Gene expression and alternative splicing dynamics are perturbed in female head transcriptomes following heterospecific copulation

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Abstract

Background: Despite the growing interest in the female side of copulatory interactions, the roles played by differential expression and alternative splicing mechanisms of pre-RNA on tissues outside of the reproductive tract have remained largely unknown. Here we addressed these questions in the context of conspecific vs heterospecific matings between Drosophila mojavensis and its sister species, D. arizonae. We analyzed transcriptional responses in female heads using an integrated investigation of genome-wide patterns of gene expression, including differential expression (DE), alternative splicing (AS) and intron retention (IR).

Results: Our results indicated that early transcriptional responses were largely congruent between conspecific and heterospecific matings but are substantially perturbed over time. Conspecific matings induced functional pathways related to amino acid balance previously associated with the brain’s physiology and female postmating behavior. Heterospecific matings often failed to activate regulation of some of these genes and induced expression of additional genes when compared with those of conspecifically-mated females. These mechanisms showed functional specializations with DE genes mostly linked to pathways of proteolysis and nutrient homeostasis, while AS genes were more related to photoreception and muscle assembly pathways. IR seems to play a more general role in DE regulation during the female postmating response.

Conclusions: We provide evidence showing that AS genes substantially perturbed by heterospecific matings in female heads evolve at slower evolutionary rates than the genome background. However, DE genes evolve at evolutionary rates similar, or even higher, than those of male reproductive genes, which highlights their potential role in sexual selection and the evolution of reproductive barriers.

Keywords: Speciation, Postmating response, Alternative splicing, Intron retention, RNA-seq, Head transcriptomes, D. mojavensis, D. arizonae

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Background

Sexual reproduction involves a set of coupled interactions affecting the performance of both sexes, such as those involved in mate-recognition, male courtship and female postcopulatory responses [1]. Females undergo a complex process of physiological changes after mating called the postmating response, induced by biochemical interactions between ejaculate components transferred during copulation and female molecules in the reproductive tract [2, 3]. The role of seminal fluid proteins in the female postmating response has been well characterized in Drosophila species, and hundreds of male-derived proteins have now been identified in other taxa [4–7]. The female side has remained more elusive and very little is known about the downstream effector genes that mediate the female postmating response, particularly those occurring outside of the female reproductive tract (e.g. genes related to female behavior).

Although transcriptional changes induced by conspecific or heterospecific matings have been explored in a number of species [8–16], most of these studies have not considered alternative splicing (AS) as an additional mechanism by which genes responsible for postmating changes might be regulated [17]. Differential regulation of spliced isoforms created by different combinations of exons (or intron retention, IR) from the same genomic loci may have substantial functional consequences not reflected in gene expression [18], which can uncover additional mechanisms within the complexity of molecular reproductive interactions. Recent comparisons of Drosophila species show that AS diversification contributes to lineage-specific adaptation [19], with sex-biased splicing and IR rates [20] in several tissues including the brain [18], suggesting that this mechanism might be important in female behavioral responses.

Changes induced by heterospecific matings can compromise gametic interactions during the fertilization process leading to postmating prezygotic or PMPZ isolation [2, 21]. Moreover, conspecific matings are often accompanied by a set of behavioral changes, such as those involved in female receptivity, exploration, diet and ovisposition [22–27]. If altered by mating with a heterospecific male, these behavioral changes can compromise the mating outcome, leading to reproductive barriers. The transcriptional bases of these responses are more likely located in tissues of the central nervous system [28]. In fact, in D. melanogaster the seminal fluid protein (SFP) Sex Peptide (SP), is not only one of the main triggers of the female postmating response, but is also gradually released into the hemolymph by cleavage [29–31], suggesting important and lasting changes outside of the reproductive tissues. Sex Peptide interacts with a sex peptide receptor (SPR) in the female, which is expressed in both reproductive organs and the nervous system [32]. Consistent with this, transcriptional changes associated with behavioral and photoreception pathways have been detected in female heads after conspecific mating [25, 33].

