory mechanism. Some stressful events, such as heat shock, colonization of new environments and interspecific hybridization, have been shown to activate transposable elements in male and female germlines, which is associated, at some level, with the piRNA regulation mechanism (LABRADOR *et al.*, 1999; FONTDEVILA, 2005; KELEHER *et al.*, 2012; VELA *et al.*, 2014; GUERREIRO, 2014; SATYAKI *et al.*, 2014; SAINT-LEANDRE *et al.*, 2015; CAPPUCCI *et al.*, 2019). In *Drosophila* ovaries, the *Piwi* protein plays a major role in heterochromatin modification by interacting with several proteins, including HP1 and H1, leading to heterochromatin formation (YIN; LIN, 2007; MOSHKOVICH; LEI, 2010; OLOVNIKOV *et al.*, 2012; SATO; SIOMI, 2020). Moreover, for some specific TEs, maternally deposited piRNAs have an important role in silencing TEs in the

somatic ovarian cells (AKKOUCHE *et al.*, 2013), and they are responsible for chromatin modification and TE regulation. Thus, for some TEs, the failure of maternal piRNA deposition might contribute to the TE activation observed in the *D. arizonae*-*D. mojavensis* hybrids that did not show antisense piRNAs in the maternal lines. Nonetheless, in hybrids of *D. melanogaster* and *D. simulans*, interspecies hybridization is responsible for TE activation in germ cells and in somatic follicle cells of ovaries (THOMAE *et al.*, 2013; SATYAKI *et al.*, 2014). In these hybrids, although they have normal amounts of piRNAs, their heterochromatin genes are downregulated, which is associated with *Hmr* (*Hybrid male rescue*) and *Lmr* (*Lethal male rescue*) genes that are divergent among the parental lines. These genes, which are responsible for hybrid incompatibility in *D. melanogaster* $\times$ *D. simulans*, exert a positive effect on heterochromatin genes, such as *HP1a*. Thus, in the *D. arizonae*-*D. mojavensis* hybrids, which present regular amounts of piRNAs and conserved expression of genes that participate in the piRNA pathway, other factors, such as chromatin changes induced by gene misregulation, may alter TE expression.

Differential expression in the hybrid male reproductive tissues and TE post-transcriptional control by piRNA and siRNA pathways

Overall, male gonads showed higher differential expression of TEs than ovaries. Hybrid males obtained from *D. arizonae* $\times$ *D. mojavensis* crosses are sterile, although they show different phenotypes in sperm motility, depending on the cross direction (RUIZ *et al.*, 1990; REED; MARKOW, 2004).

One of the potential mechanisms affecting the hybrid incompatibility is increased TE expression. TEs are thought to contribute to reproductive isolation through transcriptional misregulation, leading to sterility or inviability (CASTILLO; MOYLE, 2019). Here, hybrids obtained from the same cross direction (HBm and HBw) that exhibit the same phenotype of immotile sperm displayed different amounts of deregulated TEs, with very few being equally

misregulated between them. On the other hand, HA_w, hybrids obtained from crosses between *D.m. wrigleyi* females x *D. arizonae* males that are sterile and have motile sperm, showed more deregulated TE families than HB_w. Thus, the two subspecies of *D. mojavensis* likely have different TE contents, and when interspecies crosses occur, TE misregulation is detected in their hybrids at different levels. Differences in TE contents are often observed in *Drosophila*, even for different populations of the same species or for different species (SONG *et al.*, 2014; FABLET *et al.*, 2019), and the consequences of these differences under hybridization events is often observed as hybrid dysgenesis (PICARD, 1976; KIDWELL *et al.*, 1977; BLACKMAN *et al.*, 1987; YANNOPOULOS *et al.*, 1987; PETROV *et al.*, 1995; HILL *et al.*, 2016; FUNICOV *et al.*, 2018). According to Castillo and Moyle (2019), the occurrence of hybrid sterility and hybrid inviability might result from the misregulation of TEs at different developmental stages, and as shown in the present study, it may be related to the misregulation in different tissues. Therefore, the rapid evolution of TEs in different lineages potentially explains the presence of polymorphic incompatibilities in many systems (CASTILLO; MOYLE, 2019).

In our hybrids, most of the deregulated TEs were overexpressed, in contrast to the results reported by Romero-Soriano *et al.* (2017), who described underexpression of TEs in hybrid males from *D. buzzatii* and *D. koepferae*. Some studies have examined the location of overexpressed TEs in hybrid testes and found that the new copies were expressed in the primary spermatocytes near the apical and basal regions of the testes (CARNELOSSI *et al.*, 2014; GUERREIRO, 2015). The apical region is composed of germline stem cells and primary and secondary spermatocytes, while the basal region contains transcripts for many spermatogenesis genes (FULLER, 1998; VEDELECK *et al.*, 2018). Therefore, we speculated that in *D. mojavensis*-*D. arizonae* hybrids, the higher expression of TE families observed in testes might influence the hybrid incompatibility, which is stronger in males. Additionally, higher TE expression may be a product of divergence of some specific regulatory genes, such as piRNA pathway genes or genes involved in the transcriptional control of TEs (histone markers), or

differences in the TE content in the parental genomes, leading to a lack of piRNAs for some specific TE families.

In testes, the regulation of TEs by piRNA post-transcriptional silencing is complex. For some deregulated TE families in HA_w, HB_w and HB_m, the relationship between the TE overexpression and lack of *ping-pong* amplification was verified. However, for several TEs, regardless of the presence of sense and antisense mapping piRNAs, upregulation was verified. In *Drosophila* testes, the piRNA pathway functions is differently from ovaries (NAGAO *et al.*, 2010), and it may be related to a specific developmental stage and hybrid sterility (KOTOV *et al.*, 2019). In fact, according to Quénerch' du *et al* (2016), different populations of piRNA are present in the spermatogonia or primary spermatocytes. In spermatogonia, piRNAs are predominantly transposon-mapping piRNAs, and the *ping-pong* signature has been verified for several of these sequences. However, in testes enriched in primary spermatocytes, *Ago3* is not expressed, but the *ping-pong* signature is observed, indicating that a noncanonical *ping-pong* cycle functions in spermatogenesis. In our analyses of piRNA-related genes, no misregulation or absence of *Ago3* was observed in testes; nevertheless, this protein was expressed at lower levels in testes than in ovaries. Moreover, in testes enriched in primary spermatocytes, most of the piRNAs function to suppress the repeats and TEs (QUÉNERCH'DU *et al.*, 2016). However, in the testes of *aub* (*Aubergine*) mutants, where *Ago3* expression is also perturbed, the number of transposon-mapping piRNAs was reduced, while piRNA biogenesis was affected in *aub* heterozygous testes (QUÉNERCH'DU *et al.*, 2016).

