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The seminal proteome of a monandrous fly, *Drosophila subobscura*

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When females mate with multiple males, ejaculates from different partners compete within the female reproductive tract. Males gain a competitive advantage by transferring seminal fluid proteins (Sfps) that manipulate female post-mating responses, such as reducing female propensity to remate. But in monandrous species, where females typically mate only once, post-mating sexual selection is absent, raising questions about the complexity and identity of Sfps in monandrous seminal proteomes. To address this, we characterized the seminal proteome of the monandrous fly *Drosophila subobscura*. Using a label-free quantitative proteomics approach, we identified 172 Sfps, comparable in number to the 153 Sfps previously reported in polyandrous *Drosophila melanogaster* using a similar approach. The *D. subobscura* seminal proteome contains all the *D. melanogaster* sex peptide network—proteins that control the female post-mating response—and many proteins known to be involved in post-copulatory sexual selection. We also found a surprising over-abundance of proteins previously shown to be downregulated under monogamy selection in *D. melanogaster*. Collectively, the patterns suggest that the *D. subobscura* seminal proteome is characterized more by conflict than cooperation and, despite differences in mating systems, is compositionally similar to that of *D. melanogaster,* with a complex proteome rich in receptivity-inhibiting Sfps.

1. Introduction

When females are polyandrous, a male's ejaculate faces competition with sperm from rival males. A vast body of theory [1–3] and empirical work [4–8] shows that males often respond to heightened competition by increasing the number of sperm in an ejaculate [9]. Yet, a large proportion of the ejaculate is composed of non-sperm components, the seminal fluid. Seminal fluid allows males to maximize competitive advantage through the complex mixture of proteins transferred to females together with sperm [10]. Moreover, males adjust seminal fluid components in response to the risk of sperm competition (e.g. [11–13]). Seminal fluid proteins (Sfps) have myriad functions

that increase male competitive advantage through reducing female re-mating, influencing sperm storage and usage, stimulating ovulation and interfering with rival sperm [10,14,15]. The sex peptide (SP) network is a well-characterized protein group (consisting of 13 proteins) in *Drosophila* [16], known to control the female post-mating response. Males transfer SP to females to stimulate egg production and reduce female receptivity to re-mating [17,18]. SP transfer improves male reproductive success but can reduce female lifespan and fitness, highlighting how the SP network mediates sexual conflict ([18,19], but see [20]).

Sfp characterizations have primarily focused on polyandrous species [11,21–23]. As a result, we have some understanding of the composition of seminal proteomes in species where male reproductive success is shaped by sperm competition and cryptic female choice post-copulation. Yet much less is known about the composition of the seminal proteome in monandrous species, in which these processes are absent and where monandry typically means that females mate only once in their lifetime. If monandry is female-controlled, we might expect selection to favour Sfps that support female fertility, whereas proteins that function primarily in sperm competition or in wresting control from female sperm choice are expected to be reduced or lost. Moreover, the manipulative proteins that reduce female receptivity in many insects and that are associated with sexual conflict are expected to be lost from the seminal proteome of monandrous species because females 'choose' to mate only once in their lifetime. However, if monandry represents a form of conflict, with males manipulating female propensity to remate against the female's evolutionary interests, the seminal proteome could be complex and rich in receptivity-inhibiting Sfps. Understanding the composition and function of a monandrous seminal proteome would give us clues as to whether monandry represents either cooperation and is under female (or mutual) control, or conflict where male manipulation is driven to an extreme.

Here, we test these ideas using the fly *Drosophila subobscura*, an ideal species in which to explore the characteristics of a monandrous seminal fluid proteome because it has extremely low levels of polyandry, with only 4% of females re-mating when sufficient sperm is received in the first mating [24]. Despite low levels of female re-mating, males increase copulation duration when rival males are present [25], a behavioural response typically associated with perceived sperm competition risk [24,26,27]. *Drosophila subobscura* also has a gene duplication of *SP*, which has diverged at least partially under adaptive evolution [28]. Moreover, the seminal fluid proteome of *Drosophila melanogaster*—a polyandrous distantly related species that diverged from *D. subobscura* roughly 25 million years ago [29]—is well characterized [30], providing an informative comparative proteomic framework. By using an identical method of Sfp characterization to that used in a study of *D. melanogaster* [22], we are able to make direct comparisons and test predictions about how monandry shapes seminal proteome composition relative to polyandry.

Under the assumption that monandry aligns female and male interests, we predict that:

(1) There will be fewer Sfps in *D. subobscura* than in *D. melanogaster*. If a monandrous seminal proteome has fewer functional requirements, fewer proteins are needed.
(2) Sfps involved in post-copulatory sexual selection will be lower in abundance or absent in *D. subobscura* because post-copulatory sexual selection is absent.
(3) Sfps within the SP network will be lower in abundance or missing from the *D. subobscura* seminal proteome because males do not need to transfer receptivity-inhibiting Sfps, such as SP, in a monandrous species.
(4) There will be differences in the molecular functions of the seminal proteome between *D. subobscura* and *D. melanogaster*. If monandry has a cooperative basis, fewer proteins that regulate the onset of the female post-mating response (e.g. peptidases) are required.
(5) Sfps with a promiscuity role will be lower in abundance or absent in *D. subobscura*. Proteins with a promiscuity role have previously been identified using an experimental evolution experiment in *D. melanogaster*, which identified genes with reduced expression in selection lines with monogamous mating [31].

However, if these predictions are not met, this would support the alternative hypothesis that monandry represents conflict between the sexes, such that males force monandry against female fitness interests. Under this scenario, protein groups controlling female re-mating and post-copulatory sexual selection are expected to remain in the Sfps of a monandrous species. We test these predictions using a proteogenomic approach by identifying and characterizing the seminal proteome of *D. subobscura* (electronic supplementary material, figure S1).

2. Material and methods

(a) Stock and fly maintenance

We used mixed Morocco populations [32] of *D. subobscura* maintained at 18°C on a 12 : 12 light : dark cycle. Adult flies were fed double yeast and maize medium (10 g agar, 85 g sucrose, 40 g yeast extract, 60 g maize, 1000 ml H_2O, 25 ml 10% nipagin) [33]. Laying flies were kept at a density of 25 males and 25 females on 200 ml of food in glass *Drosophila* bottles. A total of 5–6 bottles were kept and flipped every 3–4 days, yielding a total population size of 250–300 flies. Experimental flies for matings were collected from bottles every 24 h to ensure they were unmated [34]. Females and males were initially aged to 6 days in groups of 10 single-sex individuals. Females and males were then housed individually for 24 h to avoid the adverse effects previously observed of keeping males together prior to mating [35]. All mating trials were carried out at 23°C.

(b) RNA sequencing on male reproductive tracts

(i) Sample collection and preparation

Seven-day-old *D. subobscura* males were allowed to mate freely with females for 2 days (electronic supplementary material, figure S1A). Males were then removed from mating vials, frozen and stored at −80°C. Accessory glands (AGs) and ejaculatory ducts (EDs) were dissected in Trizol into three batches of 10 males. RNA was extracted using the phenol–chloroform method. Samples were made DNA-free, converted to cDNA and processed for library preparation and PacBio sequencing (for further details see electronic supplementary material).

(ii) Reference protein database for *Drosophila subobscura*

To create a reference protein database for *D. subobscura*, the mRNA sequence (ISO-seq) data were translated into protein sequences via three-frame translation using the Biopython package [36]. Protein sequences with a length of >50 amino acids were retained in the list. The translated transcriptome was merged with the published protein reference for *D. subobscura* (GCF_008121235.1) [37] and was used as a reference for the proteomic analysis.

To cross-validate our new protein database, the public reference protein sequences (GCF_008121235.1) were searched against the translated transcriptome using BLASTP [38]. In total, 73% of the reference proteins were detected in our transcriptomic sample, confirming that a large proportion of expressed genes were captured.