It is well known that interacting male and female reproductive genes evolve rapidly [34], however it is unclear whether genes governed by AS dynamics or expressed outside of the reproductive tissues follow this evolutionary path. We addressed these questions by exploring head transcriptomes in cons- vs heterospecific matings between Drosophila mojavensis and D. arizonae. Perturbation of the female transcriptional response by heterospecific matings was first demonstrated in female reproductive tracts of Drosophila mojavensis when mated to D. arizonae males [8]. These species diverged ~ 0.5Mya [35] and display strong PMPZ isolation as fertilization success is reduced after heterospecific matings [21, 36]. Although heterospecific matings occur in both directions [37], transcriptional responses have not been previously explored in D. arizonae females. Here we implemented an integrated approach to explore the conspecific context of each species and demonstrate that the postcopulatory response involves functionally different roles played by DE, AS and IR dynamics. These responses are substantially perturbed by heterospecific matings and some of these genes evolve rapidly.

Results

Pervasive perturbation of DE and AS following heterospecific matings

Experimental design consisted of conspecific and heterospecific matings between the species D. mojavensis and D. arizonae considering three biological replicates composed of 20 pooled dissected heads (Fig. 1). After trimming and filtering of sequence reads, we obtained an average of 20 million mapped reads across the 30 RNA-seq libraries. Minimum count filtering was applied independently to all different subfeatures (e.g. exon, junction, intron) at the beginning of each analysis performed. We found evidence for gene expression changes when comparing mated with virgin samples (Fig. 2a). These changes were consistently detected at different hierarchies of gene expression such as at gene-wide (DE, using FDR = 0.05) (Fig. 3) as well as at exon and junction features (AS, using FDR = 0.01), including up to 16% of the AS genes exhibiting differential IR (using FDR = 0.05). Although the DE - AS overlap never exceeded 5% of genes, as expected from their distinct molecular regulatory mechanisms, both generally followed similar patterns in postmating experiments (Fig. 2a). However, the relative contribution of DE and AS showed large variation across the different conditions of the experiment. The overlap of genes responding to cons- vs heterospecific matings was very low for all experiments, ranging from 14
to 28% (Fig. 2a), indicating that copulation between either ♀\textit{Dmoj} or ♀\textit{Dari} with a heterospecific male induces a very different transcriptional response in female heads.

The female’s species (♀\textit{Dmoj} or ♀\textit{Dari}) seems to define the main patterns for expression responses when crossing \textit{D. mojavensis} and \textit{D. arizonae}. It defines the strength of the con- vs heterospecific response (Fig. 3) as well as when such changes are induced in the heads (45 min vs 6 h postmating periods). ♀\textit{Dmoj} crosses exhibited a larger response at 45 min (Fig. 3a) and higher number of genes when compared to ♀\textit{Dari}, which then tended to decrease over time. ♀\textit{Dari} matings generated a response to the heterospecific matings that was slower and tended to increase over time, but decreased for female heads of conspecific matings (Figs. 2a and 3b).

The direction of expression changes in mated females compared to female virgin samples showed an overrepresentation of up- vs downregulated genes (Fig. 2b). Thus, ♀\textit{Dmoj} matings exhibited up to four times more upregulated than downregulated genes (Fig. 2b), while ♀\textit{Dari} matings had over twice as many downregulated genes as upregulated. The number of genes and the distribution of the expression response in \textit{DE} genes were substantially perturbed by the heterospecific matings (Fig. 3). The response of ♀\textit{Dmoj} was stronger for conspecific matings in terms of the number of genes involved (Fig. 2b, Fig. 3) when compared to that of heterospecific matings, while ♀\textit{Dari} involved more genes in the heterospecific matings than that of the conspecific matings (Fig. 2b, Fig. 3). Expression of most of the significant \textit{DE} genes common to con- and heterospecific responses was highly correlated, suggesting that these genes follow similar directions regardless of the species identity of the male (Fig. 3). However, a few of these genes show interesting and opposing patterns between con- and heterospecific matings, which make them additionally interesting candidates to further investigate in the context of the reproductive isolation.
between the cactophilic Drosophila species (Supplementary Tables 1S and 2S).