Unlike ovaries, three genes involved in the piRNA pathway displayed differential expression in the testes: *cuff*, *aub* and *zuc*. The *Cutoff* (*cuff*) gene has an essential role in piRNA production from dual-strand clusters (PANE *et al.*, 2011). Moreover, their interactions with other piRNA proteins, such as *Rhino* (*rhi*) and *Deadlock* (*del*), form the RDC complex, which licenses the transcription of dual-strand piRNA clusters (MOHN *et al.*, 2014). On the other hand, the gene *Zucchini* (*zuc*) is necessary for the biogenesis of *Piwi*-bound piRNAs (HAN *et al.*, 2015),

whereas *Aubergine* (*aub*) participates in the secondary biogenesis of piRNAs, together with *Ago3*, by binding mature piRNAs and initiating the *ping-pong* amplification cycle (review in LUO; LU, 2017). In the HA_w males, two genes (*cuff* and *aub*) presented differential expression compared with at least one parental line, which might affect the piRNA silencing mechanism to some extent. Interestingly, differential *Aub* expression was also verified in hybrids of *D. buzaatti* x *D. koepferae*, but only for one cross direction (GÁMEZ-VISAIRAS *et al.*, 2020). Moreover, differential expression was verified for *cuff* and *zuc* in *D. arizonae*, the two *D. mojavensis* subspecies, and in the HB_m and HB_w hybrids. Indeed, some genes with functions in genome maintenance, such as piRNA or siRNA pathway genes, display signatures of positive selection (OBBARD *et al.*, 2009; KOLACZKOWSKI *et al.*, 2011; FABLET, 2014), and among these genes, *aub* and *zuc* present evidence of adaptations, since deviations in neutrality have been verified (OBBARD *et al.*, 2009; GÁMEZ-VISAIRAS *et al.*, 2020). Thus, differential expression of piRNA pathway genes may affect the large amount of deregulated TEs in hybrid testes.

Based on our data, the siRNA control pathway is quite complex in the male reproductive tissues, since several TEs overexpressed in HB_m, HA_w and HB_w testes exhibited larger amounts siRNAs mapping to deregulated TE families. Surprisingly, most of these TEs also showed complementary piRNAs and the *ping-pong* signature. Thus, they are not efficiently regulated by the siRNA or piRNA pathway. A potential explanation for the higher amount of homologous siRNAs for these TEs is a compensatory mechanisms, as was previously reported in *D. melanogaster*. When problems in the piRNA pathway were detected, an increase in siRNA production has been reported for numerous elements (ROZHKOV *et al.*, 2013). However, for these specific TEs, no decrease in piRNA production was detected, suggesting that epigenetic changes may play a role in TE derepression. At the same time, several TEs that were upregulate in male reproductive tissues presented lower amounts of complementary siRNAs than the parental lines. Based on this result, the overexpression of these elements likely occurs in the soma. Surprisingly, most of these elements belong to the *Gypsy* superfamily. *Gypsy*

retrotransposons are endogenous retroviruses (ERVs), and their expression in *Drosophila* is mainly controlled by siRNAs (WEBER *et al.*, 2006; GHILDIYAL *et al.*, 2008), suggesting that the lack of control by siRNAs in the somatic cells of the testes might promote the overexpression of this type of TE. Furthermore, in the present study, some TEs did not present complementary siRNAs or piRNAs in hybrids or the parental lines. These elements were likely transcriptionally regulated in the parental lines, possibly in heterochromatin regions, but due to divergence of regulatory sequences, they lost their regulatory mechanism in hybrids and were upregulated, since post-transcriptional regulatory mechanisms are absent. However, additional analyses must be performed to confirm this hypothesis.

Similar to the situation in ovaries, we are unable to exclude other factors that play a potential role in TE derepression in testes. In fact, the hybrids HBm and HBw showed decreased expression of a heat shock protein, *Hsp70-1*. Proteins of this family have been shown to play roles in the siRNA, miRNA and piRNA regulatory pathways (IWASAKI *et al.*, 2010; KARAM *et al.*, 2017; CAPPUCCI *et al.*, 2019). The *Hsp70* chaperone has recently been shown to interact with components of chaperone machinery involved in piRNA biogenesis, and the disruption of these proteins decrease the efficacy of TE repression (CAPPUCCI *et al.*, 2019). Therefore, in testes, the post-transcriptional and transcriptional silencing mechanisms are likely more complex and prevent TE derepression, which in hybrid testes, is disturbed due to gene misexpression and regulatory incompatibilities.

CONCLUSIONS

The findings from this study make several contributions to the current literature. Here, the genome of female hybrids of *D. arizonae* and *D. mojavensis* is likely to be more stable than the genome of males. In hybrid ovaries, a few TEs showed dysregulated expression compared to that in both parental lines, which might be related to the fertile phenotypes of these hybrids. In male hybrids, substantial TE derepression was verified, regardless of the cross direction, indicating

that TE overexpression may play an important role in determining the sterile hybrid phenotype. Furthermore, in these hybrids, the silencing mechanism in ovaries and testes is complex, since some TEs were upregulated even in the presence of complementary piRNAs and *ping-pong* amplification, indicating that hybrid incompatibility might affect other components of piRNA biogenesis and result in TE derepression.