(c) Quantitative proteomics

(i) Experimental design

Our experiment to identify the *D. subobscura* seminal proteome was designed to mimic the approach used by Sepil *et al*. [22] for *D. melanogaster*, to allow for comparisons between these species. Virgin males, at one week old, were randomly assigned to the 'mated' or 'unmated' treatment. For males in the 'mated' treatment, a single male was introduced to a vial containing a female. During mating experiments, pairs were monitored, and mating was recorded if it occurred within 2 h (mean % successfully mated = 47% ± 2.7 s.e.). Once mating finished, the male was aspirated from the vial and flash frozen in liquid nitrogen within 5 min. A male assigned to the unmated treatment was also flash frozen at a similar time point to each mated male. Males were flash frozen until at least 20 individuals from each treatment were collected, providing surplus samples to ensure ten successful dissections of the AGs and EDs. The experiment was repeated four more times, resulting in five independent biological replicates for mated and unmated samples (electronic supplementary material, figure S1B).

We thawed males and dissected the AGs and ED in phosphate-buffered saline (PBS) buffer. Ten males from each treatment and replicate were dissected, and the AGs and EDs were pooled in 40 µl PBS buffer on ice, in preparation for proteomic analysis. In total, this yielded five replicates of 10x mated and 10x unmated male AGs and EDs. All samples were stored at −80°C until preparation for proteomic analysis.

(ii) Proteomic data processing and protein annotation

For proteomic analysis, cells were disrupted, and proteins quantified and digested in solution (see electronic supplementary material, methods, for details on proteomic data acquisition and analysis). Resulting peptides were separated by liquid chromatography and analysed by mass spectrometry (MS).

MS/MS spectra were searched using Mascot (via PEAKS Studio) (https://www.matrixscience.com/server.html) and MaxQuant (v. 2.4.2.0) [39] against the *D. subobscura* reference proteome (GCF_008121235.1), supplemented with the translated transcriptome. Searches were performed with trypsin as the specified enzyme, allowing up to two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionine as a variable modification. The precursor and fragment ion mass tolerances were set to ±10 ppm and ±0.01 Da, respectively. The false discovery rate (FDR) was controlled at 1% at both the peptide and protein levels.

We excluded sparsely quantified proteins—having zero values in more than two samples (out of 10). The remaining missing values (0) were imputed using the MissForest package [40]. Label-free quantification values were normalized using variance-stabilizing normalization. In total, 1389 proteins were identified and given a unique ID from either the reference genome or transcriptome. Mapping to both the official annotation and the translated transcriptome increased the number of proteins detected by 7.7% (1389 versus 1290 using the official annotation alone).

(d) Statistical analysis

All analyses were conducted using R v. 4.4.1 [41]. Briefly, we first identified *D. subobscura* Sfps and then annotated proteins using *D. melanogaster* homology. We next identified Sfp content and abundance and compared the seminal proteomes of *D. subobscura* with those of *D. melanogaster*.

(i) Identification of *Drosophila subobscura* seminal fluid proteins

To identify sample outliers and to test for overall protein abundance differences within and between mating treatments, we used principal component analysis using the prcomp function and hierarchical cluster analysis in the pvclust package [42]. Differential expression of protein abundance was calculated using the limma package [43], contrasting unmated and mated samples. A linear model was applied with empirical Bayes [43] to smooth standard errors (electronic supplementary material, figure S2). Proteins with differential abundance between mating treatments were identified using Benjamini–Hochberg FDR correction, and those with FDR < 0.05 were considered significantly different (n = 256). Among these, 172 proteins had higher abundance in unmated males than mated males ($\log_2$ fold change (FC) >0) and were categorized as proteins transferred during mating. These are the candidate Sfps in *D. subobscura*.

To visualize the characteristics of the transferred proteins, we plotted the $\log_2$ FC of the difference between unmated and mated males and the negative $\log_{10}$ of the FDR-corrected p-value in a volcano plot. To visualize protein abundance for the 1389 proteins in the AGs and EDs of mated and unmated males, we used a heatmap generated with the pheatmap package [44], annotating candidate Sfps across rows. Proteins were ranked based on their mean abundance across unmated samples, with rank 1 representing the most abundant protein and rank 1389 the least abundant protein, and each of the five unmated samples was plotted according to this rank order. The ranked abundance of transferred proteins compared with proteins with no differential abundance or proteins with higher abundance in mated samples was assessed using a Kruskal–Wallis rank sum test and *post hoc* Dunn tests.

(ii) Protein annotations of sex peptides and sex peptide receptors in *Drosophila subobscura*

Each candidate Sfp was annotated using the *D. subobscura* genome (XP_ protein IDs) or transcriptome (IDs beginning '28271_HQ_transcript') and assigned a unique protein identifier. To identify homologues in *D. melanogaster*, we used BLASTP, or if no hit was found, we used TBLASTN [45]. Among the 172 candidate *D. subobscura* Sfps, 23 lacked one-to-one Blast matches and shared a *D. melanogaster* annotation with at least one other *D. subobscura* protein. This resulted in some *D. subobscura* proteins sharing the same protein name (e.g. CG9997 occurs twice in *D. subobscura*). Six *D. subobscura* proteins had no homology to *D. melanogaster*.

To search for the SP and sex peptide receptor (SPR) homologues in *D. subobscura*, the DNA and protein sequences of the two SPs [46] and SPR (FlyBase [47]) from *D. melanogaster* were searched against the DNA and protein references of the official annotation (GCF_008121235.1) and our newly generated protein database using TBLASTN, BLASTP, BLASTP-short, BLASTN and BLASTN-short [45].

A heatmap was used to visualize protein abundance levels of the 172 transferred proteins (i.e. the candidate Sfps, hereafter referred to as Sfps) in mated and unmated males. Proteins belonging to the SP network and those involved in post-copulatory sexual selection (as collated by [30]) were annotated across rows.

(iii) Comparison of *Drosophila* seminal fluid proteomes

To understand the features of the seminal fluid proteome of *D. subobscura*, we compared the 172 Sfps that we identified to the predicted homologues of other *Drosophila* species with characterized seminal proteomes: *D. melanogaster, Drosophila montana* and *Drosophila pseudoobscura* [21–23]. To provide a 'fair' comparison with the *D. melanogaster* seminal proteome, we focused on Sfps that had been identified using the same quantitative proteomics approach, totalling 153 [22], as opposed to more extensive Sfp lists collated over a range of approaches (292 Sfps [30] and 220 Sfps [48]). The number of core *Drosophila* Sfps (those shared between pairs of species) and the number of species-specific proteins were identified using Venn diagrams.

Drosophila pseudoobscura and *D. montana* Sfps were identified using a quantitative proteomics and bioinformatics approach, and *D. melanogaster* orthologues were identified via FlyBase [21,23,47]. *Drosophila melanogaster* and *D. subobscura* Sfps were identified by comparing the quantitative proteomics of virgin and mated accessory glands [22]. The *D. melanogaster* dataset identified 1333 high-confidence proteins, similar to the 1389 identified in *D. subobscura*. Since the seminal fluid proteome of *D. melanogaster* is well characterized [30] and the methods for identifying Sfps are comparable with this study [22], the following analyses focus solely on comparisons between *D. subobscura* and *D. melanogaster* seminal fluid proteomes.

(iv) Presence of sex peptide network and post-copulatory sexual selection proteins

The presence of Sfps known to be associated with post-copulatory sexual selection (n = 11) [30] and Sfps within the SP network (n = 9) [17] was identified within the shared proteins of *D. subobscura* and *D. melanogaster*. The over- or under-representation of these protein groups within the shared proteins of *D. melanogaster* and *D. subobscura* was assessed using hypergeometric tests comparing the number present in the shared proteins with the numbers present in *D. melanogaster*.

