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Quantitative proteomics reveals rapid divergence in the postmating response of female reproductive tracts among sibling species

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Fertility depends, in part, on interactions between male and female reproductive proteins inside the female reproductive tract (FRT) that mediate postmating changes in female behaviour, morphology, and physiology. Coevolution between interacting proteins within species may drive reproductive incompatibilities between species, yet the mechanisms underlying postmating–prezygotic (PMPZ) isolating barriers remain poorly resolved. Here, we used quantitative proteomics in sibling *Drosophila* species to investigate the molecular composition of the FRT environment and its role in mediating species-specific postmating responses. We found that (i) FRT proteomes in *D. simulans* and *D. mauritiana* virgin females express unique combinations of secreted proteins and are enriched for distinct functional categories, (ii) mating induces substantial changes to the FRT proteome in *D. mauritiana* but not in *D. simulans*, and (iii) the *D. simulans* FRT proteome exhibits limited postmating changes irrespective of whether females mate with conspecific or heterospecific males, suggesting an active female role in mediating reproductive interactions. Comparisons with similar data in the closely related outgroup species *D. melanogaster* suggest that divergence is concentrated on the *D. simulans* lineage. Our study suggests that divergence in the FRT extracellular environment and postmating response contribute to previously described patterns of PMPZ isolation and the maintenance of species boundaries.

1. Introduction

In species with internal fertilization, fertility depends on complex and potentially protracted interactions between the sexes that take place within the female reproductive tract (FRT) [1–3]. These interactions are expected to be particularly dynamic in species in which females mate with multiple males, because postcopulatory sexual selection (including sexual conflict) drives the evolution of sex-specific traits that influence male competitive fertilization success and female control over paternity [4–9]. In turn, the coevolution of interacting male and female reproductive traits within populations [10] may generate postmating–prezygotic (PMPZ) incompatibilities between populations [11], potentially contributing to the formation of new species [12–15].

Interactions between male and female reproductive proteins mediate a suite of postmating changes in female behaviour, morphology, physiology, and gene expression, collectively known as 'postmating responses' [1,2,16,17]. In particular, male-derived seminal fluid proteins (SFPs) transferred during mating trigger multiple postmating responses, including changes in female receptivity to remating, rates of ovulation and oviposition, patterns of sperm storage and usage, and female lifespan (reviewed in [1,17]). Studies in a growing number of insect taxa have characterized the transcriptomic and/or proteomic changes that occur in females after mating (e.g. fruit flies [18–22], mosquitoes [23,24], honeybees [25], butterflies and moths [26,27]). Because SFPs are among the most rapidly evolving proteins known [12,13], coevolution between functionally interacting male and

female reproductive proteins is likely to result in species-specific molecular interactions that are needed to coordinate a 'successful' female postmating response [22,26,28]. Theory predicts that divergence in these traits may represent a taxonomically widespread 'engine of speciation' [2,14], yet it is currently unknown whether male–female molecular interactions evolve sufficiently fast to be relevant to the speciation process. Few studies have examined how divergent ejaculate–female interactions may result in different female postmating responses among closely related species or between conspecific and heterospecific crosses [22,26,28]. Consequently, our understanding of how molecular interactions between the sexes mediate reproductive outcomes relevant to the formation of PMPZ reproductive isolation remains limited.

Drosophila simulans and *D. mauritiana* are a model system for studying the evolution of PMPZ reproductive isolation due to their very recent evolutionary divergence and phylogenetic proximity to the genetic model *D. melanogaster* [29–36]. The two species diverged from a common ancestor approximately 240 000 years ago, and the common ancestor of the *D. simulans* clade diverged from *D. melanogaster* approximately 3 million years ago [37]. *Drosophila simulans* is a cosmopolitan, human commensal, yet contemporary populations are geographically isolated from those of *D. mauritiana*, which is endemic to the islands of Mauritius [29]. Previous studies have identified several physiological and behavioural mechanisms underlying PMPZ barriers in these species. First, many matings between *D. simulans* females and *D. mauritiana* males are of abnormally short duration, which interrupts sperm transfer and results in a low oviposition rate [29,34,36]. Second, although *D. simulans* females who do copulate for long enough with *D. mauritiana* males receive as many sperm as in conspecific matings [34,36], they tend to eject heterospecific ejaculates more rapidly than conspecific ejaculates [36]. Third, when *D. simulans* females mate with both *D. simulans* and *D. mauritiana* males, progeny are sired predominantly by the conspecific male, regardless of the order of matings, due to species-specific patterns of sperm transfer, storage, and usage [32,35,36,38]. Because our knowledge of PMPZ isolating mechanisms in this sibling species pair exceeds that of any other taxa, it is an ideal system for studying how divergence in female postmating responses may contribute to PMPZ reproductive isolation.