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SUPPLEMENTARY MATERIAL

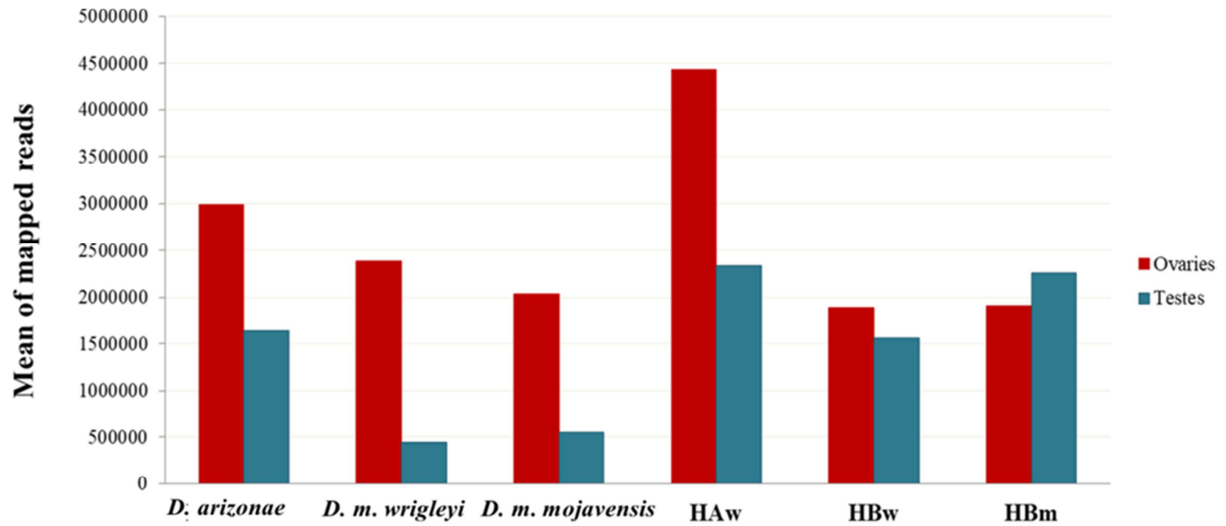


Figure S1. Mean number of raw reads aligning into the TE library according to parental line and interspecific hybrids by tissue. Source: prepared by the author.

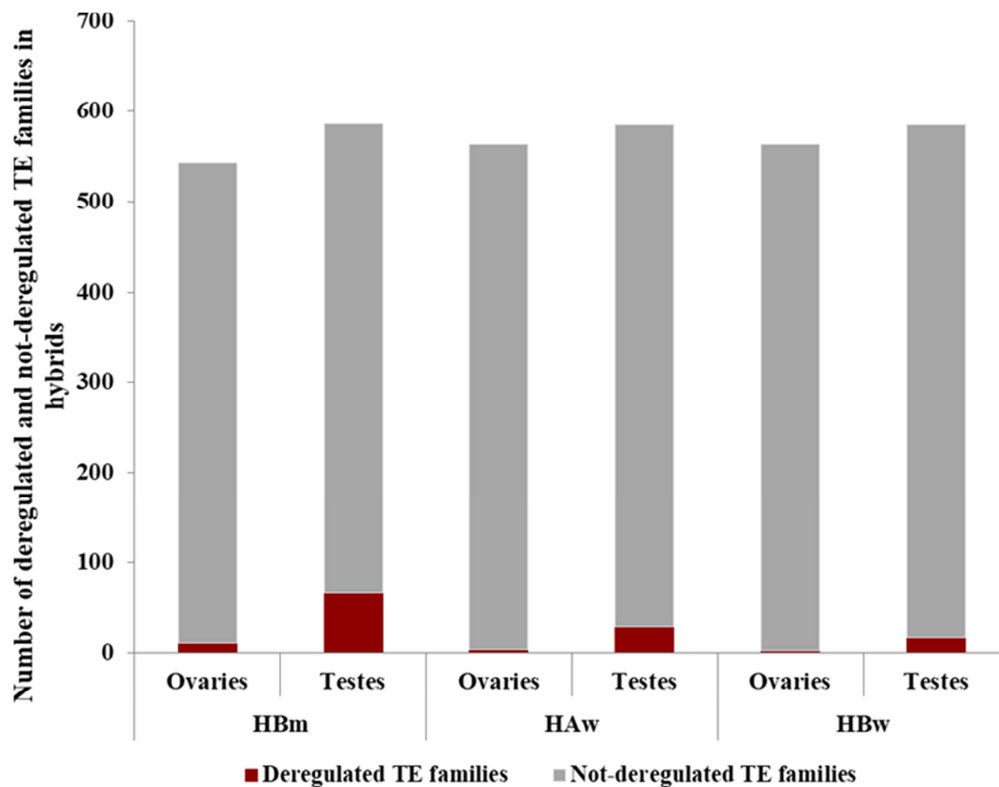


Figure S2. Number of deregulated TE families in hybrids of *D. arizonae* and *D. mojavenensis* by reproductive tissue. Source: prepared by the author.

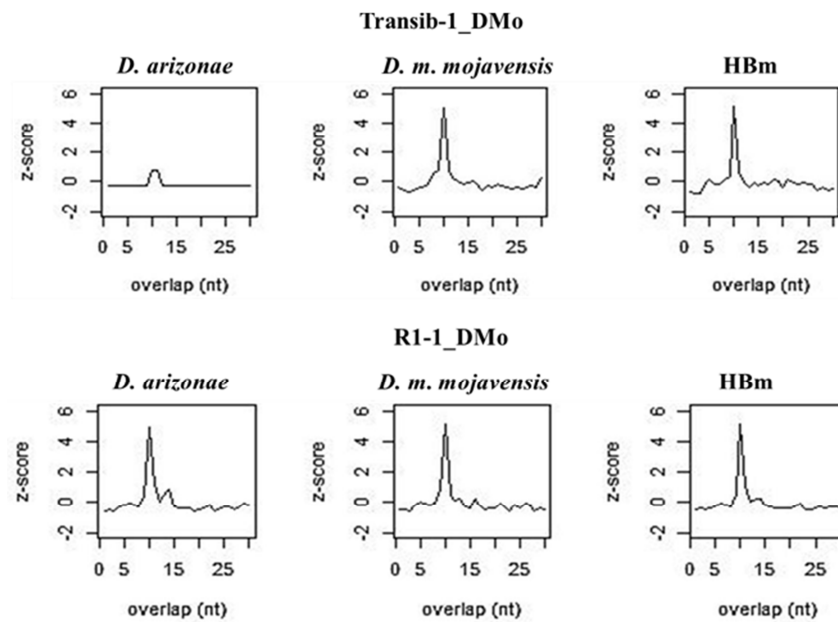


Figure S3. *Ping-pong* signature of deregulated TEs in HBm ovaries. Y-axis: z-score; X-axis: overlap (piRNA length in nt). *Ping-pong* amplification occurs when there is an enrichment in 10 nt overlaps. Z-score is considered significant when presented values > 2.58. Source: prepared by the author.

