Introduction

In addition to sperm, males of many internally fertilizing species transfer seminal fluid proteins (Sfps) to their mates during copulation. These proteins function in a variety of reproductive processes, including sperm capacitation, sperm storage and competition, and fertilization, and in some cases they affect female behavior and physiology [1]. Like other reproductive proteins, Sfps often evolve rapidly between species, underscoring their relevance to reproductive success [2]. Sfps are thought to interact with several classes of molecules, including other Sfps (which may originate from seminal fluid of the same male or from a competitor), proteins native to the female reproductive tract, and pathogens that may be introduced during the course of mating. These interactions create opportunities for coevolution, leading to speculation that sperm competition, sexual conflict, sexual selection, and host–pathogen interactions could drive the rapid, adaptive evolution of many Sfps [3]. Because of their rapid evolution and their critical importance to reproductive fitness, Sfps may also be involved in the formation of new species [3–5]. As such, researchers have sought to identify and characterize Sfps in such diverse taxa as mosquitoes, crickets, honeybees, rodents, and primates [6–10].

Although Sfps are being studied in many species, they are best characterized in Drosophila melanogaster. Because the Drosophila mating system features multiple matings by females and sperm competition between males [11–14], Sfps are thought to be especially important for reproductive success and for mediating conflict and competition. Previous studies have focused on three areas: (a) the effects of the full set of Sfps (and especially of accessory gland proteins, or Acps) on male and female fitness; (b) identification of putative Acps by expressed sequence tag (EST) sequencing, comparative genomics, and proteomics; and (c) functional analysis of specific Acps. Whole-organism work revealed that Acps mediate a “cost of mating” to females. The repeated receipt of Acps through multiple matings lowers female reproductive output by reducing female life span without a corresponding increase in egg production [15–17]. Furthermore, when a population of males harboring natural genetic variation was allowed to adapt to a static female genotype, male sperm competitive ability, mating success, and harm caused to females increased in 30–40 generations [18]. These dramatic evolutionary outcomes sparked much interest in identifying the specific proteins of seminal fluid. Screens for genes expressed specifically in the male accessory glands (and encoding proteins predicted to be secreted) identified ~70 putative Acps [19,20], several of which have been genetically or biochemically characterized (reviewed in [21]). Work in related Drosophila species has revealed that many predicted Acps are subject to lineage-specific gene gain, gene loss, and/or copy number variation [22–24]. Additionally, several
**Author Summary**

Across many species, males transfer both sperm and seminal proteins to their mates. These proteins increase male reproductive success by improving sperm competitive ability and modifying female behavior. In *Drosophila*, seminal proteins increase female rates of egg-laying and sperm storage and reduce a female’s willingness to mate with subsequent suitors. Several male seminal proteins have been extensively characterized, and others have been predicted based on gene expression patterns, yet the full set of proteins that is transferred to females has not been defined. Here we introduce a new proteomic method that identifies transferred seminal proteins in recently mated females and quantifies their relative abundance. We confirm many of the predicted seminal proteins and discover a number of novel seminal fluid components. Some of these proteins show elevated rates of evolution, consistent with their involvement in sexual selection or sexual conflict, and many have arisen by tandem gene duplication. By using this method in three species of *Drosophila*, we identified lineage-specific components of seminal fluid. Additionally, we developed and validated a method to identify completely new genes in the *D. melanogaster* genome. These transferred proteins are now targets for follow-up genetic, biochemical, and evolutionary analyses.

Proteomic studies have examined both Acps and sperm proteins found in males [25,26], and whole-genome, tissue-specific microarray analysis has increased the number of predicted Acps to 112 [21,27].

In spite of this considerable progress, less than one-third of the predicted *D. melanogaster* Sfps have been detected in mated females [21,28]. Furthermore, prior work to predict Sfps has often required that candidate genes show tissue-specific expression in the male reproductive tract. Identifying the set of transferred Sfps in an unbiased fashion is of critical importance, since it is these proteins that are the most likely to influence post-mating processes like sperm competition and sexual conflict. We have developed a mass spectrometry (MS) method that specifically detects male Sfps in mated female *D. melanogaster*. In addition to confirming the transfer of many predicted Sfps, we identified dozens of new seminal fluid components, including completely new classes of proteins. Evolutionary analyses show that positive selection and tandem gene duplication drive the evolution of seminal fluid between species, and comparative proteomics with additional species identified lineage-specific Sfps. We also used our MS data to estimate the relative abundance of each Sfp in seminal fluid and to discover previously unannotated genes encoding additional Sfps. Taken together, our experiments illustrate the power of combining proteomics with evolutionary biology to address fundamental questions about reproduction.

**Results**

**Isotopic Labeling of Female Flies Allows Transferred Sfps to Be Identified**

To distinguish between transferred Sfps and proteins native to the female reproductive tract, we metabolically labeled female flies using a diet enriched in $^{15}$N isotopes to create an isotopically “heavy” form of the female proteins [29]. Females were reared on yeast that was grown in media enriched in $^{15}$N. After one full generation of labeling, the $^{15}$N enrichment in detected fly peptides was ~98 atom percent excess, and no peptides from whole female flies were identified with natural abundance nitrogen isotopes. These data confirm that isotopic labeling can be readily achieved in *D. melanogaster* and other drosophilids (see below). Therefore, we reasoned that by mating unlabeled males to labeled females and then analyzing proteins found in the female reproductive tract by MS, transferred male Sfps could be identified by those peptides that showed natural abundance isotope distributions. We chose to label females instead of males, because MS resolution is best for unlabeled peptides, and we were interested in identifying male Sfps.