In this study, we take a quantitative proteomics approach to investigate the molecular mechanisms underlying male–female postmating interactions within and among species, and their potential role in mediating species-specific postmating responses. Specifically, we used highly accurate isobaric labelling to compare the protein composition of the FRT before and after mating, and after both conspecific and heterospecific inseminations in *D. simulans* and *D. mauritiana*. We then used semi-quantitative proteomics in the closely related outgroup species *D. melanogaster* to polarize the relative divergence of postmating responses observed on the *D. simulans* and *D. mauritiana* lineages. Our results shed light on how divergence in the FRT molecular environment may have contributed to mechanisms of conspecific sperm precedence that underlie PMPZ reproductive isolation in these closely related species [32,36].

2. Material and methods

Detailed descriptions can be found in the electronic supplementary material.

(a) Sample collection and preparation

We collected FRT samples from the following five conditions: *Drosophila simulans* virgins (hereafter called *sim* virgins), *D. mauritiana* virgins (*mau* virgins), *D. simulans* females mated to *D. simulans* males (*sim* × *sim*), *D. mauritiana* females mated to *D. mauritiana* males (*mau* × *mau*), and *D. simulans* females mated to *D. mauritiana* males (*sim* × *mau*). It was not possible to collect sufficient tissue from the reciprocal hybrid cross (*D. mauritiana* females mated to *D. simulans* males) due to the high frequency of female rejection [30]. Mated samples were collected 6 h after the end of a successful copulation. We chose 6 h as the postmating timepoint to maximize our chances of detecting proteomic differences between virgins and mated females. *D. melanogaster* females exhibit a peak in differential gene expression at 6 h postmating [19], and 6 h roughly corresponds to the end of the first postmating phase of FRT maturation, in which females switch to a sustained, elevated level of ovulation and fertilization [19,39].

FRTs (i.e. bursa, oviduct, seminal receptacle, spermathecae, paroavaria, and associated fat body) of approximately 100 females from each condition were dissected and pooled in PBS per replicate. Two replicates were collected per condition resulting in 10 samples. Samples were solubilized in 100 µl 1 M HEPES with 2% SDS and 5% b-mercaptoethanol. Thirty micrograms of each sample was prepared and labelled with 10-plex tandem mass tags (TMT, Thermo Scientific). Samples were reduced with TCEP, alkylated with iodoacetamide, digested with trypsin, individually labelled with TMT reagents, and combined at equal amounts. The peptide mixture was fractionated using high pH reverse-phase chromatography and profiled with a linear gradient of 5–60% acetonitrile + 20 mM ammonium formate. Thirty-six fractions were initially collected and combined into 18 fractions for analysis by LC–MS/MS.

(b) LC–MS/MS analysis

LC–MS/MS was performed using a Dionex Ultimate 3000 and Lumos Orbitrap mass spectrometer (Thermo Scientific). One microlitre of each fraction was separated with a gradient of 1.6–32% acetonitrile in 0.1% formic acid. Peptides were quantified using a synchronous precursor selection MS3 method [40]. Each full MS1 scan was followed by data-dependent MS2 scans to isolate and fragment the most abundant precursor ions by collision-induced dissociation (35% normalized collision energy), and then the 10 most abundant fragment ions were selected for MS3 analysis for further fragmentation by higher-energy collisional dissociation (65% normalized collision energy).

Mass spectra were searched against the *D. simulans* protein database (dsim-all-translation-r2.02, FlyBase.org) using PEAKS X (Bioinformatics Solutions Inc.). Although protein divergence between *D. simulans* and *D. mauritiana* is very low (non-synonymous substitution rate, or $dN = 0.007$; see electronic supplementary material), we used the SPIDER algorithm [41] which allows single amino acid substitutions to account for the potential limitation of cross-species database searches. Reporter ion intensities were calculated by summing the centroided reporter ions, and protein abundances were calculated by summing the reporter ion intensities in each channel. Peptide identifications were accepted if the false discovery rate (FDR) < 1% based on the decoy-fusion approach [42], and protein identifications were accepted if the FDR < 1%.

(c) Protein database annotation and analyses

Drosophila simulans FlyBase gene (FBgn) identifiers were converted to their orthologous *D. melanogaster* FBgn identifiers using the FlyBase Drosophila Orthologs database (dmel_orthologs_in_drosophila_species_fb_2019_01). To compare mating-induced abundance changes of FRT proteins, we identified and removed putative male-derived proteins. Proteins were classified as male-derived if they had been previously identified as *D. melanogaster* sperm proteins [43,44] or *D. melanogaster* SFPs (N Brown, JL Sitnik, MF Wolfner 2020,

personal communication), or if they showed signatures of being male-derived ejaculate proteins unique to *D. simulans* and/or *D. mauritiana* (see supplemental methods for details). Our final dataset included 3287 FRT proteins in *D. simulans* and *D. mauritiana* (available on Dryad [45]).