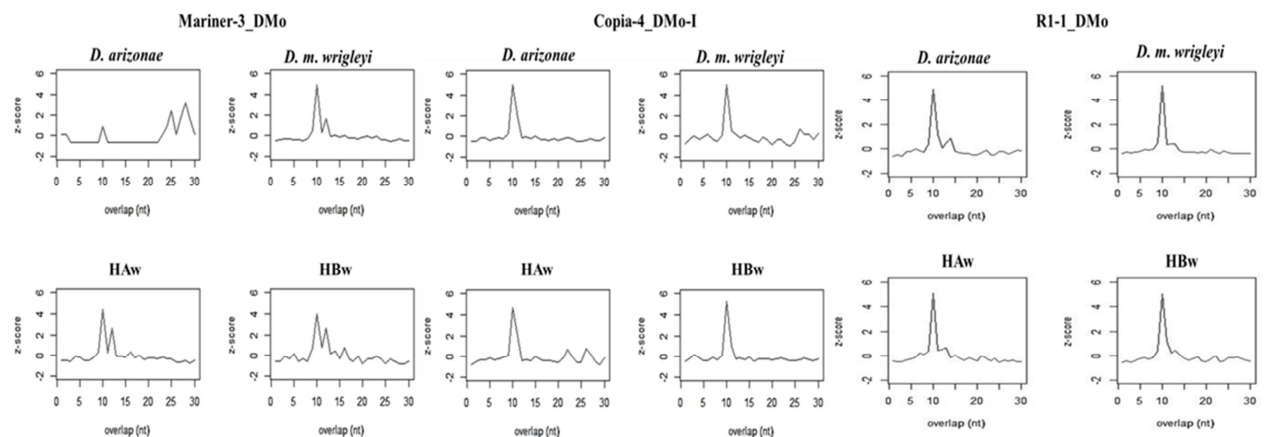


Figure S4. *Ping-pong* signature of deregulated TEs in HAw and HBw ovaries. Y-axis: z-score; X-axis: overlap (piRNA length in nt). *Ping-pong* amplification occurs when there is an enrichment in 10 nt overlaps. Z-score is considered significant when presented values > 2.58. Source: prepared by the author.

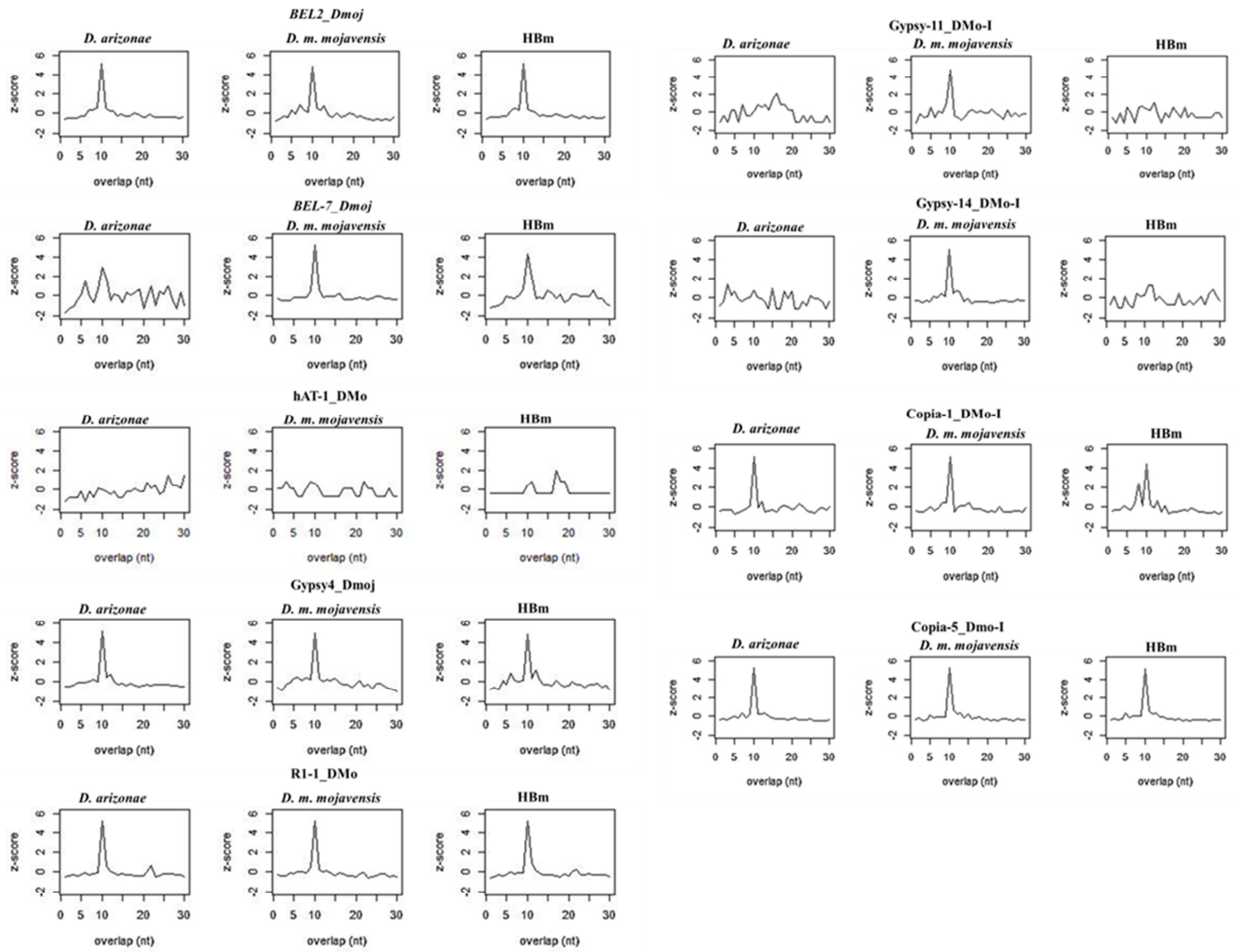


Figure S5. Ping-pong signature for deregulated TEs in HBm testes. Y-axis: z-score; X-axis: overlap (piRNA length in nt). Ping-pong amplification occurs when there is an enrichment in 10 nt overlaps. Z-score is considered significant when presented values > 2.58 . Source: prepared by the author.