We performed multiple biological replicates of mating experiments with different strains of males: Canton S (a standard lab strain), sons of *tudor* females (spermless males) [30], and, as a negative control, DTA-E males (which are spermless and do not produce main cell accessory gland proteins) [31]. In two DTA-E experiments, we detected 11 transferred proteins, including several known to be produced outside of the accessory glands (Table S1). Six total experiments with Canton S and *tudor* males identified a set of 138 high-confidence Sfps (Table 1 and Table S2), using a peptide-level $q$-value $\leq 0.01$ within each experiment [32]. Just over half (75/138) of the transferred Sfps were previously predicted through tissue-specific expression profiling or other experimental or comparative genomic methods [21], but only 19 were confirmed previously to be transferred at mating. Notably, we found only five previously documented sperm proteins [25], confirming that our protein preparation protocol effectively selected for soluble, extracellular proteins. We did not detect 49 predicted Sfps [21]. These proteins may be transferred at low levels, immediately cleaved or degraded in the female, or have certain peptide sequences or post-translational modifications that complicate detection by shotgun proteomics. Alternatively, some may not be transferred at mating.

We identified 63 novel Sfps, 45 of which were found in at least two biological replicates. Many of these proteins fell into the same functional categories as the previously predicted set, including proteases, protease inhibitors, mediators of an

| Table 1. Classes of Seminal Fluid Proteins Detected by MS in Recently Mated *D. melanogaster* Females |
| Protein Class | Number of Proteins Detected (Number Newly Identified) |
|---------------|------------------------------------------------------|
| Unknown function | 47 (23) |
| Protease | 15 (5) |
| Protease inhibitor | 14 (6) |
| Defense/immunity | 12 (4) |
| Lipid metabolism | 9 (2) |
| Carbohydrate interactions | 7 (2) |
| Odorant binding | 7 (7) |
| Other functions | 7 (5) |
| Post-mating behavior | 5 (0) |
| Sperm protein | 5 (0) |
| Chitin binding | 4 (4) |
| DNA interactions | 3 (3) |
| Protein modification | 3 (2) |
| TOTAL | 138 (63) |



doi:10.1371/journal.pbio.0060178.t001
immune response, and proteins involved in lipid metabolism (Table 1). We discovered several new classes of proteins among the transferred Sfps. Most intriguing were six members of the odorant binding protein (Obp) family [33]. Obps are thought to shuttle small molecules through aqueous solutions by binding them in a small, hydrophobic pocket; they are traditionally associated with the olfactory nervous system [34]. We confirmed that these Obps are transferred in seminal fluid by performing MS on protein digests from dissected accessory glands and by confirming each gene’s expression in the male reproductive tract with FlyAtlas [27].

Positive Selection and Tandem Gene Duplication Drive the Evolution of Sfps

Reproductive proteins of diverse species often evolve under positive Darwinian selection, which may indicate involvement in a coevolutionary process such as sexual selection, sexual conflict, or host–pathogen recognition [2]. We used coding sequence alignments from the 12 *D. melanogaster* genomes project [35,36] to calculate the rates of nonsynonymous substitution (d_S) and synonymous substitution (d_N) for all Sfps for which an ortholog was identified (116 of the total 138). For each Sfp, we determined the whole-gene, pairwise d_N/d_S (ω) ratio between the *D. melanogaster* gene and an ortholog from a closely related species (Figure 1). By this conservative test, five Sfps showed evidence of adaptive evolution (ω > 1). However, prior studies have shown that when the whole-gene pairwise ω ratio exceeds 0.5, or when the nonsynonymous substitution rate (d_S) is elevated, there are often specific sites within the protein for which adaptive evolution can be detected with more sensitive methods [7,37]. Therefore, we used multiple species alignments to search for specific residues under selection for all genes with pairwise ω > 0.5 and/or pairwise d_N > 0.05. (We did not test all Sfps, in order to minimize the number of statistical tests.) We found evidence for adaptive evolution at specific sites for 16 of 36 proteins (Figure 1 and Table S3), including four proteins that were unidentified previously as Sfps. Nine of these tests for selection remained significant after applying a strict Bonferroni correction for multiple tests. These rapidly evolving proteins are attractive targets for future study.

Previous studies found that some predicted Sfps are clustered throughout the genome [21,24]. We examined the chromosomal locations of the transferred Sfps and found similar patterns. We defined a cluster as genes with start codons located within 10 kb of each other. We identified 19 clusters of 2–5 transferred Sfps, which contain one-third (46/138) of the detected Sfps (Figure 2A). For 17 clusters, all member genes are transcribed in the same direction, and 15 clusters contain genes that encode proteins with full-length homology to one another. Thus, most of the observed clustering can be attributed to tandem gene duplication. Four paralogous clusters contain at least one gene that was under selection in the sites analysis above. Previous work also found a dearth of Acps on the X chromosome. Consistent with this finding, the 13 transferred Sfps on the X chromosome were significantly fewer than would be expected by chance (χ^2 = 4.68, 1 degree of freedom [df], p = 0.03), given the proportion of annotated genes on the X.