Raw protein abundances were $\log_2$-transformed and median-normalized using the MSnbase package in Bioconductor [46]. Protein abundances were highly correlated between replicates, with Pearson's $r > 0.97$ for all pairwise comparisons. Differential protein abundances were evaluated with empirical Bayes moderated *t*-tests using the LIMMA package in Bioconductor [47]. Proteins were classified as differentially abundant if the absolute $\log_2$-fold change was greater than 1 and FDR-adjusted *p*-value was less than 0.05, unless otherwise noted. To reduce the dimensionality of the data and identify the major axes of variation in FRT protein composition among conditions, we conducted a principal component analysis (PCA) on average protein abundances ($\log_2$-transformed, median-normalized) of all 3287 FRT proteins using the DEP package in Bioconductor [48].

Functional annotation was conducted using the Database for Annotation, Visualization, and Integrated Discovery v. 6.8 [49]. The full list of FRT proteins was specified as the background dataset for comparison, and enrichment was considered significant if the FDR < 5%. SignalP v. 5.0 was used to identify proteins that contained a secretory signal peptide (hereafter called secreted proteins).

(d) Comparison to semi-quantitative proteomic data from *D. melanogaster*

After discovering substantially different postmating responses in *D. simulans* and *D. mauritiana*, we decided to compare our TMT results to analogous, semi-quantitative proteomic results from virgin and mated *D. melanogaster* FRTs to polarize the observed evolutionary differences on the *D. simulans* and *D. mauritiana* lineages. Because the goal of this additional experiment was to gain insight on the possible ancestral FRT postmating response, rather than to conduct detailed quantitative comparisons between all three species, we decided that a label-free approach was appropriate.

Consistent with the *simulans*/*mauritiana* samples, mated *D. melanogaster* samples were collected 6 h after copulation. FRTs from approximately 150 females were dissected and pooled in PBS per replicate, and two replicates were collected per condition. Unlike the *simulans*/*mauritiana* samples, *D. melanogaster* females were mated to males raised on media containing isotopically labelled arginine and lysine, so mass spectrometry searches could be conducted exclusively for unlabelled female-derived proteins [50]. Samples were solubilized in 100 µl 2× Laemmli buffer with 10% TCEP. Fifteen micrograms of each sample was separated on a 1.5 mm 12% SDS–PAGE gel. Each sample was sliced into 10 bands, and then reduced with DDT, alkylated with iodoacetomide, digested with trypsin, and eluted with 0.1% formic acid for LC–MS/MS analysis.

LC–MS/MS was performed using a Dionex Ultimate 3000 and Q Exactive Orbitrap mass spectrometer (Thermo Scientific). Peptides were separated with a gradient of 1.6–32% acetonitrile in 0.1% formic acid. MS1 scans were followed by data-dependent MS2 scans to isolate and fragment the most abundant precursor ions by higher-energy collisional dissociation (25% normalized collision energy). Mass spectra were searched against the *D. melanogaster* protein database (dmel-all-translation-r6.30, FlyBase.org) in PEAKS X.

Because male-derived proteins were isotopically labelled, all proteins identified by PEAKS are known to be female-derived. However, to be consistent with our analyses in *simulans*/*mauritiana*, we removed proteins that had previously been identified as sperm proteins or SFPs. Our final dataset in *D. melanogaster* included 1909 FRT proteins (available on Dryad [45]).

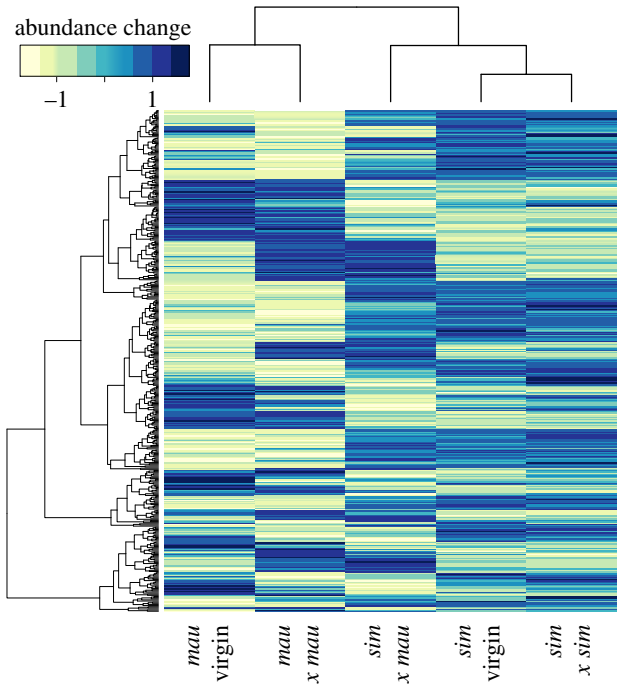

Figure 1. Clustering of FRT protein composition. For illustrative purposes, the heatmap shows average protein abundances for the 442 FRT proteins identified as significantly different between virgins, mated females, and/or after mating in *D. simulans* and *D. mauritiana*. Differentially abundant proteins have absolute $\log_2$-fold change greater than 1 and FDR-adjusted *p*-value < 0.05 in at least one pairwise comparison. The hierarchical clustering of conditions (columns) and proteins (rows) is based on Euclidean distance. Heat map colours indicate relative change in protein abundance normalized for each protein (yellow, decrease; blue, increase). (Online version in colour.)