(v) Seminal fluid proteome abundance

To facilitate comparisons between species, the abundance of the Sfps within each species was ranked. In *D. subobscura*, the average abundance level for each transferred protein was calculated from samples of unmated males. For *D. melanogaster* [22], ranks were calculated from average abundance levels for one-week-old unmated males that were housed in groups of 12

males. Proteins were ranked in descending order, such that rank 1 refers to the most abundant protein. Proteins common to both species were identified, and the abundance of shared proteins versus species-specific proteins was compared within each species using Kruskal–Wallis rank sum tests. Next, the rank order of Sfps found in both *D. subobscura* and *D. melanogaster* (n = 55) was correlated, and the significance of the relationship was calculated using Spearman's rho. Some *D. melanogaster* proteins had a best Blast hit to more than one protein in *D. subobscura*, so although 42 proteins overlap between species, we have abundance data for 55 proteins in *D. subobscura* that are shared with *D. melanogaster*. We also tested whether proteins within the SP network (n = 9) [17] and those thought to be involved in post-copulatory sexual selection (n = 11) [30] had a higher rank than other proteins within the *D. melanogaster* and *D. subobscura* seminal fluid proteome, using Kruskal–Wallis rank sum tests.

(vi) Gene Ontology term molecular function

We compared the molecular functions of Sfps in *D. subobscura* and *D. melanogaster* using Gene Ontology (GO) term analysis with FlyBase (release FB2025_02) [47]. The Sfps of *D. subobscura* (n = 172) and *D. melanogaster* (n = 153) [22] were uploaded to FlyBase to find information on GO Molecular Function. Some proteins had more than one GO term, and all the GO terms listed per protein were included in the analysis. In total, 213 GO terms were returned for *D. subobscura* and 223 for *D. melanogaster*. GO terms with more than four proteins associated per species were filtered ($n_{\text{Go terms}}$ = 12), and the proportion of proteins for each of these 12 GO terms within each species was calculated. Chi-square tests, with FDR correction for multiple testing, were used to test for differences in the proportion of proteins associated with each GO term for each species (i.e. number of proteins per GO term divided by the total number of proteins for that species).

(vii) Number and abundance of 'promiscuity proteins'

A previous study in *D. melanogaster* used experimental evolution to establish monogamous and polygynandrous (control) mating lines. They identified *Sfp* genes that had reduced expression after more than 160 generations of enforced monogamous mating [31]. We refer to the proteins that are translated from these genes as 'promiscuity proteins' because their expression was maintained in polygynandrous lines but downregulated in monogamous lines. To test the idea that *D. subobscura*, as a monandrous species, would lose or de-prioritize promiscuity-linked Sfps, we identified the number and abundance of these *D. melanogaster* promiscuity proteins in *D. subobscura*.

First, we identified proteins in the Hollis *et al.* [31] dataset that are Sfps, using the overlap with the Sfps identified by Sepil *et al.* [22]. This comparison yielded 133 Sfps, with abundance FC levels between polygynandry and monogamy selection lines. From this set, 51 proteins had reduced abundance in the monogamy lines compared with the polygynandry lines, using a threshold of logFC <−0.3548 h post-eclosion (see electronic supplementary material for analyses using thresholds of logFC <−0.25 and logFC <−0.15). This list was used to estimate the expected proportion of promiscuity proteins in *D. subobscura* seminal fluid, under the null hypothesis that selection did not act differently on protein composition under monogamy.

The distribution of the null hypothesis was calculated by sampling with replacement from this list (*D. melanogaster* total Sfps, n = 133; number of promiscuity proteins, n = 51) a total of 53 times – the number of *D. subobscura* Sfps that are also found in the *D. melanogaster* proteome. This overlap in proteins between the two datasets was used to avoid bias in the proteins that can be identified as 'promiscuity proteins'. The proportion of promiscuity proteins in each bootstrap sample was recorded, and the resulting distribution was used to calculate 95% confidence intervals. This bootstrapped distribution was then compared with the observed proportion in *D. subobscura*, calculated as the number of promiscuity proteins (n = 37) divided by the number of *D. subobscura* Sfps shared with *D. melanogaster* (n = 53). Bootstrap p-values were calculated using a permutation test by summing the number of null values that were equal to or more extreme than the observed value. The abundance of promiscuity proteins in *D. subobscura* was compared with the abundance of other proteins that are shared between the *D. subobscura* and Hollis *et al.* [31] Sfps using Kruskal–Wallis tests.

3. Results and discussion

(a) Identification of *Drosophila subobscura* seminal fluid proteins

To identify proteins that have lower abundance in mated than unmated males, we first verified that mating changes the abundance of Sfps in the AGs and EDs. Mated and unmated samples clustered separately within a principal component analysis (electronic supplementary material, figure S3A). Hierarchical cluster analysis, which can be used to test overall protein abundance similarity across samples, clustered samples from the same mating treatment together, and p-values computed by bootstrapping strongly supported clusters by mating treatment (electronic supplementary material, figure S3B, bootstrap probability for mating treatment, bp = 100%). Of the 1389 proteins identified in the male reproductive glands, 172 were significantly more abundant in unmated than in mated males and were therefore predicted to be transferred during mating (electronic supplementary material, figure S3B and C). These candidate Sfps also had significantly higher abundance in the reproductive glands of unmated males compared with other proteins (electronic supplementary material, figure S4; Kruskal–Wallis rank sum test = 209.79, d.f. = 2, $p < 0.0001$, *post hoc* comparison between candidate Sfps and proteins with higher abundance in mated males 'mated up', *post hoc* Dunn tests, $Z = 10.56$, p.adj < 0.0001, and proteins with no difference between virgin and mated males 'not different' proteins; *post hoc* Dunn tests, $Z = 14.02$, p.adj <0.0001). Together, these analyses show

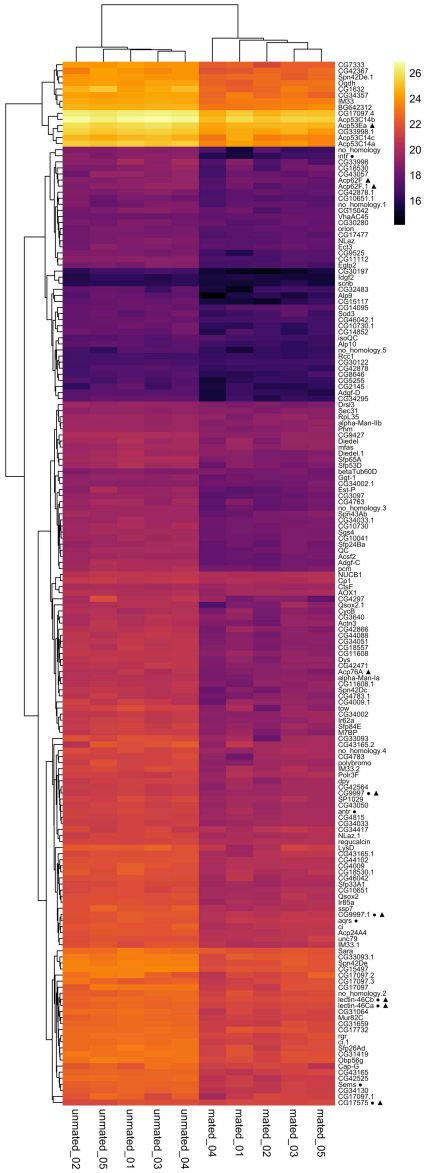

Figure 1. Heatmaps showing the abundance of 172 identified Sfps in mated and unmated males. Both rows and columns are clustered using hierarchical clustering with average linkage. Proteins in the 'sex peptide network' are annotated with circles, and those involved in post-copulatory sexual selection with triangles. Protein names are the best Blast hit to *D. melanogaster*.

that mating alters overall protein abundance in the male reproductive glands and that candidate Sfps are, on average, more abundant in unmated males but decline significantly during mating (figure 1).

Although we expected to find fewer Sfps in the seminal fluid of monandrous species, the 172 *D. subobscura* Sfps are comparable to the 153 found in *D. melanogaster* using a similar approach [22]. Loss events are expected through redundancy or loss of benefit under different mating systems [49] and have occurred across the *Drosophila* genus within accessory gland proteins [50], including within the SP network [51]. However, instead of detecting a loss of proteins in monandrous Sfps, our finding supports the idea that many Sfps are required for core fertility roles and the post-mating female response [14,52,53].