One example of rapidly evolving tandem duplicates is the gene pair CG17472 and CG31680. Across five species, CG17472 has evolved adaptively, with 21.3% of sites predicted to be under positive selection (estimated ω = 3.36, PAML M8 versus M8a comparison: χ^2 = 15.38, 1 df, p < 0.0001). These duplicates flank a transposition hot spot and a third, pseudogenized copy of the locus (Figure 2B). Additionally, CG17472 has duplicated along the lineage leading to *D. simulans* and *D. sechellia* (Figure 2C). Examining the ω ratio on each branch of the phylogeny reveals a burst of positive selection on the CG31680 lineage immediately after duplication. Indeed, a branch model allowing for variable selective pressures along each branch (shown in Figure 2C) fit the data significantly better than a model with a uniform ω for all branches (χ^2 = 29.04, 14 df, p = 0.01).

Relative Quantification Confirms the Importance of the Newly Identified Sfps

We used our MS data to estimate the relative abundance of each Sfp in seminal fluid. By counting the number of spectra associated with each Sfp in a given experiment and standardizing by the length of the protein and the total number of Sfp spectra detected in the experiment, we calculated a normalized spectral abundance factor (NSAF) [38,39] for each protein, which could then be averaged across all experiments (Figure 3 and Table S2). Notably, NSAF values were positively associated with the number of biological replicates in which a protein was found (Figure 3). Several of the most abundant proteins were previously characterized Sfps, such as Acp62F (a protease inhibitor) and Acp70A (the sex peptide). However, several novel proteins were also in the top quartile for abundance, including Obp56f, Obp56g, and the tandem duplicate CG17472. Although these NSAF measurements are only approximate, these data provide the first proteomic-scale view of the relative amount of each transferred Sfp, which may be useful for selecting candidates for further investigation.

Comparative Proteomics Reveals Lineage-Specific Changes in Seminal Fluid Content

To examine the cross-species evolution of seminal fluid content, we used the predicted protein annotations of *D.
and D. yakuba [35,36] to repeat our mating experiments with a wild-type strain of each species (Figure S1). Of the 63 Sfps detected in all three species, 19 were not reported previously as seminal fluid components. For Sfps that were detected in only one or two species, we investigated whether these proteins could be called as either lineage-specific gene gain or loss events. Most of the proteins had identifiable orthologs in the other species; our failure to detect these proteins may be due to changes in expression patterns, sequence substitutions that render MS identification more difficult, changes in the amounts of proteins transferred at mating, or the lower number of replicates (two per species) performed for D. simulans and D. yakuba. However, our data identify 13 lineage-specific Sfps across the three species (Table S5). For example, in D. melanogaster, CG6289 (a predicted serine protease inhibitor) has duplicated to form the lineage-specific gene CG6663. Also, in D. yakuba, Acp76A (another serine protease inhibitor) has duplicated, and several other proteins appear to be either lineage-specific to D. yakuba or rendered nonfunctional in other species (Table S5).

Some proteins detected for D. simulans and D. yakuba lacked
annotated orthologs in *D. melanogaster*. For seven such proteins, we identified the syntenic region in *D. melanogaster* and performed reverse-transcriptase PCR (RT-PCR) to determine whether transcripts of the region were made. In five cases, we detected a transcript in *D. melanogaster* (see Table S5), and three of these putative loci were detected as proteins in *D. melanogaster* when searching for unannotated proteins in the *D. melanogaster* genome (see below). Curiously, one of these genes, which we have annotated as *Sfp53D*, showed male-specific expression in *D. yakuba* and male-biased expression in *D. simulans*, but no sex expression bias in *D. melanogaster* (data not shown). *Sfp53D* is therefore an example of the type of protein that would have been omitted from previous sets of predicted Sfps due to its lack of sex-specific expression.

**A Novel Proteomic Method Identifies Unannotated Reproductive Genes in *D. melanogaster***

Based on these results, we reasoned that other Sfps may not be annotated as genes in *D. melanogaster*, which would make them impossible to detect by searching mass spectra against the annotated proteome. To detect additional unannotated Sfps, we first constructed a six-reading frame translation of the *D. melanogaster* euchromatic genome, which produced >5.8 million potential open reading frames (ORFs). Then, to reduce computational search time, we applied the Hardklör algorithm [40] to predict which MS2 spectra from a *tudor* experiment came from male peptides containing only natural abundance isotopes. These spectra were searched against the six-frame database, and those that matched an ORF corresponding to an annotated protein were discarded. This procedure left 23 novel, putative ORFs that did not match any *D. melanogaster* gene annotation in FlyBase. For each putative ORF, we used rapid amplification of cDNA ends (RACE) and RT-PCR to confirm transcription of the region encoding the peptide and to define the full-length transcript. Through this method, we discovered 19 unannotated genes (Table S6; GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) accession numbers EU755332–EU755350), most of which showed no significant identity to the predicted proteins of the other sequenced *Drosophila* species.