**Transcriptional correlation between crosses and postmating periods**

We next examined the level of transcriptional correlation between con- vs heterospecific matings for genes showing significant DE and AS responses. Overall, DE and AS correlations show similar tendencies, with a strong initial correlation between con- vs heterospecific matings at 45 min that tended to decrease substantially at 6 h (Fig. 4). One exception was the case of ♀Dmoj (con vs hetero), where DE genes (Fig. 4a) showed very low correlation even at 45 min postmating (Spearman’s \( \rho = -0.16 \)), indicating substantial transcriptional perturbation in heterospecifically-mated females (Fig. 4a). ♀Dari (con vs hetero) on the other hand showed a stronger transcriptional correlation at 45 min (Spearman’s \( \rho = 0.86 \)) which then decreased at 6 h (Spearman’s \( \rho = 0.70 \)). This pattern was opposite to that observed in AS genes (Fig. 4b), where ♀Dmoj showed a strong con vs hetero correlation at 45 min, which was disrupted at 6 h, while transcriptional perturbation appeared earlier (at 45 min postmating) in ♀Dari crosses (Fig. 4b).
We next investigated the level of correlation for genes responding to mating dynamics between the species (♀\textit{Dmoj} vs ♀\textit{Dari}). With the exception of \textit{DE} genes at 45 min (Fig. 4a), which showed a moderate correlation in the conspecific-mating response between the species (♀\textit{Dmoj} con vs ♀\textit{Dari} con, Spearman’s \( \rho = 0.51 \)), the rest of the comparisons (\textit{DE} and \textit{AS}) were not correlated between the species (con- or heterospecific) (Fig. 4a and b).

Most of \textit{AS} dynamics detected through \textit{junctionSeq} reflected differential isoform regulation. However, the specific case of intron retention, as detected using \textit{IRFinder}, more likely indicates gene regulation by nonsense-mediated decay (\textit{NMD}) or a similar pathway. We tested this hypothesis as a possible mechanism of transcriptional response to mating by estimating intron retention changes between mated vs virgin samples (\textit{IR change}), for up and down-regulated genes (Fig. 5). We discovered that \textit{IR change} consistently increased for down-regulated genes, while it decreased for up-regulated genes in response to all mating experiments (Fig. 5). This finding is consistent with \textit{IR} serving as a mechanism of gene expression downregulation.

### Evidence of positive selection in postmating responsive genes

We investigated rates of molecular evolution (\( \omega = dN/dS \)) of \textit{DE} and \textit{AS} genes for each experimental cross. Results on
evolutionary rates revealed two main patterns of molecular evolution in these genes (Fig. 6a). Firstly, AS genes seem to evolve at a much lower evolutionary rate than DE genes, even lower than the genome background. Secondly, DE genes exhibited substantial differences in the evolutionary rates between both species and crosses. The average ω ratio was substantially higher than the genome background for conspecific matings in D. arizonae (ω = 0.38, Fig. 6a), while D. mojavensis genes evolve at background rates. The heterospecific matings show exactly the opposite pattern, with DE genes from heterospecifically-mated D. mojavensis females evolving more rapidly than background (ω = 0.30), while heterospecifically-mated D. arizonae DE genes evolve at genome background rates (ω = 0.18, Fig. 6a). Rapidly evolving DE genes appear to change at similar rates or even higher than those of seminal fluid proteins (SFP) previously reported by Kelleher et al. [38] in D. mojavensis (Fig. 6).

Functional specialization played by DE and AS genes
To analyze the functional pathways associated with female genes responding to mating experiments in female heads, we performed gene ontology (GO) enrichment analysis. We found that detected genes were enriched in four main functional pathways (Fig. 6b and c): i) nutrient homeostasis, ii) chitin metabolism, iii) photoreception and iv) muscle assembly (Fig. 6). Pathways associated with i) nutrient homeostasis are particularly interesting for their implications in the female postmating response. These pathways are all associated with amino acid balance in brains: L-Carnitine from Lysine and Methionine (Carnitine biosynthetic process and Gamma-butyrobetaine dioxygenase activity, Fig. 6b), and the production of Threonine and Leucine (Threonine and Leucine biosynthetic process, Fig. 6b). Furthermore, some of these metabolic functions have been previously detected in conspecific mating experiments in D. melanogaster [28, 39]. Physiological functions associated with these specific amino acids include energy balance in brain tissues (for Carnitine) [40, 41], insulin secretion following food intake, increasing cellular uptake of nutrients (for Leucine) [42] and female behaviors such as sleep suppression (for Threonine) [43, 44].
We found functional specialization between DE and AS genes, with DE (Fig. 6b) patterns being more associated with pathways of i) nutrient homeostasis and ii) chitin metabolism, while AS genes (Fig. 6c) were dominated by functional networks related to iii) photoreception and iv) muscle assembly. Both mechanisms of gene regulation showed dramatic functional differentiation between con- vs heterospecific crosses (Fig. 6b and c). Most of the enriched pathways were detected in a conspecific context, but only a subset remained significant in the heterospecific matings (Fig. 6b and c). Genes associated with i) nutrient homeostasis and iii) photoreception, activated in conspecific matings, were not activated in heterospecific matings (Fig. 6b and c). Genes associated with i) nutrient homeostasis and iii) photoreception, activated in conspecific matings, were not activated in heterospecifically-mated ♀Dmoj. Similarly, all proteolytic pathways activated in conspecific ♀Dari were not activated in heterospecific matings. Genes that have been previously reported as related with the i) female behavior were exclusive to D. mojavensis and were not enriched in D. arizonae (Fig. 6b).