Protein abundances were quantified as spectral counts corrected for protein length [51], and analysed using identical methods in Bioconductor. There was a strong correlation in protein abundances between replicates, with Pearson's $r > 0.95$ for all pairwise comparisons. Due to differences in quantitative accuracy between semi-quantitative and TMT proteomics, we evaluated the magnitude of interspecific postmating responses by comparing the distributions of mating-induced fold changes in FRT protein abundance for each species.

3. Results

Hierarchical clustering of average protein abundances for each condition (i.e. *sim* virgin, *mau* virgin, *sim* × *sim*, *mau* × *mau*, and *sim* × *mau*) indicate that the two species have distinct protein abundance patterns, with *sim* virgin clustering with *sim* × *sim*, and *mau* virgin clustering with *mau* × *mau*, respectively (figure 1). The heterospecific mated condition (*sim* female × *mau* male) clusters as an outgroup to *sim* virgin and *sim* × *sim*. The first two principal components from our PCA collectively explain nearly 90% of the variation in average protein abundances (figure 2) and are sufficient to separate the conditions by species (PC1: 63.5% of variance explained) and mating status (PC2: 26.1% of variance explained).

(a) Protein compositions of virgin and mated female reproductive tracts differ between sibling species

We identified 244 proteins as differentially abundant between *D. simulans* and *D. mauritiana* virgin FRTs. For simplicity, we refer

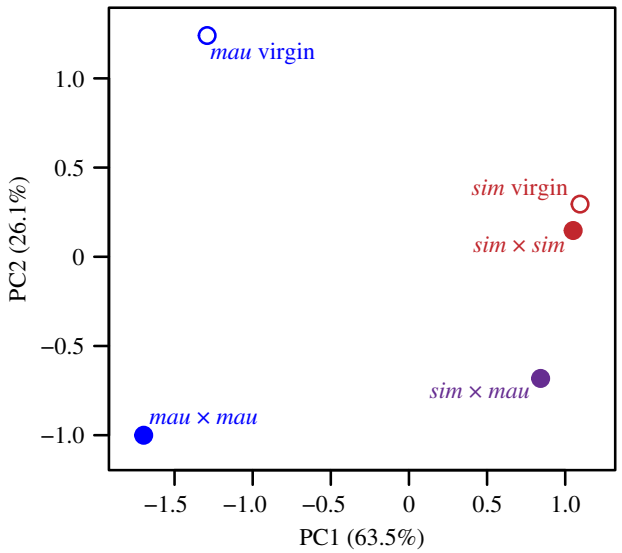

Figure 2. Principal component analysis of FRT protein composition. The first two principal components explain nearly 90% of the variation in average protein abundances and are sufficient to separate conditions based on species (PC1) and mating status (PC2). (Online version in colour.)

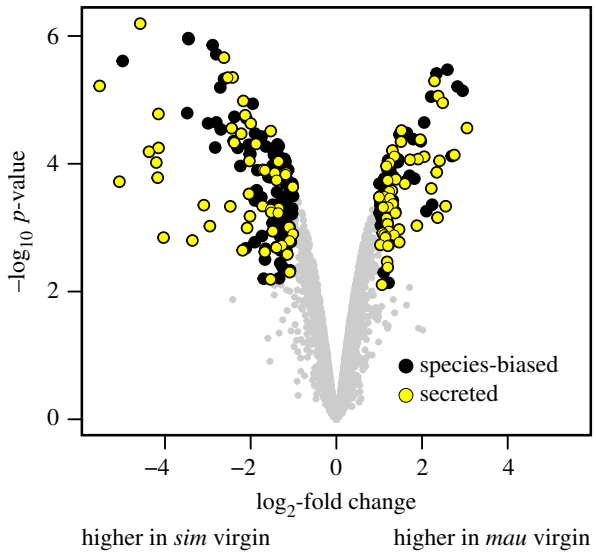

Figure 3. Enrichment of secreted species-biased proteins in *D. simulans* and *D. mauritiana* virgin FRTs. Proteins that are species-biased (i.e. significantly more abundant in either *sim* virgin or *mau* virgin) are highlighted in black. Species-biased proteins that are secreted are highlighted in yellow. (Online version in colour.)

to the proteins that are significantly more abundant in either *sim* virgin or *mau* virgin, respectively, as species-biased in virgins. Among these, there is a significant enrichment of secreted proteins (figure 3; FDR < 0.001% for both *sim* virgin-biased and *mau* virgin-biased). Almost half (42%) of the proteins that are species-biased are secreted proteins, while only 14% of the remaining proteins are secreted proteins ($\chi^2 = 128.12$, $p < 0.0001$). These results indicate that the virgin FRT of *D. simulans* and *D. mauritiana* have distinct compositions of secreted proteins, which may differentiate the FRT environments in each species. The proteins that are species-biased in virgins are also enriched for distinct biological categories. Specifically, the proteins that are significantly more abundant in *sim* virgins are enriched for cytochrome p450 enzymes (FDR < 0.01%) and serine proteases (FDR = 1.4%), whereas the proteins that are