All 19 proteins have predicted signal sequences for secretion; many are encoded by only one or two exons, and all produce short polypeptides (median length: 93 residues). Consistent with our clustering analysis, half of the genes were found in regions of the genome containing other annotated Sfps. Most of the novel proteins had no recognizable domains based on BLAST and structural homology searches, but we identified one C-type lectin and three enzyme inhibitors, including a putative protease inhibitor, *Sfp24Ba*. This protein was identified by three peptides, one of which is indicated in Figure 4A. *Sfp24Ba* is adjacent to another previously unannotated gene, *Sfp24Bb* (an apparent tandem duplicate), and lies 25 kb upstream of the gene that encodes a transferred protease inhibitor, *Acp24A4* (Figure 4B). Comparative structural modeling (Figure 4C) suggests that this protein is a Kunitz-type protease inhibitor. The discovery of these 19 new Sfp genes in a model system that has been studied for over a century and for which comparative genomic analysis is now straightforward underscores the limitations of both computational gene prediction programs
Discussion

Our study provides a proteomic-scale view of the transferred Sfps in *D. melanogaster*. While we confirmed that 75 predicted Sfps are truly transferred at mating, we also identified a total of 82 genes (63 already annotated, 19 newly discovered) previously unknown to encode seminal fluid products. By using data from the genome sequencing projects and by performing comparative experiments in *D. simulans* and *D. yakuba*, we identified many instances of positive selection, tandem gene duplication, and lineage-specific changes in seminal fluid content between species. Taken together, our experiments demonstrate how new proteomic methods can be combined with the vast amounts of genomic sequence data that are now available to encode seminal fluid products. By using data from the genome sequencing projects and by performing comparative experiments in *D. simulans* and *D. yakuba*, we identified many instances of positive selection, tandem gene duplication, and lineage-specific changes in seminal fluid content between species. Taken together, our experiments demonstrate how new proteomic methods can be combined with the vast amounts of genomic sequence data that are now available to encode seminal fluid products. By using data from the genome sequencing projects and by performing comparative experiments in *D. simulans* and *D. yakuba*, we identified many instances of positive selection, tandem gene duplication, and lineage-specific changes in seminal fluid content between species. Taken together, our experiments demonstrate how new proteomic methods can be combined with the vast amounts of genomic sequence data that are now available to encode seminal fluid products.
identification method was straightforward to perform, and because it was experimentally based, it offered automatic verification for the new genes and allowed us to immediately assign them to a specific biological process: male reproduction.

One striking result from these experiments is that seminal fluid content in D. melanogaster appears to be considerably more complex than was previously predicted. The Obp genes identified reproducibly and at high abundance by MS are particularly attractive targets for further characterization. One hypothesis for the origin of these reproductive Obps is based on the fact that Obps are a large, 51-member family in D. melanogaster [33]. If some members of this family were functionally redundant, selection on the regulatory and coding sequences of some Obps might have been relaxed, allowing them to be co-opted from an olfactory function into a male reproductive function. Indeed, several of the identified Obps show accessory gland–specific patterns of expression, while others are expressed in both the accessory glands and the head [27]. The function of these reproductive Obps remains to be determined; they may present odorants or pheromones to odorant receptors in the female reproductive tract or play some other role, such as transferring small molecules to the female to elicit a behavioral response. If some of the Obps interact with a receptor in the female tract, the Or10a odorant receptor is one possible target, since its expression is up-regulated in female reproductive tracts in response to the receipt of Sfps [44].

While the selective pressures driving the evolution of Sfps (and of reproductive proteins in general) remain unclear, the important roles of tandem gene duplication and positive selection in the evolution of Sfps are consistent with the predictions made by models of sexual selection/conflict [45]. If males are engaged in a coevolutionary chase with females, driven by sexual selection or sexual conflict, duplication of an Sfp locus could allow males to better adapt to a particular allele or paralog of a female receptor [45]. Indeed, gene Sfp locus could allow males to better adapt to a particular receptor, and male-female coevolution and conflict. The challenge ahead will be to apply the combination of genetic, biochemical, and evolutionary methods that have already yielded many insights into Drosophila reproduction to this novel collection of transferred proteins. Functional tests of individual Sfps are essential for understanding the causes of the dramatic post-mating changes in female behaviors. For example, several studies have used gene knockouts or RNA interference to identify the post-mating effects of specific Sfps [50–55]. Other experiments have associated naturally occurring variants in several Sfps with different measures of sperm competition [12,56]. We expect that both of these approaches will become more effective in the future, since they can now be targeted to those transferred Sfps identified here.

Materials and Methods

**Flies.** Fly stocks were maintained on standard media at 25 °C, except during isotopic labeling (see below). D. melanogaster stocks included a wild-type lab strain, Canton S, and the strain used for genome sequencing, y; en. To produce males, homozygous tudl bw sp females [30] were mated to either Canton S or y; en bw sp males, and male progeny were retained for use in mating experiments. The DTA-E stock was used to produce males lacking both sperm and main-cell accessory gland proteins [51]. D. simulans strain W89 and D. yakuba strain Tai6 were used in additional mating experiments.