Discussion
The cactophilic D. mojavensis and D. arizonae are promiscuous flies, mating multiple times a day in a laboratory setting, even to heterospecífics (Diaz et al unpublished data). Yet these species have remained isolated for ~ 0.5 My, suggesting the presence of multiple reproductive barriers preventing introgressive hybridization [36, 45, 46]. PMPZ isolation has been confirmed for crosses involving D. mojavensis females [21], where the reaction mass is more evident and molecular interactions in the female lower reproductive tract were found altered by the heterospecific ejaculate [8]. Here, we demonstrate that copulation induces substantial transcriptional changes in head tissues of females that are substantially perturbed when mating with a heterospecific male in both species. These changes compromise functional pathways important for the female postcopulatory physiology and behavior that normally would be expressed in conspecifically-mated females. Moreover, some of these genes evolve rapidly, which might have implications for the extent of sexual selection and sexual conflict [47].

Our results indicate that mating induces not only gene expression changes in female heads soon after mating, but also that a great part of the female postmating response involves a change in alternative splicing. The number of genes responding through AS often exceeded that of DE genes, but both mechanisms appear to be involved in the female postcopulatory response. We examined AS patterns caused by multiple mechanisms, including intron retention (IR) [18], when comparing mated vs virgin females. Differential usage of examined gene features showed substantial consequences for the postmating response that were not reflected in gene expression of head transcriptomes. The role of AS in the postmating response has not been previously evaluated, but is consistent with the complexity of interactions related to sexual traits [34]. Interestingly, in this study, genes experiencing DE or AS appear to be almost mutually exclusive (less than 5% overlap). Consequently, the female postmating response seems to target different functions through each of these mechanisms. DE genes are mainly linked to pathways of proteolysis and nutrient homeostasis, while AS genes are more related to those involved in photoreception and muscle assembly changes.

IR is a particular case of AS that, although it has been associated with some active functional changes, is most likely linked to negative gene regulation resulting from the degradation of mRNA by the nonsense-mediated mRNA decay (NMD) pathway [20, 48]. We demonstrate that the extent of IR is not only different between con- and heterospecific matings but also seems to be an active mechanism of gene regulation, as IR rates increased for down regulated genes, but decreased for up-regulated genes.

The transcriptional response to mating has been studied in a few insect species [9–16], showing biologically meaningful pathways common to the postmating response across different species. In fact, some of the mating-activated genes that we found in female heads are associated with functional pathways previously reported in different tissues and species. Proteolytic pathways for example, are within the most common and strongly activated genes that are part of the female response, found in both reproductive tissues and whole female bodies [10, 39, 49]. However, this is a complex reproductive response, given that a great part of the male ejaculate is also composed of a diverse cocktail of both proteases and their inhibitors [2].

A great array of proteases with diverse functions are associated with the postcopulatory female response [4, 12, 50]. Most of these are related to earlier postmating processes and molecular interactions occurring in the female tract [e.g. sperm storage and cleavage of seminal fluid proteins (SFP)] [50–52], but it is unclear whether the proteases or inhibitors expressed by the female are involved in the same functions. However, the lasting effects, even several days after mating, and the fact that they have been detected in several species, from insects to mammals [4, 52], suggest that these proteolytic cascades are involved in multiple functions of the whole organism mating response. In this study, the expression of proteolytic cascades we observed in heads of mated females does not seem connected to functions occurring in the female reproductive tract. One possibility is that some of these cascades are involved in protein degradation for amino acid related pathways and nutrient homeostasis, a
hypothesis supported by our functional analysis. We identified two functional categories associated with Carnitine biosynthesis. This particular amino acid is known to start with the degradation of proteins containing N-methylated lysine by proteolytic cascades [53], with a major role in energy homeostasis in the nervous system [40, 41].