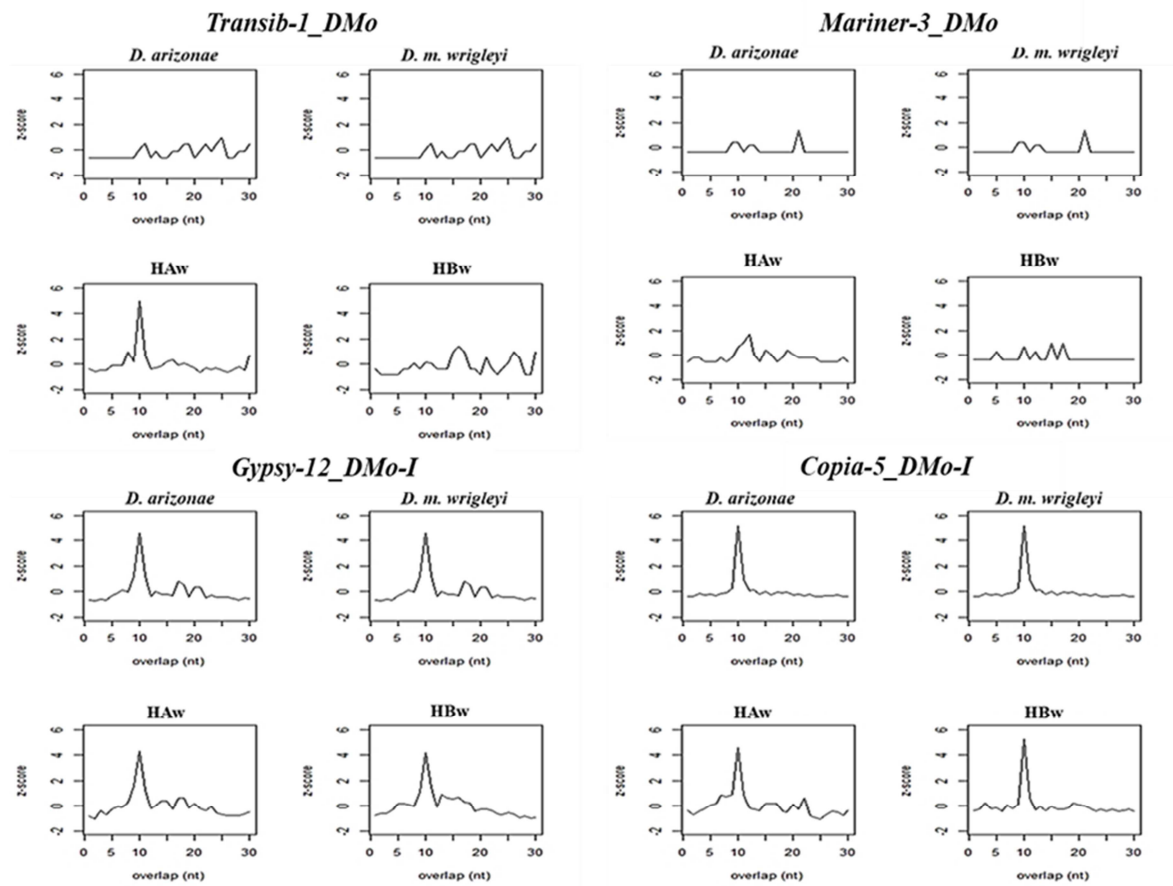


Figure S6. Ping-pong signature of deregulated TEs in HAw testes. Y-axis: z-score; X-axis: overlap (piRNA length in nt). Ping-pong amplification occurs when there is an enrichment in 10 nt overlaps. Z-score is considered significant when presented values > 2.58. Source: prepared by the author.

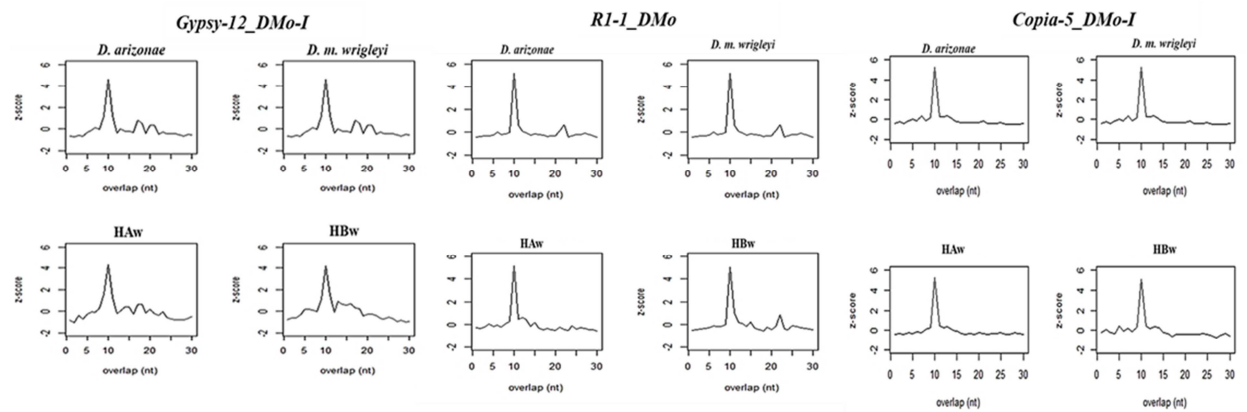


Figure S7. Ping-pong signature of deregulated TEs in HBw testes. Y-axis: z-score; X-axis: overlap (piRNA length in nt). Ping-pong amplification occurs when there is an enrichment in 10 nt overlaps. Z-score is considered significant when presented values > 2.58. Source: prepared by the author.

***Supplementary Tables are available from the author on reasonable request.**

6 DISCUSSÃO GERAL

Neste estudo foi realizada uma ampla análise do processo de hibridização interespecífica em descendentes de espécies que divergiram recentemente. As análises dos parâmetros da história de vida indicaram que híbridos provenientes de cruzamentos entre *D. arizonae* e diferentes subespécies de *D. mojavensis* apresentam viabilidade extremamente baixa. De fato, estudos mostraram que a viabilidade híbrida em *Drosophila* pode ser reduzida, contudo, esta é variável de acordo com as espécies submetidas a cruzamentos interespecíficos, bem como aos estágios de desenvolvimento avaliados (viabilidade embrionária, larval e de pupa) (MAMUTE *et al.*, 2014). As causas que levam à inviabilidade híbrida podem estar relacionadas a falhas no ciclo celular, como mitose e meiose, desapareamento cromossômico, bem como incompatibilidade de elementos regulatórios (DOBZHANSKY, 1937; MULLER, 1942; COYNE *et al.*, 1991; ORSI *et al.*, 2010; STELKENS *et al.*, 2015).