(b) The shared seminal fluid proteins of *Drosophila*

In total, 17 of the 79 Sfps identified in *D. montana* [23] were shared with *D. subobscura* (electronic supplementary material, figure S5). In total, 40 of the 163 Sfps identified in *D. pseudoobscura* [21] were shared with *D. subobscura* (electronic supplementary material, figure S5). In total, 42 of the 153 Sfps identified in *D. melanogaster* [22] were shared with *D. subobscura* (electronic supplementary material, figure S5). We predicted a strong phylogenetic signal, with greater protein overlap between the more closely related *D. subobscura* and *D. pseudoobscura*. However, we only see 15% overlap between these two species, compared with a 30% overlap between *D. pseudoobscura* and *D. montana* (electronic supplementary material, figure S5). This suggests that the method used to identify Sfps might bias results since *D. pseudoobscura* and *D. montana* Sfps were identified using a quantitative proteomics and bioinformatics approach [21,23], whereas *D. melanogaster* and *D. subobscura* used an approach based on comparing the quantitative proteomics of virgin and mated accessory glands [22]. Our further analyses, therefore, focused on comparisons between the Sfps of *D. subobscura* and *D. melanogaster*.

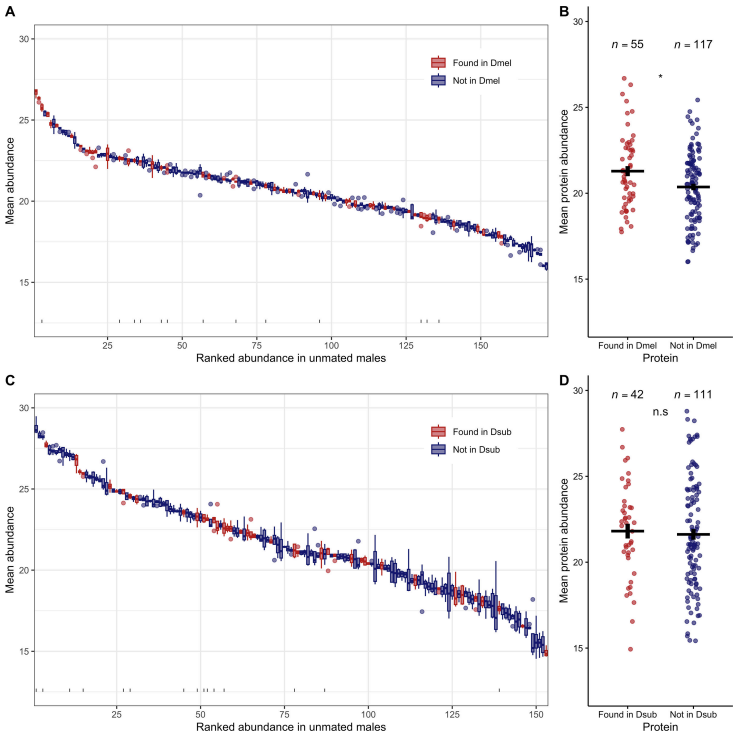

Figure 2. Abundance of proteins in the seminal fluid proteome in *D. subobscura* (Dsub) (A, B) and *D. melanogaster* (Dmel) (C, D). For A and C, ranked abundance for the average of all unmated samples was sorted in decreasing order, with proteins common to both species shown in red. Boxplots show medians and range of the replicates for each protein. Bars along the *x*-axis denote the ranking position of proteins involved in the SP network or post-copulatory sexual selection. For B and D, means and s.e. are shown as black lines and bars. * denotes significance at $p < 0.05$.

(c) The shared seminal fluid proteins of *Drosophila subobscura* and *Drosophila melanogaster*

Only 42 proteins (17%) are shared between the seminal fluid proteomes of *D. subobscura* and *D. melanogaster* (electronic supplementary material, figure S5). Within the shared proteins, we found eight out of the nine SP network proteins (aqrs, antr, CG17575, CG9997, intr, lectin-46a, lectin-46b, sems). Two copies of the *SP* gene are known to be present in the *D. subobscura* genome [46], and we were able to verify that both are expressed in the accessory glands using the RNA-sequencing data (electronic supplementary material, figure S6). However, SP was filtered out of the proteomics list during data processing because only one peptide was detected. SP was therefore not included in the subsequent proteomics analyses, despite potentially being present in the seminal fluid. The presence of the SP network in the shared proteins of *D. subobscura* and *D. melanogaster* represents a significant overrepresentation (hypergeometric cumulative probability; shared = 9/42, compared with *D. melanogaster* = 9/153, $p < 0.0001$).

Out of the 11 post-copulatory sexual selection proteins found in *D. melanogaster* [22], eight proteins are found in both *D. subobscura* and *D. melanogaster* (including SP and lectin-46Ca, lectin-46Cb, Acp53Ea, Acp62F, Acp76A, CG17575, CG9997), which represents a significant overrepresentation (hypergeometric cumulative probability; shared = 8/42, compared with *D. melanogaster* = 11/153, $p = 0.0002$). Two *D. subobscura* proteins were annotated as CG9997 (also part of the SP network) and two proteins annotated as Acp62F. Acp36DE and Spn28F are among the most highly abundant post-copulatory sexual selection proteins found in *D. melanogaster*, but they were not identified in the *D. subobscura* seminal fluid proteome.

(d) The abundance of *Drosophila subobscura* proteins

Proteins common to both *D. subobscura* and *D. melanogaster* seminal proteomes have significantly higher abundance in *D. subobscura* than proteins unique to *D. subobscura* seminal fluid (figure 2A,B; Kruskal–Wallis rank sum test = 4.98, d.f. = 1, $p = 0.026$, $n = 172$). In contrast, shared proteins do not have higher abundance within the *D. melanogaster* seminal fluid proteome (figure 2C,D; Kruskal–Wallis rank sum = 0.30, d.f. = 1, $p = 0.581$, $n = 153$). The higher abundance of shared proteins in *D. subobscura* could reflect their conserved or core functional role in fertility and reproduction, since this function is common to both species.

We next aimed to test whether the mating system affected the abundance of protein groups between species. However, comparing protein abundance between two species using MS presents methodological difficulties because sequence differences between orthologous proteins can influence peptide detectability, potentially masking true biological correlation. To partially mitigate this, we compared the ranked abundance of proteins shared between the two species. For the shared proteins, there is a weak but significant positive correlation in ranked abundance between the two species (figure 3A; Spearman's rho = 0.34, $p = 0.011$, $n = 55$). This observation suggests similarity in the importance of shared Sfps in monandrous and polyandrous species. Proteins associated with post-copulatory sexual selection had higher-ranked abundance compared with other proteins within

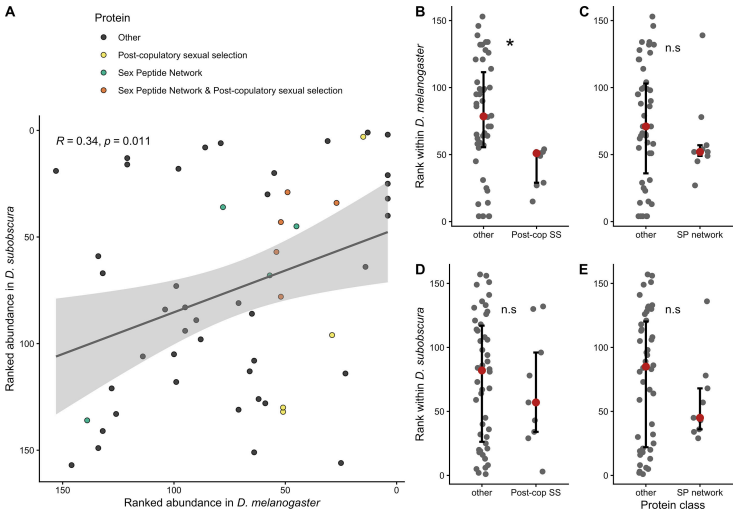

Figure 3. Ranked abundance of Sfps in *D. subobscura* and *D. melanogaster.* (A) Correlation of ranked abundance in *D. subobscura* and *D. melanogaster*, where rank 1 is a protein with the highest abundance. Ranking is plotted in reverse such that the most abundant proteins in *D. subobscura* and *D. melanogaster* are in the top right of the plot, and proteins least abundant in both species are in the bottom left of the plot. Proteins are colour coded, with those belonging to the SP network in green, those involved in post-copulatory sexual selection in yellow) or in both, orange. (B–E) Within-species comparisons of protein class ranked abundance for *D. melanogaster* (B and C) and *D. subobscura* (D and E) with medians (red) and interquartile range (bar) are presented.