**Isotopic labeling.** The isotopic labeling procedure followed a previously described method [29], with some modifications. Wild-type S. cerevisiae was grown to saturation in minimal media containing 2% glucose, yeast nitrogen base without amino acids and ammonium sulfate (Difco), and 15N-labeled ammonium sulfate (≥ 99% 15N-enrichment; Spectra Stable Isotopes). Yeast cells were pelleted, resuspended in a small volume of sterile water, and lyophilized. This dried yeast was then mixed with water to form a “heavy” (15N) yeast paste. Flies were isotopically labeled by allowing unlabeled females to lay eggs for 24–36 h onto an agar plate topped with a small amount of heavy yeast paste. Adults were then discarded, and eggs were allowed to develop to adulthood at 25 °C in a vial capped at the open end by the plate. Heavy yeast paste was added to the plate throughout development of the sole food and nitrogen source. Virgin females were collected over CO2 within 8 h of eclosion and stored in a separate vial with 15N yeast paste on an agar plate. Shotgun MS analysis of proteins from whole, first-generation 15N flies was used to confirm isotopic labeling. In parallel to 15N labeling, males of the strain to be tested were grown in standard vials. Males were collected while young (0–3 d old) and aged in isolation in a standard vial.

**Mating experiments.** We performed 12 total mating experiments: three biological replicates each of Canton S and tudor males, and two biological replicates each of DTA-E, D. simulans, and D. yakuba males. For each experiment, males and females were aged to 2–5 d before mating. On the day before an experiment, approximately 40 labeled, virgin females were divided into three vials containing agar with a small amount of heavy yeast paste. Unlabeled males, in a ≥ 1.5-fold excess relative to females, were placed into three standard vials. The next day, males were transferred to the female vials without anesthesia. Mating was allowed to proceed for 2 h; vials were inspected several times during this period to confirm that copulations occurred. At the end of the mating period, flies were sexed over CO2, males were discarded, while females were kept on ice and immediately dissected in 50 mM ammonium bicarbonate. The lower female reproductive tracts were retained and stored in cold ammonium bicarbonate, while ovaries were excluded to prevent saturating the protein sample with the highly abundant egg yolk proteins. (If ovaries had been included, a greater fraction of peptides identified by MS would have arisen from these female proteins, making it more difficult to detect peptides from lower-abundance male Sfps.) It is unlikely that the removal of the ovaries diminished our ability to detect certain Sfps, as we identified all five Sfps.
D. melanogaster samples, the proteome was taken from the version 4.3 release of the contaminants (or their shuffled counterparts). For proteins, made by randomly shuffling the amino acids in each protein D. simulans and names were later updated to version 5.2). For D. yakuba the GLEANR protein predictions from the 12-genome controls were performed by using template from cDNA reactions that lacked reverse transcriptase. Only euchromatic genes were considered and plotted, such that plots in Figure 2A do not indicate, for example, centromeric heterochromatin. Clusters were defined as genes that were within 10 kb of each other. For proteins encoding a given cluster of eight or more genes we used simulations to determine whether the proteins showed evidence of paralogy. We used simulations to estimate a null distribution of the number of clusters that would be expected for a set of 138 genes distributed across the chromosomes in the same ratio as our Sfps. We extracted coding sequence annotations from http://www.flybase.org/ (version 5.2) and noted the location of the start codon for each gene (one isoform per locus). We then generated 1,000 sets of 138 genes by randomly selecting genes from each chromosome arm in the same ratio as in our Sfps. The expected number of clusters was counted; the median was 3, and the range was 0–9 clusters. Therefore, we judged our observed 19 clusters to be significantly more than would be expected by chance.

RT-PCR. Several GLEANR-predicted proteins identified in D. simulans and D. yakuba lacked annotated orthologs in the version 4.3 release of the D. melanogaster genome. In particular, D. simulans dsim_10234 was annotated as CG12828, and another is reported [64] in GenBank under accession number BK003861, but is not yet recorded in FlyBase). We thus tested whether these genes (GLEANR numbers: dsim_2017, dsim_3417, dsim_15912, dsim_10234, dyak_792, dsim_95, dyak_11419, dyak_12348, and dyak_10591) were expressed in D. melanogaster and showed sex-specific expression. PCR primers were designed to amplify transcripts in both the species of identification and the syntenic region of D. melanogaster. Although several of these proteins were encoded by short, single-exon genes, primers were designed to span putative introns when possible. Total RNA was prepared from whole male and whole female flies of both species using the TRIzol reagent (Invitrogen) and subjected to rigorous DNase treatment using the Turbo DNase kit (Ambion). First-strand cDNA from each sex was synthesized using the SuperScript III kit (Invitrogen) according to the manufacturer’s instructions. This cDNA was then diluted and used in PCR reactions. As a positive control, we assayed for transcription of ribosomal protein L32 (RpL32) using previously published primers [12], modified as needed for D. simulans and D. yakuba. Negative PCR controls were performed by using template from cDNA reactions that lack reverse transcriptase.