We detected several biosynthetic pathways associated with the production of specific amino acids important for nutrient homeostasis such as carnitine, threonine, and leucine. Although such pathways are not directly classified as behavioral or reproductive, some of their metabolic functions have been previously detected in conspecific experiments in D. melanogaster [28, 39]. These networks regulate energy balance [40, 41] and nutritional uptake in brain tissues [42], and have been directly linked to several postcopulatory behaviors, including circadian rhythms [43, 44], nutrient sensing, exploratory behavior for specific nutrient source, consumption and posterior oviposition [26, 27, 54, 55]. Consequently, mated D. melanogaster females experience a major switch in their diet following copulation [56], consuming more amino acids during the dark phase [26]. The mechanism of this behavioral switch has been linked to neuronal signaling triggered by SP-SPR dynamics in D. melanogaster [57–59].

Transcriptional dynamics detected in female heads of both species were substantially perturbed by heterospecific matings. Thus, changes of regulatory networks related to nutrient homeostasis in brain tissues were characteristic of the conspecific postmating response in D. mojavensis, while changes detected in D. arizonae females were dominated by proteolytic pathways. The biological function of genes, and the magnitude of their expression were perturbed following copulation with a heterospecific male. Heterospecifically-mated D. mojavensis did not activate nutrient-homeostasis (DE genes) or photoreception-related (AS genes) pathways, and activated proteolytic and some muscle assembly genes instead. In contrast, heterospecifically-mated D. arizonae females showed no enriched pathways for DE genes, suggesting strong functional perturbation. These results suggest that, soon after mating, expressed genes from the female reproductive tract likely modulate the expression of behavioral genes in distant tissues such as the female head [28, 39]. The mode and mechanism of signaling for these changes in insects is still an ongoing investigation. Given the distance and heterogeneity of the tissues involved, changes induced in the female head are assumed to be the product of altered interaction networks, influencing the physiology of other tissues [29, 60]. This is more an indirect effect of neurons associated with internal reproductive tissues, which may produce signaling molecules that influence gene expression at distant sites in the fly body [61]. However, evidence from D. melanogaster suggests that seminal fluid components like the SP rapidly circulate in the female’s hemolymph and has been found associated with brain tissues [32]. Furthermore, immediate post-copulatory transcriptional responses in the nervous system could also be due to social interactions that occur during courtship, as has been shown in D. melanogaster with males that court but fail to copulate [62]. Further research is needed to disentangle the underlying mechanisms causing the activation of genes outside of the female reproductive tract and their involvement in the post-copulatory response.

Although female reproductive tract genes involved in the postmating response have only been investigated in a few species, results from D. mojavensis and D. virilis [45, 49, 63] indicate that these genes do not always evolve as rapidly as male reproductive genes. Here, we found that DE genes in female heads are evolving rapidly in D. arizonae, but not in D. mojavensis. Conspecific genes detected in D. arizonae are related to different proteolytic pathways and showed evolutionary rates even higher than SFP genes previously detected in these species [38]. These pathways were not enriched in D. mojavensis females, but some were activated when mated with D. arizonae males. Substantial postmating transcriptional differences in rapidly evolving genes connected to female behavior might have implications for the reproductive barriers between D. mojavensis and D. arizonae. For example, one possibility that could influence the expression pattern detected in female heads is the difference between the species in signals elicited during courtship [64]. In contrast to D. melanogaster, where courtship is longer and involves visual displays in front of the female [65, 66], courtship in cactophilic Drosophila likely involves chemical and auditory cues, as it occurs largely behind the female [64]. In addition, we have recently observed that D. mojavensis females are more likely to remate than D. arizonae females (Diaz et al. unpublished data). Differences in these courtship signals between D. mojavensis and D. arizonae may elicit altered transcriptional responses in female heads.