Em híbridos de *D. arizonae* e *D. mojavensis*, capazes de ultrapassar as barreiras de isolamento reprodutivo e serem viáveis, a incompatibilidade decorrente da divergência entre as espécies se manifesta na forma de esterilidade masculina, que é variável e depende da população materna. Em híbridos provenientes de cruzamentos entre fêmeas *D. arizonae* e machos *D. mojavensis*, que estão em alopatria na natureza, os machos foram sempre estéreis, uma vez que apresentam espermatozoides imóveis, e as fêmeas sempre férteis. Entretanto, considerando o cruzamento recíproco, todos os machos apresentaram espermatozoides móveis, contudo a esterilidade foi variável, dependendo da subespécie de *D. mojavensis*. Nossos achados corroboram resultados prévios sobre o grau de isolamento reprodutivo pré e pós-zigótico nesse par de espécies (RUIZ *et al.*, 1990; REED; MARKOW, 2004; REED *et al.*, 2008; CARNELOSSI *et al.*, 2014). Embora os fatores genéticos associados à imotilidade espermática e esterilidade masculina ainda não tenham sido identificados, Vigneault *et al.* (1986) e Pantazidis *et al.* (1988) sugerem que eles possam estar relacionados com interações epistáticas deletérias entre cromossomos sexuais e autossomos. Contudo, a ocorrência de distintos graus de

esterilidade em híbridos de diferentes subespécies de *D. mojavensis* cruzadas com *D. arizonae* indicam a presença de fatores polimórficos entre as espécies que estão envolvidos no processo de isolamento reprodutivo, como também proposto por Reed *et al.* (2004). É interessante ressaltar que as diferenças observadas na fertilidade entre híbridos machos e fêmeas está de acordo com a Regra de Haldane (1922), a qual postula que em cruzamentos interespecíficos os híbridos primeiramente afetados por inviabilidade ou esterilidade são os que possuem sexo heterogamético. Bono *et al.* (2009) ao analisarem híbridos de *D. arizonae* e *D. mojavensis*, quanto a alguns parâmetros de história de vida, demonstraram que as fêmeas híbridas viáveis possuem desempenho similar às espécies puras, ao passo que os machos híbridos, além de serem estéreis, apresentam menor resistência à dessecação, o que é um importante fator seletivo, considerando que essas espécies vivem em ambientes desérticos. Portanto, é possível inferir que em híbridos desse par de espécies, os fatores que levam à incompatibilidade híbrida são mais severos, e provavelmente evoluem mais rapidamente em machos do que em fêmeas, o que pode ser verificado fenotipicamente. Confirmando esse fato, Turissini *et al.* (2018) mostraram que em machos híbridos, provenientes de cruzamentos de espécies do grupo *melanogaster*, a inviabilidade e a esterilidade evoluem mais rapidamente do que nas fêmeas híbridas.

Considerando as diferenças fenotípicas, e na tentativa de identificar alguns dos fatores que estão influenciando a esterilidade híbrida, análises de expressão gênica e de elementos de transposição foram realizadas. Confirmando o fenótipo fértil de fêmeas híbridas de *D. arizonae* e das duas subespécies de *D. mojavensis*, poucos genes apresentaram expressão divergente em relação às linhagens parentais. Contudo, em testículos de híbridos o cenário foi muito diferente, isso porque, dependendo da direção de cruzamento, e, portanto, da linhagem materna, maior ou menor grau de desregulação da expressão gênica foi observado. Resultados similares foram obtidos por Llopart *et al.* (2018) e Ranz *et al.* (2003) que demonstraram que híbridos machos de *D. santomea*–*D. yakuba* e *D. melanogaster*–*D. simulans*, respectivamente, possuem mais genes desregulados em comparação às fêmeas híbridas.

Nosso estudo mostrou a ocorrência de muitos genes subexpressos em híbridos com espermatozoides imóveis (provenientes de cruzamentos entre fêmeas *D. arizonae* e machos *D. m. mojavensis* e *D. m. wrigleyi*), e que a maior parte deles apresentava funções relacionadas à espermatogênese e a motilidade espermática, o que pode ser associado ao fenótipo estéril. Híbridos de outras espécies de *Drosophila*, como os provenientes de cruzamentos entre *D. simulans* e *D. mauritiana*, mostraram o mesmo padrão, isto é, super-representação de genes subexpressos com funções relacionadas à espermatogênese em híbridos estéreis (MICHALAK; NOOR, 2003, 2004; MA; MICHALAK, 2011), evidenciando a associação entre o fenótipo e a desregulação da expressão gênica. Dentre esses genes podemos destacar alguns diretamente relacionados com o ciclo celular como *Jupiter* e *twe* (*twine*), bem com funções diretamente relacionadas à motilidade espermática, como genes de dineína (*Dnah3*, *Dhc16F* *CG10839*, *CG31275 l(2)41Ab*), entre outros. *Jupiter* é um gene que codifica uma proteína localizada na rede de microtubulos, com importante função no ciclo celular (KARPOVA *et al.*, 2006). O gene *twe*, por sua vez, codifica uma proteína necessária para o início da meiose em machos. Em híbridos machos sem a expressão da proteína *twine* foram verificadas falhas na transição G2/M, dessa forma a meiose não é completa e podem ser encontradas espermátides 4N (WHITE-COOPER *et al.*, 1998; MAINES; WASSEMAN, 1999; KARPOVA *et al.*, 2006; LINDSLEY *et al.*, 2013; JIANG *et al.*, 2013). Além disso, o correto funcionamento de *twine* depende da regulação por meio de outros genes envolvidos nas fases iniciais da meiose (JIANG *et al.*, 2013; WHITE-COOPER *et al.*, 1998). Genes relacionados com o movimento dos espermatozoides, cílios e montagem dos microtúbulos, como genes de dineína, exibiram grande enriquecimento entre os genes subexpressos de híbridos estéreis originados do cruzamento entre fêmeas *D. arizonae* e machos *D. mojavensis*. Esses genes normalmente são expressos em altos níveis em testículos (RASMUSSEN *et al.*, 1994). Sendo assim, a falta dessas proteínas podem estar relacionadas com a esterilidade masculina, como previamente foi mostrado em ratos e moscas da fruta (FOSSELLA *et al.*, 2000; FABIAN; BRILL, 2012; KARAK *et al.*, 2015). Adicionalmente,

de acordo com Wu *et al.* (1993), híbridos que não apresentam espermatozoides móveis, mas que têm meiose completa, provavelmente apresentam falhas pós-meioticas associadas à espermiogênese, ao invés de anormalidades em larga escala no desenvolvimento dos órgãos reprodutivos.