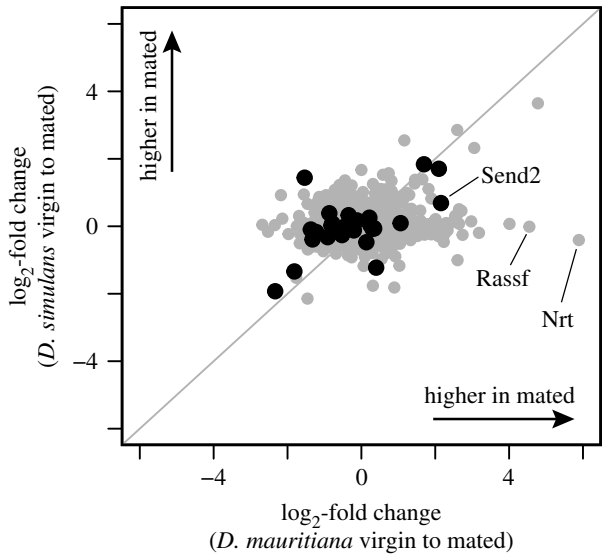

Figure 4. Relationship between the mating-induced changes in protein abundance for FRT proteins in *D. mauritiana* versus *D. simulans*. Proteins with serine-type endopeptidase activity are highlighted in black. There is greater spread along the *x*-axis than *y*-axis, indicating that there are more proteins with substantial mating-induced abundance changes in *D. mauritiana* compared to *D. simulans*. The grey line represents a 1 : 1 relationship, or perfect correlation between species in the female postmating response.

significantly more abundant in *mau* virgins are enriched for proteins involved in innate immunity (FDR = 0.73%).

We identified 320 proteins as differentially abundant between *D. simulans* and *D. mauritiana* mated FRTs. Half of these (49%) overlap with those that are species-biased in virgins. Consistent with the virgin comparisons, there is a significant enrichment of secreted proteins among the proteins that are species-biased in mated FRTs (FDR < 0.001% for *sim* × *sim* biased; FDR = 0.02% for *mau* × *mau* biased). There is a strong correlation in the level of species bias between the virgin and mated conditions ($r = 0.64$, $p < 0.001$), meaning that proteins that are significantly more abundant in *sim* virgin compared to *mau* virgin are also more abundant in *sim* × *sim* compared to *mau* × *mau*, and vice versa. These results indicate that there are differences between species in the FRT environment that are independent of mating status (see also figure 2).

(b) Mating induces substantial changes to female reproductive tract protein abundances in *D. mauritiana* but not *D. simulans*

There is a dramatic difference in the postmating response between the two species (figure 4). In *D. mauritiana*, 105 proteins (3.2% of total) are identified as significantly responsive to mating, whereas in *D. simulans*, only four proteins (0.1% of total) are identified as significantly responsive to mating. There is also significantly less variance of mating-induced fold changes in *D. simulans* compared to *D. mauritiana* (figure 5; Levene's test: $F = 532.1$, $p < 0.001$). Of the 105 proteins that are responsive to mating in *D. mauritiana*, 64 change in the same direction in *D. simulans*, which is a greater number than expected by chance (binomial test: $p = 0.03$). These results indicate that the FRT proteome in *D. simulans* responds to mating in a similar fashion as in *D. mauritiana*, just to a substantially lesser degree (see also figure 2). Thus, mating induces

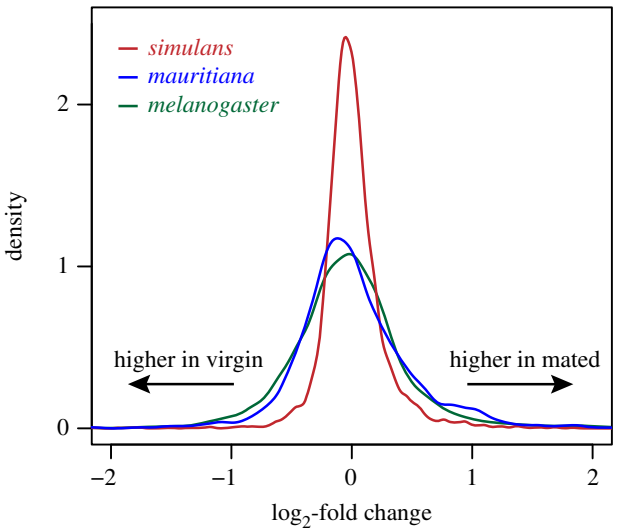

Figure 5. Distributions of mating-induced fold changes in FRT protein abundances in *D. melanogaster* (green), *D. mauritiana* (blue), and *D. simulans* (red). There is significantly less variance in the $\log_2$-fold changes between virgin and mated FRT protein abundances in *D. simulans* compared to *D. melanogaster* (Levene's test: $F = 438.9$, $p < 0.001$) and *D. mauritiana* ($F = 532.1$, $p < 0.001$), but no difference in the variance between *D. melanogaster* and *D. mauritiana* ($F = 1.77$, $p = 0.2$). (Online version in colour.)

substantial changes to FRT protein abundances in *D. mauritiana*, but limited change in *D. simulans*.