D. melanogaster (figure 3B; Kruskal–Wallis rank sum test = 6.97, d.f. = 1, p = 0.008) but not within *D. subobscura* (figure 3D; Kruskal–Wallis rank sum test = 0.187, d.f. = 1, p = 0.666). SP network proteins did not have greater abundance compared with other proteins within *D. melanogaster* (figure 3C; Kruskal–Wallis rank sum test = 1.19, d.f. = 1, p = 0.275) or *D. subobscura* (figure 3E; Kruskal–Wallis rank sum test = 0.671, d.f. = 1, p = 0.413).

(i) Post-copulatory sexual selection proteins

In line with our predictions, several proteins associated with post-copulatory sexual selection were absent from *D. subobscura*. Moreover, those that were present were not more abundant than other Sfps (median abundance post-copulatory sexual selection proteins = 21.6, other = 20.6; Kruskal–Wallis rank sum test = 1.473, d.f. = 1, p = 0.225). This contrasts with polyandrous *D. melanogaster*, where post-copulatory sexual selection proteins had higher-ranked abundance than other proteins (median abundance post-copulatory sexual selection proteins = 24.6, other = 21.1; Kruskal–Wallis rank sum test = 10.93, d.f. = 1, p = 0.0009). This finding might suggest that proteins with post-copulatory function are not as important in *D. subobscura,* potentially owing to monandrous mating. However, post-copulatory sexual selection proteins were over-represented among the shared proteins, indicating that they remain functionally important. This may be because such proteins are typically identified in *D. melanogaster* through knockout experiments (e.g. [54]) and many probably play essential roles in basic fertility, impacting post-copulatory sexual selection when their normal function is disrupted [30]. Additionally, key sperm competition proteins are known to evolve rapidly, either under adaptive evolution [55,56] or relaxed purifying selection [56–58], and they may therefore be replaced or have diverged beyond recognition in *D. subobscura*. Consequently, our ability to distinguish between a true reduction in sperm competition proteins and a failure to detect highly divergent proteins is limited.

(ii) Sex peptide network proteins

In *D. melanogaster,* the SP network plays a critical role in post-mating responses by inhibiting female receptivity to re-mating [16]. While we expected to find fewer SP network proteins or a lower abundance in a monandrous seminal proteome, we observed little difference in the number and abundance of SP network proteins compared with *D. melanogaster*. Given that females in our *D. subobscura* population rarely remate [24], the SP network may be particularly effective in this species, potentially mediated by greater transcription potential of the two copies of the *SP* gene (with the *SPR* sequence also identified in the genome). This genomic organization may indicate that monandry is driven by mechanisms of conflict rather than cooperation in this species, and that a single mating induces lifelong refractoriness in females through male manipulation.

However, females in our population do remate, although rarely (4% of females, [24]). This 4% female re-mating may be sufficient to provide selection for the maintenance of SP network proteins for their female receptivity-inhibiting effects.

Alternatively, the SP network may be conserved in *D. subobscura* because it is ancestral in *Drosophila* [51] and subject to pleiotropic constraint to maintain network function [59,60]. While proteins in a network are not easily lost because of shared selective pressures, there is evidence of copy number variation in the SP network across *Drosophila* species [51]. So, although there is conservation of the network, gene duplications and losses are possible [51]. Furthermore, the SP network may serve a different function in *D. subobscura*. In *D. melanogaster*, SP appears to have multiple roles, as it interacts with receptors beyond SPRs (e.g. [61]). Thus, the presence of an SP network in *D. subobscura* does not necessarily indicate involvement in suppressing female re-mating. Indeed, even though SP network orthologues are found across diverse *Drosophila* species [16], females of species outside of the *melanogaster* group do not respond to artificial injection of synthetic SP into the abdominal cavity of virgin

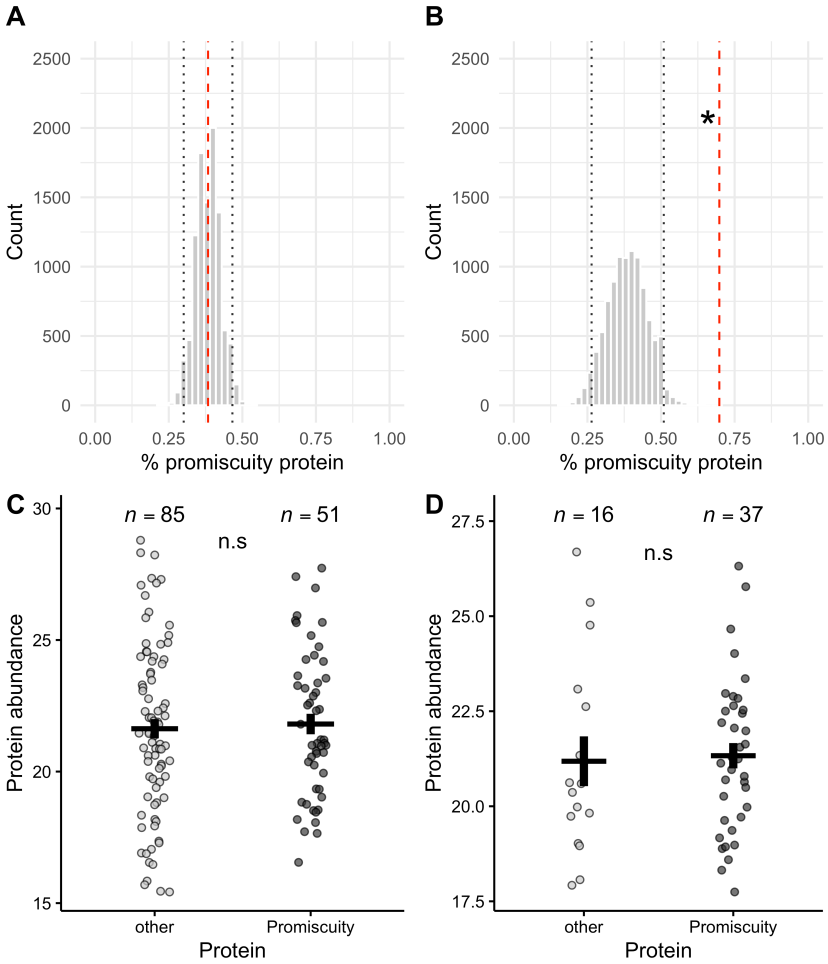

Figure 4. The number and abundance of promiscuity proteins in the seminal fluid of *D. subobscura* and *D. melanogaster*. The actual proportions of promiscuity proteins are shown by red lines, and the bootstrapped expected distributions are shown in grey for *D. melanogaster* (A), and *D. subobscura* (B). The abundance of promiscuity proteins was not significantly different from other proteins in *D. melanogaster* (C) or *D. subobscura* (D). * denotes significance at $p < 0.05$.

females [62]. The function of SPR in the wider *Drosophila* phylogeny is also likely to include functions unrelated to reproduction because SPR is expressed in non-reproductive tissues [62]. For example, SPR is also activated by myoinhibitory peptides, which probably function as ancestral ligands for SPR, illustrating how one receptor can respond to two functionally unrelated ligand systems [63]. In species lacking SP, post-mating responses in females can be mediated by other proteins (e.g. [64,65]). Taken together, the function of the SP network in *D. subobscura* remains debatable and will require functional genetics, such as the knockout of male SP network proteins and the female SPR, to resolve.