Ao contrário do cruzamento acima descrito, em híbridos do cruzamento recíproco (fêmeas *D. m. wrigleyi* x machos *D. arizonae*), que possuem espermatozoides móveis, embora sejam estéreis, menor grau de desregulação gênica foi verificado, principalmente envolvendo genes que atuam na espermatogênese. Contudo, alguns genes envolvidos com reprodução, processamento de micro-RNA e em mecanismos de silenciamento foram identificados, mostrando que outros fatores podem estar contribuindo para a esterilidade dos híbridos provenientes de cruzamentos com fêmeas *D. mojavenensis*. Hardy *et al.* (2016), ao analisarem a ultraestrutura de testículos de híbridos recíprocos de *D. arizonae* e *D. m. mojavenensis*, mostraram que híbridos de cruzamentos com fêmeas *D. mojavenensis* apresentavam defeitos no desenvolvimento do núcleo das espermátides, mas os espermatozoides tinham caudas normais, o que explica o baixo número de genes desregulados envolvidos com movimento da cauda nesses híbridos.

Considerando os TEs, foi observada uma tendência à superexpressão, em menor grau em ovários e em maior grau em testículos. Níveis diferentes de desregulação de TEs entre tecidos reprodutivos masculinos e femininos já foram identificados em espécies de *Drosophila* (CARNELOSSI *et al.*, 2014; VELA *et al.*, 2014; ROMERO-SORIANO *et al.*, 2016). Neste trabalho, verificamos ainda que a expressão diferencial de TEs não estava completamente relacionada com a direção de cruzamento, como foi observado para expressão gênica, mas sim relacionada com a subespécie de *D. mojavenensis* utilizada nos cruzamentos interespecíficos. Isso porque os diferentes híbridos apresentaram números muito diferentes de TEs desregulados, sendo a maioria deles unicamente superexpressos em híbridos específicos. Isso indica que as diferentes subespécies de *D. mojavenensis* provavelmente possuem famílias de TEs distintas com

expressão controlada em seus genomas, as quais, em face do choque genômico decorrente da hibridização interespecífica, podem ser diferencialmente ativadas devido à perda de mecanismos de regulação. De fato, diferenças no conteúdo de TEs em genomas são frequentemente observadas para diversas espécies, sendo que essas variações podem ocorrer também em nível populacional, como para *D. simulans* (SONG *et al.*, 2014; FABLET *et al.*, 2019).

Ademais, um fato interessante a ser destacado é que os híbridos machos exibem maior número de família de TEs desreguladas do que as fêmeas do mesmo cruzamento. Isso sugere que os mecanismos de regulação em níveis transcricional e pós-transcricional são sexo-específicos, podendo ser mais eficientes nas fêmeas que em machos. Confirmando esses resultados, Malone *et al.* (2009) sugerem que a regulação de determinados TEs podem ser tecido-específica e estar realcionada aos *clusters* de piRNAs presentes nos genomas parentais.

A análise da via de piRNAs mostrou que em ovários, assim como em testículos, alguns TEs não apresentavam piRNAs correspondentes na linhagem maternal. Isso poderia levar à falha de regulação desses elementos nos híbridos, devido à falta de deposição de piRNAs maternos, os quais são essenciais para iniciar o processo de regulação pós-transcricional, denominado ciclo *ping-pong*, como verificado previamente para híbridos de outras espécies de *Drosophila* (BRENNECKE *et al.*, 2008; AKKOUCHE *et al.*, 2013; LOPEZ-MAESTRE *et al.*, 2017; FUNIKOV *et al.*, 2018). Contudo, diversos TEs, em ambos tecidos reprodutivos de híbridos, apresentaram piRNAs complementares, os quais não foram capazes de controlar sua expressão. Isso poderia se explicado por um aumento de transposição em células somáticas associadas aos testículos extraídos (vesículas seminais), como proposto para o retrotransposon *Oswaldo* que tende aumentar a taxa de transposição em machos híbridos de cruzamentos entre *D. buzzatii* e *D. koepferae* (VELA *et al.*, 2014). Contudo, em nossas análises de siRNAs verificou-se que determinados TEs, que apresentam piRNAs correspondentes, também possuíam siRNAs complementares, os quais deveriam ser capazes de controlar sua expressão em células somáticas; entretanto, apesar da presença de siRNAs, esses TEs estavam superexpressos nos híbridos. Isso

significa que, de alguma forma, os mecanismos de regulação nos híbridos entre *D. arizonae* e *D. mojavensis* estão falhando, seja na via de piRNAs ou em possíveis mecanismos de regulação alternativos. Quénerch'du *et al.* (2016) mostraram que em testículos provavelmente há uma via de controle e amplificação de piRNAs não canônica, uma vez que em mutantes de proteínas essenciais para a via *ping-pong*, amplificação de piRNAs foi observada.

Em fêmeas de cruzamentos entre *D. arizonae* e *D. mojavensis*, genes que fazem parte da via de piRNAs e alguns genes associados a modificações de cromatina apresentam expressão conservada em híbridos em relação aos parentais, bem como entre as diferentes espécies parentais. Todavia, em testículos o mesmo não foi verificado para determinados genes. Isso pode ter um impacto direto na superexpressão desses elementos nas gônadas masculinas, bem como influenciar a regulação de outros genes, ou mesmo ter um papel importante no fenótipo de esterilidade observado. Desregulação na expressão gênica em híbridos masculinos é frequentemente observada em espécies de *Drosophila* e já foi associada com taxas de evolução mais rápidas em machos, que em fêmeas (MEIKLEJOHN *et al.*, 2003; RANZ *et al.*, 2003; ARTIERI *et al.*, 2007; MOEHRING *et al.*, 2007; LLOPART, 2012). Sendo assim, sugerimos que, em híbridos de *D. arizonae* e *D. mojavensis*, provavelmente diferenças de sequências regulatórias que agem de forma *cis* e *trans*, e interações epistáticas deletérias, levam à desregulação de diversos genes, incluindo aqueles relacionados aos sistemas de regulação pós-transcricional e transcricional. Como consequência, a expressão de muitos TEs não é capaz de ser controlada, o que pode ter um efeito negativo no genoma híbrido, podendo levar à incompatibilidade e ser associada ao isolamento reprodutivo. Esse efeito pode ser mais acentuado em determinadas direções de cruzamento, devido a diferentes interações epistáticas, ou dependendo da subespécie, devido a polimorfismos existentes entre elas, bem como ter maior impacto em um sexo que em outro. Isso porque, em machos, determinados genes podem apresentar maior taxa de evolução, ou mesmo devido a herança dominante de determinados alelos no cromossomo X em hemizogose em machos, que não ocorre em fêmeas (LLOPART *et*

al., 2018).