Evolutionary analyses. For each D. melanogaster protein identified, we used coding-sequence alignments generated by the 12-species genome sequencing projects [35,36] to conduct molecular evolutionary analyses. We preferentially used the more recent Fuzzy Reciprocal BLAST-based alignments of D. melanogaster coding sequences with orthologs in any other species (ftp://ftp.flybase.net/ genomes/12_species_analysis/clark_eisen/alignments); however, less than half of our Sfps were included in this set, so for the others we used the comparative assembly freeze 1 (CAF1) GeneMapper alignments produced by S. Coates and colleagues. From these sources, we were able to analyze 116 of the 138 annotated Sfps from D. melanogaster. We first made pairwise estimates of dS/dK, with model M0 of codeml in the PAML package [65]. When available, we used the D. simulans ortholog; otherwise, the D. sechellia ortholog was used. Alignments were obtained from one of the above sources and checked by eye using MEGA 4.0 [66]. For genes with pairwise dS/dK ≤ 0.5 or dS ≥ 0.05, we expanded our PAML analysis to up to five species (melanogaster, simulans, sechellia, yakuba, and erecta) in order to search for specific sites likely to have evolved under positive selection. For each gene, we considered sites for which there was no evidence of positive selection, and coding sequence alignments were checked by eye and edited in MEGA 4.0. We then tested for positive selection by comparing the likelihoods of codeml models M8 and M8a with a likelihood ratio test [67]. In model M8a, each codon is assigned to one of 11 classes, ten of which are satisfied by the codon frequencies derived from the data using maximum likelihood, and the 11th of which has α = 1, representative of neutral evolution. Model M8 differs in that the 11th class of codons can take any α value; this value is estimated from the
performed 5 primers in the genomic regions matching the identified peptides and matching annotated protein coding genes were discarded, leaving identifications were filtered by DTASelect. Identified peptides against the six-frame translation database using Sequest [62], and sample, this filtering reduced the number of spectra that needed to produce a tryptic peptide due to a lack of a K or R residue. This filtering reduced the data set to produce a tryptic peptide due to a lack of a K or R residue. This filtering reduced the data set to 5.8 million ORFs were generated. We filtered these ORFs to exclude numbers EU755332–EU755350.

**Discovery of new, unannotated proteins.** To identify unannotated Sfps, we first used wrapframe (D. States, unpublished program) to make a six-frame translation of the entire Berkeley *Drosophila* Genome Project *D. melanogaster* genome, version 5 (downloaded from ftp://hgdownload.cse.ucsc.edu/goldenPath/dm3/chrmosomes). This program translates genomic DNA in all six reading frames; each reported ORF ends with a stop codon (but does not necessarily start with a methionine). Across the four *Drosophila* chromosomes, over 7.6 million ORFs were generated. We filtered these ORFs to exclude those that contained only one type of amino acid (mono-residue repeats), those that were too short to be confidently used in MS spectrum identification (<11 residues), or those that could not produce a tryptic peptide due to a lack of a K or R residue. This filtering reduced the data set to 5.8 million ORFs. For searching this large computationally advantageous to filter the MS2A files in order to reduce the search time. We used data from three technical replicates of a *D. simulans* MS data by counting the number of spectra that positively identified each protein in a given MS run. This spectral count was normalized for the length of each protein and divided by the sum of all normalized counts for the entire MS run to produce an NSAF for each protein, as previously described [38,39]. This value was then averaged across all experiments in which a protein was detected, and identified proteins were ranked by their mean NSAFs. This rank should be interpreted as how common it was to identify ionizable and detectable spectra for a given protein, relative to the other unannotated proteins.

**Phylogenetic analyses of orthologs.** Both *D. melanogaster* and *D. sechellia* were used as outgroups for ortholog identification. First, detected unannotated genes, which were then confirmed with RT-PCR and sequencing of cDNA from whole males. SignalP was used to predict whether each novel protein is secreted [70], and we used BLAST and PHRYE [71] searches to determine whether any protein had sequence or structural homology to other proteins.

**Supporting Information**

Figure S1. Comparison of Sfps Detected in *D. melanogaster, D. simulans,* and *D. yakuba*

Table S1. Proteins Identified in Two DTA-E Mating Experiments

Table S2. High-Confidence Transferred Sfps Detected in Canton S and tudor Experiments

Table S3. Multiple-Species PAML Analyses of Sfps with Elevated dS/dD in Pairwise Comparisons

Table S4. High-Confidence Transferred Sfps Detected in *D. simulans* and *D. yakuba*

Table S5. Lineage-Specific Gene Gain and Loss Events for Proteins Detected in Only One or Two Species Analyzed

Table S6. Novel *D. melanogaster* Genes Identified by a Six-Frame Translation Database Search and Confirmed by RACE and RT-PCR

**Accession Numbers**

Sequence data has been deposited in GenBank under accession numbers EU755332–EU755350.

**Acknowledgments**

We thank B. Frewen, M. Hoopmann, and E. Hsieh for assistance with MS experiments and data processing and M. Dean, three anonymous reviewers, and the Swanson Lab for comments on the manuscript. C. Berg, M. Wolner, H. Malik, and the Bloomington Stock Center provided fly lines.

**Author contributions.** GDF, MJM, and WJS conceived and designed the experiments. GDF and XY performed the experiments. GDF and XY analyzed the data. GDF, MJM, and WJS contributed reagents/materials/analysis tools and wrote the paper.