As opposed to DE genes, AS genes exhibited evolutionary rates even lower than the genome background. These genes were linked to very specific functions of highly conserved genes. Therefore, this could be due to the conservation of those specific gene functions in these species, but more likely reflects a general trend in the molecular evolution of AS genes [67]. To date, the role of AS in the female postmating response has not previously evaluated in insect species, nor the relationship between AS and molecular evolution in Drosophila. However, this same result was also previously found when investigating AS genes across the mouse and human genomes [67], suggesting...
that the level of alternative splicing and molecular evolution at the sequence level are negatively correlated. Constitutive exons of alternative isoforms tend to evolve faster than newly alternatively spliced exons [67, 68]. It is unclear how these heterogenic patterns would affect genome-wide molecular evolution or specific gene families in insects, but highly constrained exons could decrease rates of molecular evolution at the gene level. Alternatively, spliced genes may also be more pleiotropic, as alternative isoforms can evolve without major changes in sequence through functional specialization of different exon arrays.

**Conclusions**

The female postmating response is a complex process that involves the general female physiology as well as strong tissue-specific transcriptional changes with variation in strength and direction [39]. By implementing an integrated transcriptional analysis in head tissues of mated females between *D. mojavensis* and *D. arizonae*, we were able to reveal previously unknown roles played by DE, AS and IR, and how these dynamics are integrated through functional specializations within the female postmating response. Substantial transcriptional perturbations resulting from heterospecific matings suggest a previously unknown role of these genes in postmating barriers that rely on female behavioral changes triggered during copulation. The consequences of mating interactions between the sexes have generally considered genes that are more directly linked to reproduction (i.e. SFP and female tract genes) [47]. We provide evidence showing that differentially expressed genes from distant head tissues evolve at rates that are similar, or even higher, than those of male reproductive genes. Given the functions of genes involved, these changes might be costly to the female, affecting not only fertilization, but also could alter hybrid performance (if fertilization succeeds) as well as subsequent nutritional and egg-laying decisions by the female. These types of changes have been detected in a few species [69–71], but their transcriptional basis and evolutionary rates have remained unknown. Our results indicate that transcriptional perturbation following heterospecific mating extends beyond the female reproductive tract. The extent of these interactions might be larger than previously thought, opening the door to investigate the link between head transcriptomes and reproductive barriers between species.

**Methods**

**Samples and mating experiments**

All experiments were carried out using *D. mojavensis* and *D. arizonae* isofemale lines originally collected from Anza Borrego Desert State Park, Borrego Springs, CA (in 2002) and Guaymas, Sonora, Mexico (in 2000), respectively. Inbred lines were held at 25 °C, under 12:12 h light:dark cycle and controlled density conditions in 8-drum glass vials with banana-molasses media [72] for all stocks and experiments. Experimental design consisted of conspecific (∞* D. mojavensis* x ∞* D. mojavensis* and ∞* D. arizonae* x ∞* D. arizonae*) and heterospecific matings between the species (∞* D. mojavensis* x ∞* D. arizonae* and ∞* D. arizonae* x ∞* D. mojavensis*) (Fig. 1). We refer to the reciprocal mating as ∞Dmoj for matings involving *D. mojavensis* females and ∞Dari for those involving *D. arizonae* females. All mating experiments were performed using 7–10-day old virgin flies. Males and females were paired in vials and were constantly inspected for copulation events during a 2-h window immediately after the incubator lights turned on. Males were removed from vials after copulation and females were kept in the vial until the specified postmating period was reached (45 min or 6 h), when heads were collected from both mated and control virgin females (Fig. 1). Groups of 20 dissected heads were pooled for each sample and three biological replicates were collected per experimental cross, which generated 15 samples for each direction of the cross (∞Dmoj and ∞Dari -con/-het) for a total of 30 samples. All tissues were placed immediately in TRIzol and kept at −80 °C until total RNA extractions.

**RNA extraction, cDNA library construction and sequencing**

Total RNA was extracted using Direct-zol RNA kit (Zymo Research). Both RNA quality and quantity were inspected on a Bioanalyzer (Applied Biosystems/Ambion). cDNA libraries were created using KAPA Stranded mRNA-Seq Kit according to the manufacturer’s instructions. The 30 RNA-seq libraries were sequenced at Novogene Inc. using the HiSeq SBS v4 High Output Kit on Illumina platform flow cells with runs of 2 × 150 bp paired-end reads. Illumina’s HiSeq Control Software and CASAVA software (Illumina, Inc.) were used for base calling and sample demultiplexing.