De maneira geral, ainda não se sabe se interações epistáticas deletérias que levam à desregulação de genes de vias específicas são responsáveis pela superexpressão de TEs em híbridos ou se o choque genômico que leva à mobilização de TEs afeta a expressão gênica. Contudo, podemos sugerir que ambos processos são intimamente ligados e diversos estudos vêm demonstrando isso. Satyaki *et al.* (2014) mostraram que a alelos dos genes *Lmr* (*Lethal male rescue*) e *Hmr* (*Hybrid male rescue*) interagem e controlam a expressão de sequências repetitivas e satélites. Contudo, em híbridos de *D. simulans* e *D. melanogaster*, inviabilidade híbrida e aumento da expressão de TEs ocorre devido à divergência adaptativa dessas sequências nas diferentes espécies (SATYAKI *et al.*, 2014). Moehring *et al.* (2007) sugerem que a regulação transcricional pode estar associada com incompatibilidade em híbridos, como a esterilidade. Isso porque em muitas espécies de *Drosophila*, a transcrição de genes envolvidos na espermatogênese ocorre na fase anterior à meiose (FULLER, 1998). Superexpressão e eventos de transposição já foram verificados para alguns TEs específicos em tecidos reprodutivos masculinos. Dentre eles pode-se destacar o elemento *Copia*, que possui transposição limitada a espermátócitos masculinos (PASYUKOVA *et al.*, 1997; KALMYKOVA *et al.*, 2005) e os elementos *Osvaldo* e *I* (GUERREIRO, 2015; CARNELOSSI *et al.*, 2014), os quais são expressos nas regiões apicais dos testículos, que contém células da linhagem germinativa, espermátócitos primários e secundários (VEDELECK *et al.*, 2018). Mostrou-se que híbridos intraespecíficos de *D. melanogaster* com expressão do elemento *I*, devido a falhas na via de piRNAs, apresentaram meiose anormal, sugerindo que a atividade desse elemento perturba a organização cromossômica durante o ciclo celular (ORSI *et al.*, 2010). Esse fato pode indicar que, de alguma forma, a grande desregulação de TEs (principalmente superexpressos) pode estar relacionada à esterilidade híbrida nos machos híbridos de cruzamentos entre *D. arizonae* e *D. mojavensis*. O estudo de Iwasaki *et al.* (2016), sobre as marcas repressivas da cromatina em células somáticas de ovários de *Drosophila* mutantes para a proteína Piwi, auxiliam a compreensão desses

resultados. Os autores verificaram que falhas no sistema de regulação influenciam a expressão de um subconjunto de genes codificadores de proteínas próximos a inserções de TEs. Dentre esses genes, a maioria apresentava funções relacionadas ao desenvolvimento das características sexuais primárias e diferenciação sexual. Além disso, segundo Gurrard *et al.* (2006), piRNAs podem ter um importante papel relacionado ao controle da expressão gênica durante a gametogênese, uma vez que durante esse processo muitas modificações epigenéticas ocorrem.

Sendo assim, os resultados obtidos neste trabalho e diversas evidências da literatura indicam que existe uma grande complexidade e interações de mecanismos que permeiam as barreiras do isolamento reprodutivo. Dentre esses mecanismos, podemos sugerir que, em híbridos, prejuízos nos sistemas de regulação, devido à divergência adaptativa de allos, levam à desregulação da expressão gênica e de TEs, originando uma cascata de sucessivos eventos de desregulação que contribuem para mecanismos de incompatibilidade híbrida. Os resultados apresentados nesta tese trazem contribuições inéditas para a compreensão dos efeitos da hibridização interespecífica em descendentes de espécies que divergiram recentemente, contudo, análises adicionais precisam ser realizadas para melhor entender esse processo. Dentre elas, podemos destacar estudos de regulação *cis* e *trans*, bem como análises de taxa de evolução e funcionais para determinados genes regulatórios.

7 CONCLUSÕES

Este trabalho permitiu elaborar as seguintes conclusões:

1. Redução de viabilidade nos dois sexos em híbridos recíprocos provenientes de cruzamentos entre *D. arizonae* e quatro subespécies de *D. mojavensis* e esterilidade variável dos machos híbridos dependente da população materna de *D. mojavensis*, sugerem que os fatores que levam à incompatibilidade híbrida nesse par de espécies são polimórficos, os quais podem evoluir a diferentes taxas em cada população de *D. mojavensis*, dependendo de sua origem geográfica, isto é, se estão em simpatria ou em alopatria.
2. Machos híbridos sem espermatozoides móveis apresentaram diversos genes diferencialmente expressos, os quais eram, em sua maioria, subexpressos em relação à expressão gênica nos parentais, e tinham funções reprodutivas e de motilidade dos espermatozoides. No entanto, híbridos machos que possuíam espermatozoides móveis apresentaram menos genes diferencialmente expressos, os quais tinham diversas funções, como regulação e transcrição gênica. Esses resultados indicam que a motilidade espermática está relacionada ao grau de desregulação da expressão gênica que ocorre nesses híbridos.
3. Fêmeas híbridas apresentaram poucos genes diferencialmente expressos, concordando com fenótipo fértil, e sugerindo menor interrupção de elementos regulatórios. Em machos híbridos, a maior expressão diferencial de genes pode estar relacionada com a evolução mais rápida de sequências regulatórias, principalmente para genes exclusivamente expressos em testículos, bem como a incompatibilidade decorrente de alelos localizados no cromossomo X, uma vez que em machos, alelos que normalmente se expressam em condições de recessividade podem se comportar como dominantes devido ao fato de estarem em hemizigose.

4. Em fêmeas híbridas ocorre menor desregulação de TEs do que em machos híbridos, o que também pode ter um papel importante no fenótipo fértil e estéril, respectivamente, verificados nesses híbridos.
5. Diferentes mecanismos que envolvem a via de regulação de TEs atuam em ovários e testículos, uma vez que híbridos machos e fêmeas provenientes dos mesmos cruzamentos interespecíficos não apresentaram os mesmos TEs superexpressos.
6. Os mecanismos de silenciamento por meio das vias de piRNA e siRNAs se mostraram complexos em fêmeas e machos híbridos, uma vez que TEs que tinham piRNAs e siRNAs complementares não estavam regulados, estando superexpressos, sugerindo ocorrência de falhas em outros mecanismos de regulação, tais como divergência de expressão de genes envolvidos nas vias de regulação em machos híbridos. Isso indica que, em machos, assim como verificado em análises de expressão gênica, interações epistáticas de alelos com maior taxa de evolução ou expressão dominante podem contribuir para a desregulação de TEs.

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