**Funding.** These experiments were supported by National Institutes of Health (NIH) grants to WJS (HD057974, HD42563, HD054563) and MJM (DK069386, RR011823), an NIH training grant to GDF (T32 HG009535), and National Science Foundation grant DEB-0741671 to WJS.

**Competing interests.** The authors have declared that no competing interests exist.

**References**

1. Poiani A (2006) Complexity of seminal fluid: a review. Behav Ecol Sociobiol 60: 289–310.
2. Clark NL, Augaard JE, Swanson WJ (2006) Evolution of reproductive proteins from animals and plants. Reproduction 131: 11–22.
3. Swanson WJ, Vasquez VD (2002) Reproductive protein evolution. Annu Rev Ecol Syst 33: 161–179.
4. Coyne JA, Orr HA (2004) Speciation. Sunderland (Massachusetts): Sinauer Associates. 454 p.
5. Martin OY, Hosken DJ (2003) The evolution of reproductive isolation through sexual conflict. Nature 423: 979–982.
6. Brascall WE, Andrews JA, Maroja LS, Harrison RG, Howard DJ, et al. (2006) Identification and comparative analysis of accessory gland proteins in *Orthoptera*. Genome 49: 1069–1080.
7. Clark NL, Swanson WJ (2005) Pervasive adaptive evolution in primate seminal proteins. PLoS Genet 1: e35. doi:10.1371/journal.pgen.0010035
8. Collins AM, Caperna TJ, Williams V, Garrett WM, Evans JD (2006) Proteomic analyses of male contributions to honey bee sperm storage and mating. Insect Mol Biol 15: 541–549.
9. Fujimoto N, Akimoto Y, Suzuki T, Kitamura S, Ohts S (2006) Identification of prostatic-secreted proteins in mice by mass spectrometric analysis and evaluation of lobe-specific and androgen-dependent mRNA expression. J Endocrinol 190: 795–803.
10. Dottorini T, Nicolaides L, Ranson H, Rogers DW, Crisanti A, et al. (2007) A genome-wide analysis in *Aedes aegypti* mosquitos reveals 46 male accessory gland genes, possible modulators of female behavior. Proc Natl Acad Sci USA 104: 16215–16220.
11. Clark AG, Begun DJ, Prout T (1999) Female x male interactions in *Drosophila* sperm competition. Science 285: 217–220.
12. Finnema AC, Dumont BL, Clark AG (2005) Sperm competitive ability in *Drosophila melanogaster* associated with variation in male reproductive proteins. Genetics 169: 245–257.
13. Clark AG, Aguade M, Prout T, Harshman LG, Langley CH (1995) Variation in sperm displacement and its association with accessory gland protein loci in *Drosophila melanogaster*. Genetics 139: 189–201.
14. Imhof M, Hart B, Brem G, Schlotterer C (1998) Multiple mating in wild *Drosophila melanogaster* revisited by microsatellite analysis. Mol Ecol 7: 915–917.
15. Chapman T, Liddle LF, Kalb JM, Wolfer M, Partridge L (1995) Cost of
mating in Drosophila melanogaster females is mediated by male accessory gland products. Nature 373: 214–215.

Fowler K, Partridge L (1989) A cost of mating in female fruit flies. Nature 338: 760–761.

Kuiper B, Stewart AD, Rice WR (2006) The cost of mating rises nonlinearly with copulation frequency in a laboratory population of Drosophila melanogaster. J Exp Biol 19: 1795–1802.

Rice WR (1996) Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. Nature 381: 755–757.

Swanson WJ, Clark AG, Waldrip-Dail HM, Wolfner MF, Aquadro CF (2001) Evolutionary EST analysis identifies rapidly evolving male reproductive proteins in Drosophila. Proc Natl Acad Sci U S A 98: 7575–7579.

Wolfner MF, Harada HA, Bertram MJ, Steelk TJ, Kraus KW, et al. (1997) New genes for male accessory gland proteins in Drosophila melanogaster. Insect Biochem Mol Biol 27: 825–834.

Ravi Ram K, Wolfner MF (2007) Seminal influences: Drosophila Acps and the molecular basis of sperm competition between males and females during reproduction. Integr Comp Biol 47: 427–445.

Begun DJ, Lindfors HA, Thompson ME, Holloway AK (2006) Recently evolved genes identified from Drosophila yakuba and Drosophila erecta accessory gland protein families. Genetics 172: 1675–1681.

Wagstaff BJ, Begun DJ (2005) Comparative genomics of accessory gland protein genes in Drosophila melanogaster and D. pseudoobscura. Mol Biol Evol 22: 818–832.

Holloway AK, Begun DJ (2004) Molecular evolution and population genetics of duplicated accessory gland protein genes in Drosophila. Mol Biol Evol 21: 1625–1628.

Dorus S, Busby SA, Gerike U, Shabanowitz J, Hunt DF, et al. (2006) Dorus S, Busby SA, Gerike U, Shabanowitz J, Hunt DF, et al. (2006) Integrative gene annotation and transcriptional profiling approach towards genome-scale proteomics of membrane proteins. Nat Biotechnol 19: 242–247.

Chintapalli VR, Wang J, Dow JAT (2007) Using FlyAtlas to identify better Drosophila melanogaster models of human disease. Nat Genet 39: 713–720.