Among the proteins that decrease in abundance after mating in *D. mauritiana*, there is a significant enrichment of secreted proteins (FDR = 0.10%) and proteins with serine-type endopeptidase activity (FDR = 0.06%). Decreased serine endopeptidase abundance in mated females is consistent with the hypothesis that these proteases are negatively regulated by the male ejaculate, potentially to reduce enzymatic activity in the FRT that could degrade SFPs [20,52]. Interestingly, Spermathecal endopeptidase 2 (Send2)—a well-studied serine protease that is strongly upregulated after mating in *D. melanogaster* [53,54]—is a notable exception to this pattern and exhibits dramatically different abundance patterns in *D. simulans* and *D. mauritiana*. Send2 is at low abundance in the *D. mauritiana* virgin FRT but is strongly induced after mating ($\log_2$-fold change = 2.2; $p = 0.03$), whereas Send2 is at high abundance in the *D. simulans* virgin FRT and changes very little after mating ($\log_2$-fold change = 0.7; $p = 0.68$).

We also note that the two proteins that are the most uniquely responsive to mating in *D. mauritiana* (Nrt and Rassf) are involved in cell–cell adhesion, suggesting that this molecular capability makes them important players in mediating species-specific male–female cell–cell interactions [55]. There is no evidence for functional enrichment among the proteins that increase in abundance after mating in *D. mauritiana*, and we were unable to conduct enrichment analyses for *D. simulans* due to the very small number of differentially abundant proteins.

(c) Postmating proteomic responses are not perturbed after heterospecific inseminations

To evaluate whether heterospecific inseminations elicit a unique postmating response, we compared the mating-induced fold changes in FRT protein abundances following conspecific (*sim* virgin versus *sim* × *sim*) and heterospecific (*sim* virgin versus *sim* × *mau*) inseminations. There is a significant correlation between the postmating fold changes ($r = 0.38$, $p < 0.001$; see electronic supplementary material, figure S1), indicating that an ejaculate from a *D. mauritiana* male does not elicit a substantively different postmating response in *D. simulans* females. (We note that the relatively weak correlation, albeit significant, reflects the fact that most proteins exhibit very little abundance change after mating.) Similarly, there are no proteins that are differentially abundant between mated females after conspecific (*sim* × *sim*) versus heterospecific (*sim* × *mau*) matings. These results suggest that regardless of whether a *D. simulans* female mates with a conspecific or heterospecific male, the FRT proteome remains largely unchanged.

(d) *D. melanogaster* postmating response suggests a derived evolutionarily condition in *D. simulans*

Because *D. simulans*, *D. mauritiana*, and *D. melanogaster* shared a most recent common ancestor approximately 3 Ma [37], *D. melanogaster* is informative with regard to inferring the possible ancestral FRT postmating response. There is a comparably high number of proteins that are responsive to mating (absolute $\log_2$-fold change greater than 1) in *D. melanogaster* (5.9% of total) and *D. mauritiana* (5.6%), but far fewer in *D. simulans* (1.2%). There is also no difference between *D. melanogaster* and *D. mauritiana* in the variance of mating-induced fold changes (figure 5; $F = 1.77$, $p = 0.2$), but significantly more variance in *D. melanogaster* compared to *D. simulans* ($F = 438.9$, $p < 0.001$). While the breadth and magnitude of the postmating response appears to be conserved between *D. melanogaster* and *D. mauritiana*, the specific proteins that are responsive to mating are largely different. Only 11 proteins are responsive to mating in both species (out of 112 and 183 mating-responsive proteins in *D. melanogaster* and *D. mauritiana*, respectively), but all change in the same direction. Five of these are identified as having serine-type endopeptidase activity (FDR < 0.001%). Given the similar magnitude of postmating changes in *D. melanogaster* and *D. mauritiana*, our results suggest that prominent changes to the FRT proteome is likely to be the ancestral postmating response, and the limited changes in *D. simulans* is a derived condition.