**Sequence trimming and mapping**

Nearly 700 million total paired-end read sequences were obtained from the Illumina runs, ranging from 16 to 27 million reads per sample. Reads were trimmed for quality and adapter sequences were removed using a minimum quality base of Q = 20 and minimum read length of 50 bp using the software Trimmomatic [73]. Trimmed reads were then mapped to corresponding reference genomes using splice-aware mapper GSNAP [74] with the option of new splice events detection. The *D. mojavensis* reference genome was used for samples involving ∞Dmoj, while *D. arizonae* was used as reference for ∞Dari samples. Generated *sam* files were converted to *bam* format after indexing and filtering for a minimum
mapping quality of MQ = 20 using SAMtools [75]. These mapping results were then used for all differential expression and alternative splicing downstream pipelines.

Reference genomes
Template based genomes were used for mapping RNA-seq reads. For D. mojavensis, the assembly from [76] (SRP190536) was used with updated annotations retrieved from FlyBase version FB2016.05 [77]. A template genome version of D. arizonae (https://osf.io/ukevx/?view_only=7e20375d605040e4add37f707c6aba4) was assembled using the same method as D. mojavensis in [76] with paired-end and mate pair Illumina reads from [78] (SRP278895).

Differential expression - DE
We created a gene level read count matrix for all samples using featureCounts [79]. The read count matrix was filtered for a minimum count cutoff = 3 cpm over at least two replicates per comparable group. All DE analyses were performed using the R package edgeR [80] after TMM library normalization. Normalized counts were analyzed by Generalized Linear Models (GLM) assuming a negative binomial model of read counts, followed by DE analyses. All comparisons were performed between mated females (con- and heterospecific mating) and virgin females (♀ Dmoj and ♀ Dari) at postmating periods (45 min and 6 h) using three biological replicates per condition. Features with a false-discovery rate (FDR) corrected p-value < 0.05 [81] and a log2-fold-change threshold of > 1.0 were considered significant.

Alternative splicing - AS
We used the JunctionSeq [82] pipeline in order to detect genome-wide patterns of alternative spliced genes. AS is defined as the relative regulation of isoforms belonging to a multi-isofrom gene with respect to a given biological condition [83]. The pipeline is based on differential usage calculated from both exon and junction feature coverages. The pipeline relies on the originally implemented method in DEXSeq [84], which tested differential usage of annotated exons, but extended to splice junctions usage and both annotated and non-annotated splicing events. A new flattened GTF annotation file where overlapping features are not allowed was first generated using QoRTs [83]. All overlapping genes were merged as composed by a flat set of non-overlapping exons and splice junctions with unique identifiers. QoRTs was also used to generate a read count matrix for AS analysis, including three types of read counts per gene as estimated by exons, junction and gene level counts. The generated count matrix was then used by JunctionSeq R package [82] to estimate differential exon and junction usage with respect to gene-wide expression. No read was counted more than once in the model since exon and junction dispersions are then fitted independently. As for differential expression, alternatively spliced genes were detected if at least one exon or splice junction was differentially used between mated females (con- and heterospecific mating) and virgin females (♀ Dmoj and ♀ Dari) at postmating periods (45 min and 6 h) using three biological replicates. Only features with p-values < 0.01 after FDR correction were considered significant.

Intron retention rates - IR
Intron retention is a specific type of AS that is not necessarily captured by JunctionSeq and can have different biological implications that help to better explain expression changes. An intron can be retained in the final mature mRNA, coding for a new function [85, 86] or a nonfunctional transcript that is degraded by nonsense-mediated decay (NMD) [87]. We investigated whether postmating AS events also involve mechanisms of intron retention using the IRFinder pipeline [88]. A new reference annotation was built by removing all overlapping features present in the same strain sense of individual introns and then unique identifiers were assigned to each flattened exon. Only regions with high mapping scores as estimated through simulated reads across the genome were identified and included in the flattened annotation file. A read count matrix with all reads overlapping splice junctions was generated and IR rates were estimated as: junction reads / (junction reads + intronic reads) for each sample. The count matrix was then used by IRFinder R package [88] in order to estimate GLM. This method is used to test the fold change of IR between biological conditions using the DEXSeq2 R package framework [89]. Genes with differential IR were then detected if at least one intron was differentially retained between mated females (con- and heterospecific mating) and virgin females (♀ Dmoj and ♀ Dari) at postmating periods (45 min and 6 h) using three biological replicates. Only features with p-values < 0.01 after FDR correction were considered significant.