(e) Molecular function of *Drosophila subobscura* seminal fluid proteins

We predicted a lower proportion of proteins with molecular functions associated with manipulative effects, such as peptidases and peptidase inhibitors, in a monandrous seminal proteome. However, the proportions of Sfps within each molecular function GO term are similar in *D. subobscura* and *D. melanogaster* (electronic supplementary material, figure S7; chi-square test, $p > 0.1$ for each GO term following FDR correction for multiple testing), suggesting that overall molecular functions performed by the seminal proteomes in each species are broadly conserved. Moreover, only 42 proteins (17%) are shared between the seminal fluid proteomes of *D. subobscura* and *D. melanogaster*, implying that different proteins may perform similar roles across species [66]. This pattern of functional conservation of the seminal proteome is also observed across very distantly related taxa, such as between *Drosophila* and humans [30].

(f) Promiscuity proteins identified through selection lines

A previous study identified *D. melanogaster Sfp* genes that evolved lower expression 48 h post-eclosion under selection for monogamous mating [31]. These 'promiscuity proteins' might be targets of reduced pre-mating or post-mating sexual selection. We therefore tested for a difference in the number and abundance of these proteins in *D. subobscura* seminal fluid, expecting that they might be reduced.

We calculated the proportion of 'promiscuity proteins' expected in *D. subobscura* under the null model that selection has not affected proteome composition. Bootstrapping with replacement on the Sfps of *D. melanogaster* gave the null prediction 95% confidence intervals for the proportion of promiscuity proteins in *D. subobscura* between 0.24 and 0.50. The actual proportion

of promiscuity proteins in *D. subobscura* is 0.68, which is significantly greater than expected by chance (figure 4B; bootstrap $p < 0.0001$).

Within *D. subobscura*, these promiscuity proteins ($n = 37$) had similar abundances to the proteins that did not respond to monogamy in the selection lines (figure 4D; Kruskal–Wallis rank sum = 0.274, d.f. = 1, $p = 0.601$). These results qualitatively held when the threshold for detection of promiscuity proteins was relaxed to $\log_2FC < -0.15$ and $\log_2FC < -0.25$ (electronic supplementary material, figure S8).

Contrary to our expectations, many more 'promiscuity proteins' were found than expected by chance in *D. subobscura*. The most parsimonious explanation is that monogamy selection in selection lines is different from selection acting within monogamous species. Females in monogamous selection lines were randomly allocated one male, and there was no opportunity for re-mating with rival males. The effect of this approach is to remove the potential for sexual conflict by perfectly aligning the reproductive success of males and females [67]. However, in monandrous *D. subobscura*, females select their male mating partners so pre-copulatory selection exists, and females have the opportunity to remate, but do so rarely. One hypothesis is that the genes that had reduced expression in monogamy selection are implicated in preventing female re-mating. Consistent with this idea, all the SP network proteins were downregulated in monogamy lines. Hence, the overrepresentation of 'promiscuity proteins' in the seminal proteome of *D. subobscura* might reflect strong selection on males to prevent females from ever re-mating via the SP network and associated proteins.

However, in contrast to this hypothesis, males from experimentally evolved *D. melanogaster* monogamy and polygamy lines did not differ in their capacity to elicit female post-mating responses at 16 h or 2 days post-mating [31]. This finding suggests that, despite reduced expression of *SP* network genes in monogamy selection lines, males remained unexpectedly effective at preventing female re-mating. Monogamy males were less competitive under sperm competition, and five of *D. subobscura's* eight post-copulatory sexual selection proteins (Acp53Ea, lectin-46Cb, lectin-46Ca, CG17575 and CG9997's two homologues) were in the 'promiscuity proteins' of *D. subobscura*. However, as discussed previously, these post-copulatory sexual selection proteins are also likely to impact basic fertility—less fertile males also do poorly in sperm competition—and so these proteins may be present in *D. subobscura* for their basic fertility function.

4. Conclusion

To summarize, despite *D. subobscura* exhibiting monogamy, it does not appear to have a reduced or less complex seminal proteome compared with polyandrous *D. melanogaster*. *Drosophila subobscura* retains all proteins from the SP network, as well as many proteins associated with post-copulatory sexual selection, and retains more 'promiscuity proteins' than expected by chance. These proteins may function to support male fitness by promoting post-copulatory success, such as preventing female re-mating in *D. subobscura*: the 4% rate of female re-mating [24] may be sufficient selection pressure for the maintenance of many of the types of Sfps seen in polyandrous species. Therefore, our data suggest that monandry in this species represents sexual conflict rather than cooperation. However, we cannot discount the possibility that proteins within the SP network have been maintained for some other reason or have a different function within *D. subobscura* [20]. These data add to the growing evidence that, despite the rapid evolution of Sfps [68,69] and differences in mating systems, the seminal proteomes of different species share many core compositional characteristics. Perhaps this is not surprising, as the primary function of seminal fluid in promoting sperm transfer and fertilization success is a fundamental feature of internal fertilization. The mechanisms underlying this function appear to be evolutionarily conserved, even across divergent taxa. This finding adds to the idea that knowledge of the fundamental roles of Sfps in model species is probably translatable, if not in terms of homologous genes and proteins then at least in terms of principles, across broad taxa.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [70] partner repository with the dataset identifier PXD072146. The data and code underlying this article are available at the Oxford University Research Archive (ORA) [71].

Supplementary material is available online [72].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. R.D.: conceptualization, formal analysis, visualization, writing—original draft, writing—review and editing; K.S.: formal analysis, visualization, writing—review and editing; R.V.: conceptualization, investigation, writing—review and editing; J.T.-H.: investigation, writing—review and editing; I.S.: conceptualization, methodology, writing—review and editing; T.A.R.P.: conceptualization, writing—review and editing; M.H.: investigation, writing—original draft; S.H.: formal analysis, investigation, writing—original draft; S.F.G.: conceptualization, writing—review and editing; S.P.: conceptualization, methodology, supervision, writing—review and editing; C.E.: conceptualization, methodology, supervision, writing—review and editing; A.R.J.: conceptualization, formal analysis, methodology, supervision, writing—review and editing; S.W.: conceptualization, funding acquisition, investigation, supervision, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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References