4. Discussion

Resolving the molecular basis of interactions between the sexes is important for understanding fundamental reproductive processes, as these interactions impact fertility and competitive fertilization success within populations [56–59], as well as reproductive isolation between divergent populations and species [13–15]. Here, we used quantitative proteomics in a well-studied speciation model system [29–31] to compare the molecular composition of the FRT environment in virgin and mated females and its role in mediating species-specific postmating responses. Our findings reveal substantial divergence in the virgin FRT proteomes between sibling *Drosophila* species, as well as dramatic differences among species in the molecular postmating female response. Specifically, we found that the FRT proteomes in *D. simulans* and *D. mauritiana* virgin females express unique combinations of secreted proteins and are enriched for distinct functional categories. Furthermore, at 6 h after mating, there were substantial differences in FRT protein abundances between virgin and mated females in

D. mauritiana and *D. melanogaster*, but very few changes in *D. simulans*. These differences are particularly striking given that *D. simulans* and *D. mauritiana* diverged from a common ancestor only 240 000 years ago [37], but are consistent with the hypothesis that female postmating responses diverge rapidly among lineages and contribute to PMPZ isolation [2,52].

Below, we discuss how the molecular composition of the FRT environment and postmating female responses differ between *D. simulans* and *D. mauritiana*, and we postulate how such differences may contribute to previously described mechanisms of PMPZ reproductive isolation between these species [32,36]. Before doing so, we note, first, that the observed differences in protein abundances reflect the combined effects of changes in gene expression, post-transcriptional regulation, and protein degradation. Due to enriched proteolytic activity in the FRT [52,60] and among SFPs [1,17], we predict that protein cleavage, and ultimately degradation, underlies at least some of the mating-induced decreases in protein abundance. For mating-induced increases in protein abundance, we predict that both gene expression and post-transcriptional regulation are important. Previous studies in *D. melanogaster* have shown that many genes are significantly upregulated after mating, but also that gene expression levels may not be strongly correlated with protein abundance [19,20,61].

Second, we note that interspecific variation in postmating proteomic responses may reflect differences in both the number of mating-responsive proteins and the timing of the transition from an 'unmated' to 'mated' state. That is, the limited changes observed in the *D. simulans* FRT proteome are consistent with a scenario in which the temporal progression of postmating events is significantly faster (or slower) in *D. simulans* females compared to *D. mauritiana* or *D. melanogaster* females. We consider differences in the postmating timeline to be an exciting (non-mutually exclusive) alternative for how postmating responses may differ among species. The fact that these species differ in how long it takes females to become receptive to remating ([35], see also below) suggests that differences in the timing of postmating transitions are indeed an important axis of evolutionary change. The current study greatly expands our understanding of how molecular postmating female responses differ among species, but future studies that compare additional postmating timepoints are needed to fully resolve the postmating response timeline and contextualize the 6 h timepoint in each species.

Lastly, we note that it is not possible to determine the extent to which divergence in postmating responses is attributable to sexual selection, natural selection, and/or genetic drift. We find that interspecific differences in the FRT environment (both in virgin and mated females) are significantly enriched for functional categories that are known to play important roles in reproduction and sexual selection (e.g. serine proteases, immunity proteins) [52,62,63], which seems inconsistent with a model of drift. However, although our results suggest a role for sexual selection, the specific agent(s) of selection underlying this divergence is unknown.

Species-specific combinations of secreted proteins may be critical to the coordination of postmating processes [39]. For example, the specialized secretory glands of the *Drosophila* FRT produce proteins necessary for recruiting sperm to the sperm-storage organs (i.e. seminal receptacle and spermathecae), maintaining sperm mobility during storage and moving eggs through the reproductive tract [54,64,65]. We find that secreted proteins are over-represented among the species-biased proteins in both the virgin and mated conditions and are also over-represented among the proteins that are responsive to mating. These findings indicate that the extracellular environment of the FRT has diverged between *D. simulans* and *D. mauritiana* and suggest that secreted proteins contribute to the successful coordination of postmating responses in a species-specific manner.

Previous studies have indeed shown that *D. simulans* and *D. mauritiana* females store and use sperm differently when females mate with multiple males: *D. simulans* females exhibit opposing patterns of fertilization bias between the seminal receptacle (first-male bias) and spermathecae (second-male bias), whereas *D. mauritiana* females exhibit no fertilization bias in either storage organ [35,38]. Consequently, following both conspecific and heterospecific matings, *D. simulans* females are able to strategically alter their use of sperm from different sperm-storage organs to bias paternity in favour of conspecific sperm [36,38]. To the extent that different combinations of secreted proteins result in different patterns of sperm storage and usage, the species-specific compositions of secreted proteins observed here in *D. simulans* and *D. mauritiana* may represent the molecular mechanism underlying the female mediation of conspecific sperm precedence [36,38]. The fact that male-derived proteins directly interact with these female secretions also sets the stage for a potential coevolutionary arms race. For example, sperm competition is expected to favour the evolution of male proteins that manipulate the female's use of sperm to enhance the male's paternity, and sexual conflict may drive the counter-adaptation of female proteins in order for females to maintain their control of fertilization outcomes [5,13,66]. Sexually antagonistic coevolution of interacting male and female reproductive proteins may, therefore, ensue and result in the evolution of ejaculate–female incompatibilities that contribute to PMPZ reproductive isolation [8,14,15].