Ravi Ram K, Ji S, Wolfner MF (2005) Fates and targets of male accessory gland proteins in mated female Drosophila melanogaster. Insect Biochem Mol Biol 35: 1059–1071.

Krugsveld J, Ketting RF, Mahmoudi T, Johansen J, Artal-Sanz M, et al. (2005) Metabolic labeling of C. elegans and D. melanogaster for quantitative proteomics. Nat Biotechnol 23: 927–931.

Booswell RE, Mahowald AP, Clark AG (2007) Associations between sperm competition and natural variation in male reproductive genes on the third chromosome of Drosophila melanogaster. Genetics 178: 1605–1614.

Aagaard JR, Xi YH, MacCoss MJ, Swanson WJ (2006) Rapidly evolving zona pellucida domain proteins are a major component of the vitelline envelope of abalone eggs. Proc Natl Acad Sci U S A 103: 17392–17397.

Washburn MP, Wolters D, Yates JR (2001) Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat Biotechnol 19: 242–247.

Wu CC, MacCoss MJ, Howell KE, Yates JR (2005) A method for the comparative proteomic analysis of membrane proteins. Nat Biotechnol 23: 552–558.

McDonald WH, Tabib DL, Sadygov RG, MacCoss MJ, Venable J, et al. (2004) MSI, MS2 and SQT - three unified, compact, and easily parsed file formats for the storage of shotgun proteomics data. J Am Soc Mass Spectrom 5: 976–989.

Klannmer AA, Wu CC, MacCoss MJ, Noble WS (2005) Peptide charge state determination for low-resolution tandem mass spectra. Proc IEEE Comput Syst Bioinform Conf: 175–185.

Bennett-Lovsey RM, Herbert AD, Sternberg MJ, Kelley LA (2008) Exploring the extremes of sequence/structure space with ensemble fold recognition in the Drosophila genome. Genome Biol 5: R3.

Yang Z (1997) PAML: a program package for phylogenetic analysis by maximum likelihood. CABIOS 13: 555–556.

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol Biol Evol 24: 1596–1599.

Swanson WJ, Nielsen R, Yang QF (2003) Pervasive adaptive evolution in mammalian fertilization proteins. Mol Biol Evol 20: 18–20.

Felsenstein J (2005) PHYLIP - Phylogenetic Inference Package, version 3.6. Evolutionary Analysis of Continuous and Discrete Characters. Sinauer Associates, Sunderland, Massachusetts.

Yang ZH (1998) Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. Mol Biol Evol 15: 568–573.

Ravi Ram K, Sirot LK, Wolfner MF (2006) Predicted seminal astacin-like protease is required for processing of reproductive proteins in Drosophila. Proc Natl Acad Sci U S A 103: 18674–18679.

Swanson WJ, Wong A, Wolfner MF, Aquadro CF (2004) Evolutionary expressed sequence tag analysis of Drosophila female reproductive tracts identifies genes subjected to positive selection. Genetics 168: 1457–1466.

Hayashi TI, Vose M, Gavrilovs S (2007) Genetic differentiation by sexual conflict. Evolution 61: 516–529.

Lawniczak MK, Begun DJ (2007) Molecular population genetics of female-expressed mating-induced serine proteases in Drosophila melanogaster. Mol Biol Evol 24: 1941–1951.

Chapman T, Bangham J, Vinti G, Seifried B, Lung O, et al. (2003) The sex peptide of Drosophila melanogaster: Female post-mating responses analyzed by using RNA interference. Proc Natl Acad Sci U S A 100: 9923–9928.

Fiumera AC, Dumont BL, Clark AG (2007) Associations between sperm competition and natural variation in male reproductive genes on the third chromosome of Drosophila melanogaster. Genetics 178: 1605–1614.

Wigby S, Chapman T (2005) Sex peptide causes mating costs in female Drosophila melanogaster. Curr Biol 15: 316–321.

Herdon LA, Wolfner MF (1995) A Drosophila seminal fluid protein, Acps2aA, stimulates egg growth in females for 1 day after mating. Proc Natl Acad Sci U S A 92: 10114–10118.

Mueller JL, Linklater JR, Ravi Ram K, Chapman T, Wolfner MF (2008) Targeted gene deletion and phenotypic analysis of the Drosophila melanogaster seminal fluid protease inhibitor Acps62F. Genetics 168: 1605–1614.

Fonseca AC, Dumont BL, Clark AG (2007) Associations between sperm competition and natural variation in male reproductive genes on the third chromosome of Drosophila melanogaster. Genetics 178: 1605–1614.

Filion DJ, Findlay GD, Yi X, MacCoss MJ, Swanson WJ (2007) Duplication and selection on abalone sperm lysin in an allopatric population. Mol Biol Evol 24: 2081–2090.

Yang ZH (1998) Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. Mol Biol Evol 15: 568–573.

Emanuelsson O, Brunak S, von Heijne G, Nielsen H (2007) Locating protein families in the cell using TargetP, SignalP, and relabeled tools. Nat Protocols 2: 953–971.

Bennett-Lovsey RM, Herbert AD, Sternberg MJ, Kelley LA (2008) Exploring the extremes of sequence/structure space with ensemble fold recognition in the program Phyre. Proteins 70: 611–625.