1. Parker GA. 1970 Sperm competition and its evolutionary consequences in the insects. *Biol. Rev.* **45**, 525–567. (doi:10.1111/j.1469-185X.1970.tb01176.x)
2. Parker GA. 1993 Sperm competition games: sperm size and sperm number under adult control. *Proc. R. Soc. B* **253**, 245–254. (doi:10.1098/rspb.1993.0110)
3. Parker GA, Lessells CM, Simmons LW. 2013 Sperm competition games: a general model for precopulatory male–male competition. *Evolution* **67**, 95–109. (doi:10.1111/j.1558-5646.2012.01741.x)
4. Short RV. 1979 Sexual selection and its component parts, somatic and genital selection, as illustrated by man and the great apes*. In *Advances in the study of behavior* (eds JS Rosenblatt, RA Hinde, C Beer, MC Busnel), pp. 131–158. London, UK: Academic Press. (doi:10.1016/s0065-3454(08)60035-2)
5. Wedell N, Gage MJG, Parker GA. 2002 Sperm competition, male prudence and sperm-limited females. *Trends Ecol. Evol.* **17**, 313–320. (doi:10.1016/s0169-5347(02)02533-8)
6. Pizzari T, Cornwallis CK, Løvlie H, Jakobsson S, Birkhead TR. 2003 Sophisticated sperm allocation in male fowl. *Nature* **426**, 70–74. (doi:10.1038/nature02004)
7. Pound N, Gage MJG. 2004 Prudent sperm allocation in Norway rats, *Rattus norvegicus*: a mammalian model of adaptive ejaculate adjustment. *Anim. Behav.* **68**, 819–823. (doi:10.1016/j.anbehav.2004.02.004)
8. Lüpold S, de Boer RA, Evans JP, Tomkins JL, Fitzpatrick JL. 2020 How sperm competition shapes the evolution of testes and sperm: a meta-analysis. *Phil. Trans. R. Soc. B* **375**, 20200064. (doi:10.1098/rstb.2020.0064)
9. Birkhead TR, Møller AP (eds). 1998 *Sperm competition and sexual selection*. London, UK: Academic Press. (doi:%2010.1016/B978-0-12-100543-6.X5022-3)
10. Ramm SA. 2020 Seminal fluid and accessory male investment in sperm competition. *Phil. Trans. R. Soc. B* **375**, 20200068. (doi:10.1098/rstb.2020.0068)
11. Ramm SA, Edward DA, Claydon AJ, Hammond DE, Brownridge P, Hurst JL, Beynon RJ, Stockley P. 2015 Sperm competition risk drives plasticity in seminal fluid composition. *BMC Biol.* **13**, 87. (doi:10.1186/s12915-015-0197-2)
12. Bartlett MJ, Steeves TE, Gemmell NJ, Rosengrave PC. 2017 Sperm competition risk drives rapid ejaculate adjustments mediated by seminal fluid. *eLife* **6**, e28811. (doi:10.7554/elife.28811)
13. Hopkins BR *et al*. 2019 Divergent allocation of sperm and the seminal proteome along a competition gradient in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* **116**, 17925–17933. (doi:10.1073/pnas.1906149116)
14. Perry JC, Sirot L, Wigby S. 2013 The seminal symphony: how to compose an ejaculate. *Trends Ecol. Evol.* **28**, 414–422. (doi:10.1016/j.tree.2013.03.005)
15. Hopkins BR, Sepil I, Wigby S. 2017 Seminal fluid. *Curr. Biol.* **27**, R404–R405. (doi:10.1016/j.cub.2017.03.063)
16. McGeary MK, Findlay GD. 2020 Molecular evolution of the sex peptide network in *Drosophila*. *J. Evol. Biol.* **33**, 629–641. (doi:10.1111/jeb.13597)
17. Chen PS, Stumm-Zollinger E, Aigaki T, Balmer J, Bienz M, Böhlen P. 1988 A male accessory gland peptide that regulates reproductive behavior of female *D. melanogaster*. *Cell* **54**, 291–298. (doi:10.1016/0092-8674(88)90192-4)
18. Thorson A, Snook RR, Wiberg RAW. 2025 Sexually antagonistic co-evolution at the molecular level predicts phenotypic outcomes of mating interactions. *bioRxiv* 2025.11.04.686516. (doi:10.1101/2025.11.04.686516)
19. Wigby S, Chapman T. 2005 Sex peptide causes mating costs in female *Drosophila melanogaster*. *Curr. Biol.* **15**, 316–321. (doi:10.1016/j.cub.2005.01.051)
20. Hopkins BR, Perry JC. 2022 The evolution of sex peptide: sexual conflict, cooperation, and coevolution. *Biol. Rev. Camb. Phil. Soc.* **97**, 1426–1448. (doi:10.1111/brv.12849)
21. Karr TL, Southern H, Rosenow MA, Gossmann TI, Snook RR. 2019 The old and the new: discovery proteomics identifies putative novel seminal fluid proteins in *Drosophila* *. *Mol. Cell. Proteomics* **18**, S23–S33. (doi:10.1074/mcp.RA118.001098)
22. Sepil I, Hopkins BR, Dean R, Thézénas ML, Charles PD, Konietzny R, Fischer R, Kessler BM, Wigby S. 2019 Quantitative proteomics identification of seminal fluid proteins in male *Drosophila melanogaster*. *Mol. Cell. Proteom.* **18**, S46–S58. (doi:10.1074/mcp.ra118.000831)
23. Garlovsky MD, Evans C, Rosenow MA, Karr TL, Snook RR. 2020 Seminal fluid protein divergence among populations exhibiting postmating prezygotic reproductive isolation. *Mol. Ecol.* **29**, 4428–4441. (doi:10.1111/mec.15636)
24. Fisher DN, Doff RJ, Price TAR. 2013 True polyandry and pseudopolyandry: why does a monandrous fly remate? *BMC Evol. Biol.* **13**, 157. (doi:10.1186/1471-2148-13-157)
25. Lizé A, Doff RJ, Smaller EA, Lewis Z, Hurst GDD. 2012 Perception of male–male competition influences *Drosophila* copulation behaviour even in species where females rarely remate. *Biol. Lett.* **8**, 35–38. (doi:10.1098/rsbl.2011.0544)
26. Bretman A, Fricke C, Chapman T. 2009 Plastic responses of male *Drosophila melanogaster* to the level of sperm competition increase male reproductive fitness. *Proc. R. Soc. B* **276**, 1705–1711. (doi:10.1098/rspb.2008.1878)
27. Bretman A, Fricke C, Hetherington P, Stone R, Chapman T. 2010 Exposure to rivals and plastic responses to sperm competition in *Drosophila melanogaster*. *Behav. Ecol.* **21**, 317–321. (doi:10.1093/beheco/arp189)
28. Cirera S, Aguade M. 1998 Molecular evolution of a duplication: the sex-peptide (Acp70A) gene region of *Drosophila subobscura* and *Drosophila madeirensis*. *Mol. Biol. Evol.* **15**, 988–996. (doi:10.1093/oxfordjournals.molbev.a026014)
29. Russo CA, Takezaki N, Nei M. 1995 Molecular phylogeny and divergence times of drosophilid species. *Mol. Biol. Evol.* **12**, 391–404. (doi:10.1093/oxfordjournals.molbev.a040214)
30. Wigby S, Brown NC, Allen SE, Misra S, Sitnik JL, Sepil I, Clark AG, Wolfner MF. 2020 The *Drosophila* seminal proteome and its role in postcopulatory sexual selection. *Phil. Trans. R. Soc. B* **375**, 20200072. (doi:10.1098/rstb.2020.0072)
31. Hollis B, Koppik M, Wensing KU, Ruhmann H, Genzoni E, Erkosar B, Kawecki TJ, Fricke C, Keller L. 2019 Sexual conflict drives male manipulation of female postmating responses in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* **116**, 8437–8444. (doi:10.1073/pnas.1821386116)
32. Verspoor RL, Smith JML, Mannion NLM, Hurst GDD, Price TAR. 2018 Strong hybrid male incompatibilities impede the spread of a selfish chromosome between populations of a fly. *Evol. Lett.* **2**, 169–179. (doi:10.1002/evl3.55)
33. Lyth S, Gill T, Manser A, Tanianis J, Hurst GDD, Betancourt AJ, Price TAR, Verspoor RL. 2025 Suppression of sex-ratio drive in *Drosophila subobscura*. *Evolution* **79**, 2394–2405. (doi:10.1093/evolut/qpaf153)
34. Holman L, Freckleton RP. 2008 What use is an infertile sperm? A comparative study of sperm-heteromorphic *Drosophila*. *Evolution* **62**, 374–385. (doi:10.1111/j.1558-5646.2007.00280.x)
35. Lizé A, Price TAR, Heys C, Lewis Z, Hurst GDD. 2014 Extreme cost of rivalry in a monandrous species: male–male interactions result in failure to acquire mates and reduced longevity. *Proc. R. Soc. B* **281**, 20140631. (doi:10.1098/rspb.2014.0631)
36. Cock PJA *et al*. 2009 Biopython: freely available Python tools for computational molecular biology and bioinformatics. *Bioinformatics* **25**, 1422–1423. (doi:10.1093/bioinformatics/btp163)