Differences in females' ability to process SFPs also may have contributed to the divergent postmating responses observed between *D. simulans* and *D. mauritiana*. If *D. simulans* females are more efficient at processing SFPs than *D. mauritiana* females, then *D. simulans* SFPs may have a more restricted time to act and thus limited scope to elicit postmating responses [1]. We find that the proteins that are more abundant in virgin *D. simulans* FRTs compared to *D. mauritiana* FRTs are over-represented by serine-type endopeptidases and cytochrome p450 enzymes—two classes of enzymes that may be involved in the degradation of male proteins. Serine-type endopeptidases regulate the cleavage of SFPs to their active forms [67,68] and are important in SFP degradation [52,69]. Cytochrome p450 enzymes are associated with oxidative degradation of endogenous and exogenous toxins [70], so their enrichment may aid in the oxidative cleavage of male-derived compounds introduced during mating [18,71–73]. We hypothesize that greater endopeptidase and cytochrome p450 abundance, and hence activity, in virgin *D. simulans* FRTs underlies its unique, relatively limited postmating response.

In further support of this 'differential SFP-processing' hypothesis, we find that (i) the proteins that decrease after mating in *D. mauritiana* are over-represented by serine-type endopeptidases, and (ii) the abundance of an important spermathecal endopeptidase (Send2) is higher in virgin *D. simulans* females than in mated *D. mauritiana* females. SFPs are known to reduce a female's receptivity to remating in *D. melanogaster* [1,17,18], so it is noteworthy that the

remating interval for *D. simulans* females (mean ± s.e.: 2.7 ± 0.04 days) is shorter than for either *D. mauritiana* females (3.5 ± 0.07 days) or *D. melanogaster* females (3.5 ± 0.03 days) [35]. Although targeted enzymatic experiments should confirm whether species differ in their rates of SFP degradation, future studies should also focus more broadly on unravelling the complexity of female × male molecular interactions by resolving protein interaction networks, including those between proteolytic enzymes and SFPs.

SFPs are known to be important agents for eliciting postmating changes in female behaviour, morphology, and physiology [1,17], so there was an *a priori* expectation that rapid evolution of seminal fluid composition could contribute to differences in postmating responses between *D. simulans* and *D. mauritiana*. However, there are several reasons why the divergence of male-transferred proteins cannot fully explain the observed divergence in female postmating responses. First, divergence in male-transferred proteins cannot account for the substantive interspecific differences observed in the FRT environment of virgin females. Second, the postmating response of *D. simulans* FRTs was largely the same irrespective of whether females mated to conspecific or heterospecific males. Third, there has been very little amino acid sequence divergence of SFPs between *D. simulans* and *D. mauritiana* (dN = 0.01; [45]). Nonetheless, we cannot rule out the possibility that even limited SFP divergence contributes to the interspecific differences in postmating responses. Previous studies in *D. melanogaster* have shown that intraspecific SFP allelic variation can result in different postmating outcomes, including patterns of sperm displacement, male mating rate, female postmating refractorines, and female fecundity [74–76].

Our finding that the *D. simulans* FRT proteome exhibited limited changes after mating, regardless of whether the female received a conspecific or heterospecific ejaculate, suggests that the *D. mauritiana* ejaculate alone is not responsible for the observed divergence in postmating responses. This study, therefore, advances our general understanding about the role that females play in mediating reproductive interactions [77]. Specifically, our results suggest females control their developmental switch from an 'unmated' to 'mated' state once it has been induced by mating. Our findings contrast with those from a transcriptomics study on the sibling species *D. mojavensis* and *D. arizonae* that found very few genes are differentially expressed in the *D. mojavensis* FRT after conspecific matings, but many genes are differentially expressed after heterospecific matings [22]. We do not know if the discrepancy reflects different methodologies (i.e. expression of transcripts versus proteins) and/or different mating systems between the species pairs. Future studies are needed to determine how species differ in (i) the intensity of postcopulatory sexual selection in natural populations, (ii) the degree to which females determine reproductive outcomes, (iii) the potency of male-derived proteins in eliciting female postmating responses, and (iv) how male–female molecular interactions are disrupted following heterospecific crosses.

By applying a comprehensive proteomic approach to compare female postmating responses among species, our study represents a step forward in unravelling the molecular mechanisms underlying postmating interactions between the sexes and their potential role in mediating species-specific postmating responses. Our research highlights the value of this approach to resolving complex ejaculate–female interactions, their divergence among populations and species, and their contribution to PMPZ reproductive isolation.

Data accessibility. Mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifiers PXD17708 (*simulans*/*mauritiana* dataset) and PXD17730 (*melanogaster* dataset). Additional data and R code are available at the Dryad Digital Repository: https://doi.org/10.5061/dryad.8cz8w9gm8 [45].

Authors' contributions. All authors designed the study; E.L.M. and C.E.M. collected the samples; E.L.M. analysed the data; E.L.M. wrote the initial manuscript; all authors revised the manuscript and approved the final version.

Competing interests. The authors declare no competing interests.

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