Statistical analysis of genes under DE, AS and IR
A Spearman’s correlation matrix comparing relative expression levels of significant DE genes was generated in order to investigate the relationship of gene expression changes between con- vs heterospecific matings as well as between postmating periods (45 min vs 6 h) and the two directions of the cross (♀ Dmoj vs ♀ Dari). Because IR changes are more likely linked to mechanisms of downregulation by transcript degradation, we tested this hypothesis by estimating IR changes between mated vs virgin samples—IR change, while comparing up vs downregulated genes. A GLM analysis was performed using
categories of up and down regulation as independent variables and the level of IR change as the dependent variable for each mating experiment. GLM analysis was performed after square root transformation to normalize the error distribution and to achieve homoscedasticity.

Functional and evolutionary analyses
Overrepresentation of specific categories of biological functions were then investigated for DE and AS genes using GOseq R package framework [90]. Additionally, we investigated signatures of positive selection on genes responding to con- vs heterospecific matings as well as for each of the overrepresented categories detected. For this, we estimated evolutionary rates (\(w = d_{\alpha}/d_{\beta}\)) using codeml, part of PAML 4.9 [91]. CDS alignments between D. mojavensis and D. arizonae were produced with MUSCLE 3.8.31 [92]. Any alignments with internal stop codons or framenshifts were removed before analysis. Codeml was run using model 0 with default values. Raw synonymous and nonsynonymous polymorphism counts were generated with Kaks Calculator 1.2 [93]. We further extracted putative seminal fluid genes identified in a proteomic analysis of D. mojavensis male accessory glands [38] and compared their rate of molecular evolution with those that were differentially regulated in heads. A GLM analysis was performed to compare the average evolutionary rates under each mating experiment against the genome background rates for each mating experiment. GLM analysis was performed after square root transformation to normalize the error distribution and to achieve homoscedasticity.

Abbreviations
DE: Differential expression; AS: Alternative splicing; IR: Intron retention; \(\hat{D}\): D. mojavensis female D. mojavensis; \(\hat{D}\): D. arizonae female D. arizonae

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12864-021-07669-0.

Additional file 1: Table S1. Differentially expressed genes in head transcriptomes (FDR with alpha = 0.05) of females following con- and heterospecific matings between D. mojavensis and D. arizonae. Results are showing significant DE genes for each female species (Dmoj and Dari) and postmating period (45 min and 6 h). Significant (Consp. or Hetero) and overlapping genes (Consp. and Hetero) are indicated. Overlapping genes showing substantial differences between con- and heterospecific crosses are indicated (Consp. and Hetero. Different responses). Gene IDs and Gene symbols are based on D. mojavensis genome as extracted from D. melanogaster orthologous. Gene IDs based on D. mojavensis genome, while Gene symbols are extracted from D. melanogaster orthologous. (NA: not applicable).

Table S2. Alternatively spliced genes in head transcriptomes (FDR with alpha = 0.01) of females following con- and heterospecific matings between D. mojavensis and D. arizonae. Results are showing significant AS genes for each female species (Dmoj and Dari) and postmating period (45 min and 6 h). Significant (Consp. or Hetero) and overlapping genes (Consp. and Hetero) are indicated. Gene IDs based on D. mojavensis genome, while Gene symbols are extracted from D. melanogaster orthologous. (NA: not applicable).

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Authors’ contributions
JMB, LMM and TAM conceived the initial idea. The project was designed by FD, JMB and LMM. FD performed most of the fly work, library construction and transcriptomic analyses. CWA participated in fly and molecular work and performed molecular evolution analyses. All authors participated in the data analysis and the writing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
A template genome version of D. arizonae and D. mojavensis was deposited in public repository (https://osf.io/ukexv/?view_only=ze02075dd60504e4ad377fd7cbab84), while all RNA-seq reads have been deposited in the Sequence Read Archive under accession number (https://dataview.ncbi.nlm.nih.gov/object/PRJNA693826?reviewer=krjn5ptdjeuerehirhhhekkcc).

Declarations
Ethics approval and consent to participate
This section is not applicable to the present study.

Consent for publication
This section is not applicable to the present study.

Competing interests
The authors declare that they have no competing interests.

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