37. Karageorgiou C, Gámez-Visairas V, Tarrío R, Rodríguez-Trelles F. 2019 Long-read based assembly and synteny analysis of a reference *Drosophila subobscura* genome reveals signatures of structural evolution driven by inversions recombination-suppression effects. *BMC Genom.* **20**, 223. (doi:10.1186/s12864-019-5590-8)
38. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL. 2009 BLAST+: architecture and applications. *BMC Bioinform.* **10**, 421. (doi:10.1186/1471-2105-10-421)
39. Cox J, Mann M. 2008 MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* **26**, 1367–1372. (doi:10.1038/nbt.1511)
40. Stekhoven DJ, Bühlmann P. 2012 MissForest—non-parametric missing value imputation for mixed-type data. *Bioinformatics* **28**, 112–118. (doi:10.1093/bioinformatics/btr597)
41. R Core Team. 2024 R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. Vienna, Austria https://www.R-project.org
42. Suzuki R, Shimodaira H. 2006 Pvclust: an R package for assessing the uncertainty in hierarchical clustering. *Bioinformatics* **22**, 1540–1542. (doi:10.1093/bioinformatics/btl117)
43. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. 2015 limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic. Acids. Res.* **43**, e47. (doi:10.1093/nar/gkv007)
44. Kolde R. 2025 pheatmap: pretty heatmaps [R package v. 1.0.13]. See https://github.com/raivokolde/pheatmap.
45. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990 Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410. (doi:10.1016/S0022-2836(05)80360-2)
46. Cirera S, Aguadé M. 1998 The sex-peptide gene (*Acp70A*) is duplicated in *Drosophila subobscura*. *Gene* **210**, 247–254. (doi:10.1016/s0378-1119(98)00069-9)
47. Larkin A *et al*. 2021 FlyBase: updates to the Drosophila melanogaster model organism database. *Nucleic Acids Res.* **49**, D899–D907. (doi:10.1093/nar/gkaa1026)
48. Hurtado J, Almeida FC, Belliard SA, Revale S, Hasson E. 2022 Research gaps and new insights in the evolution of *Drosophila* seminal fluid proteins. *Insect Mol. Biol.* **31**, 139–158. (doi:10.1111/imb.12746)
49. Carlisle JA, Bickell AL, Hart MW. 2025 Sexual selection and pseudogenization in primate fertilization. *Evolution* **79**, 1670–1680. (doi:10.1093/evolut/qpaf072)
50. Begun DJ, Lindfors HA. 2005 Rapid evolution of genomic *Acp* complement in the *melanogaster* subgroup of *Drosophila*. *Mol. Biol. Evol.* **22**, 2010–2021. (doi:10.1093/molbev/msi201)
51. Carlisle JA, McCormick BK, Verbakel L, von Philipsborn AC, Wolfner MF, Clark AG. 2025 Correlated gene copy number changes in a seminal fluid protein network in *Drosophila*. *bioRxiv* 2025.10.27.684572. (doi:10.1101/2025.10.27.684572)
52. Avila FW, Sirot LK, LaFlamme BA, Rubinstein CD, Wolfner MF. 2011 Insect seminal fluid proteins: identification and function. *Annu. Rev. Entomol.* **56**, 21–40. (doi:10.1146/annurev-ento-120709-144823)
53. Gilany K, Minai-Tehrani A, Savadi-Shiraz E, Rezadoost H, Lakpour N. 2015 Exploring the human seminal plasma proteome: an unexplored gold mine of biomarker for male infertility and male reproduction disorder. *J. Reprod. Infertil.* **16**, 61–71.
54. Avila FW, Cohen AB, Ameerudeen FS, Duneau D, Suresh S, Mattei AL, Wolfner MF. 2015 Retention of ejaculate by *Drosophila melanogaster* females requires the male-derived mating plug protein PEBme. *Genetics* **200**, 1171–1179. (doi:10.1534/genetics.115.176669)
55. Torgerson DG, Kulathinal RJ, Singh RS. 2002 Mammalian sperm proteins are rapidly evolving: evidence of positive selection in functionally diverse genes. *Mol. Biol. Evol.* **19**, 1973–1980. (doi:10.1093/oxfordjournals.molbev.a004021)
56. Dapper AL, Wade MJ. 2016 The evolution of sperm competition genes: the effect of mating system on levels of genetic variation within and between species. *Evolution* **70**, 502–511. (doi:10.1111/evo.12848)
57. Patlar B, Jayaswal V, Ranz JM, Civetta A. 2021 Nonadaptive molecular evolution of seminal fluid proteins in *Drosophila*. *Evolution* **75**, 2102–2113. (doi:10.1111/evo.14297)
58. Dapper AL, Wade MJ. 2020 Relaxed selection and the rapid evolution of reproductive genes. *Trends Genet.* **36**, 640–649. (doi:10.1016/j.tig.2020.06.014)
59. Mank JE, Hultin-Rosenberg L, Zwahlen M, Ellegren H. 2008 Pleiotropic constraint hampers the resolution of sexual antagonism in vertebrate gene expression. *Am. Nat.* **171**, 35–43. (doi:10.1086/523954)
60. Wagner GP, Zhang J. 2011 The pleiotropic structure of the genotype–phenotype map: the evolvability of complex organisms. *Nat. Rev. Genet.* **12**, 204–213. (doi:10.1038/nrg2949)
61. Ja WW, Carvalho GB, Madrigal M, Roberts RW, Benzer S. 2009 The *Drosophila* G protein-coupled receptor, methuselah, exhibits a promiscuous response to peptides. *Protein Sci.* **18**, 2203–2208. (doi:10.1002/pro.221)
62. Tsuda M, Peyre JB, Asano T, Aigaki T. 2015 Visualizing molecular functions and cross-species activity of sex-peptide in *Drosophila*. *Genetics* **200**, 1161–1169. (doi:10.1534/genetics.115.177550)
63. Poels J, Van Loy T, Vandersmissen HP, Van Hiel B, Van Soest S, Nachman RJ, Vanden Broeck J. 2010 Myoinhibiting peptides are the ancestral ligands of the promiscuous *Drosophila* sex peptide receptor. *Cell. Mol. Life Sci.* **67**, 3511–3522. (doi:10.1007/s00018-010-0393-8)
64. Kingan TG, Bodnar WM, Raina AK, Shabanowitz J, Hunt DF. 1995 The loss of female sex pheromone after mating in the corn earworm moth *Helicoverpa zea*: identification of a male pheromonostatic peptide. *Proc. Natl Acad. Sci. USA* **92**, 5082–5086. (doi:10.1073/pnas.92.11.5082)
65. Duvall LB, Basrur NS, Molina H, McMeniman CJ, Vosshall LB. 2017 A peptide signaling system that rapidly enforces paternity in the *Aedes aegypti* mosquito. *Curr. Biol.* **27**, 3734–3742. (doi:10.1016/j.cub.2017.10.074)
66. Ravi Ram K, Wolfner MF. 2007 Seminal influences: *Drosophila* Acps and the molecular interplay between males and females during reproduction. *Integr. Comp. Biol.* **47**, 427–445. (doi:10.1093/icb/icm046)
67. Holland B, Rice WR. 1999 Experimental removal of sexual selection reverses intersexual antagonistic coevolution and removes a reproductive load. *Proc. Natl Acad. Sci. USA* **96**, 5083–5088. (doi:10.1073/pnas.96.9.5083)
68. Swanson WJ, Vacquier VD. 2002 The rapid evolution of reproductive proteins. *Nat. Rev. Genet.* **3**, 137–144. (doi:10.1038/nrg733)
69. Wilburn DB, Swanson WJ. 2016 From molecules to mating: rapid evolution and biochemical studies of reproductive proteins. *J. Proteom* **135**, 12–25. (doi:10.1016/j.jprot.2015.06.007)
70. Perez-Riverol Y *et al*. 2025 The PRIDE database at 20 years: 2025 update. *Nucleic Acids Res.* **53**, D543–D553. (doi:10.1093/nar/gkae1011)
71. Dean R, Wigby S. 2026 *The seminal proteome of a monandrous fly, Drosophila subobscura*. Oxford, UK: University of Oxford. (doi:10.5287/ora-r8mqbr2o4)
72. Dean R, Sue-obc K, Verspoor R, Tannianis-Hughes J, Sepil I, Price T *et al*. 2026 Supplementary material from: The seminal proteome of a monandrous fly, *Drosophila subobscura*. Figshare. (doi:10.6084/m9.figshare.c.8498920)