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The *Drosophila* seminal proteome and its role in postcopulatory sexual selection

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Postcopulatory sexual selection (PCSS), comprised of sperm competition and cryptic female choice, has emerged as a widespread evolutionary force among polyandrous animals. There is abundant evidence that PCSS can shape the evolution of sperm. However, sperm are not the whole story: they are accompanied by seminal fluid substances that play many roles, including influencing PCSS. Foremost among seminal fluid models is *Drosophila melanogaster*, which displays ubiquitous polyandry, and exhibits intraspecific variation in a number of seminal fluid proteins (Sfps) that appear to modulate paternity share. Here, we first consolidate current information on the identities of *D. melanogaster* Sfps. Comparing between *D. melanogaster* and human seminal proteomes, we find evidence of similarities between many protein classes and individual proteins, including some *D. melanogaster* Sfp genes linked to PCSS, suggesting evolutionary conservation of broad-scale functions. We then review experimental evidence for the functions of *D. melanogaster* Sfps in PCSS and sexual conflict. We identify gaps in our current knowledge and areas for future research, including an enhanced identification of PCSS-related Sfps, their interactions with rival sperm and with females, the role of qualitative changes in Sfps and mechanisms of ejaculate tailoring.

This article is part of the theme issue 'Fifty years of sperm competition'.

1. Introduction

When females mate with two or more males (polyandry), and the ejaculates of the different males overlap spatially and temporally, the potential for postcopulatory sexual selection (PCSS) arises [1]. Here, we use the term PCSS to specify sperm competition and cryptic female choice (CFC), as is common usage [2]. Selection typically favours male adaptations that provide an advantage in sperm competition, because the carriers of those traits sire more offspring. PCSS also generates the opportunity for females to choose among the sperm of different males after mating (CFC; [3]), which in turn creates selection that favours male ejaculate traits that are preferred by females. While much of the research on PCSS has focused on behavioural and sperm traits, sperm require the support of seminal fluid for optimal fertility [4–6]. Seminal fluid composition is a key player in PCSS across a broad range of taxa (reviewed in [7–10]).

Drosophila melanogaster is an extremely powerful genetic model for studying seminal fluid molecules and their functions [11]. Although females have a sexual refractory period after mating, they are polyandrous and typically remate before they completely deplete their sperm stores. This results in the mixing of sperm from rival males and the potential for PCSS [12,13]. *Drosophila melanogaster* therefore represents an ideal system for dissecting the role of seminal fluid molecules in PCSS [7,14].

Here, we focus on seminal fluid proteins (Sfps) and their roles in PCSS. We recognize that other types of molecules in the seminal fluid also play important roles, but we do not attempt to cover them here. We first synthesize the current data to provide a comprehensive list of known and candidate *D. melanogaster* Sfps. To explore the generality of the *D. melanogaster* seminal proteome as a model, we compare to another well-characterized seminal proteome, that of humans, and examine similarities and differences between the species. We then review the experimental data for Sfp functions in PCSS and explore the representation of these Sfps among those that show similarities to human Sfps. Finally, we suggest key future research directions for the field.

2. The *D. melanogaster* seminal proteome

Seminal fluid proteins are the non-sperm proteins in the ejaculate. They are synthesized in the male's accessory glands (which contain secretory main and secondary cells), ejaculatory duct, ejaculatory bulb, testes and seminal vesicles [15–20]. Identification of Sfps ultimately requires demonstrating their transfer from males to females, which is a non-trivial experimental task. Early Sfp identification focused on genes whose expression is exclusive to—or highly enriched in—the male accessory glands and had predicted secretion signal sequences (e.g. [20–27]; electronic supplementary material, figure S1). A proteomic analysis on the reproductive tract of females, following matings to isotopically labelled, spermless males, has since provided a much higher throughput identification of additional Sfps [18]. This approach identified male-derived transferred proteins, while excluding female-derived proteins and sperm proteins. An additional high-throughput approach used quantitative proteomics to identify additional Sfps, by finding proteins that deplete in the male and increase in the female reproductive tract after mating [16].

(a) Characterization of the *D. melanogaster* seminal proteome

To establish the best current estimate of the complete set of *D. melanogaster* Sfps, we combined data from those past studies to generate a centralized database of the *D. melanogaster* seminal proteome using a specific set of criteria that we developed (electronic supplementary material, table S1). We provide a conservative 'high-confidence' list of 292 proteins based on convincing biochemical and bioinformatic data (electronic supplementary material, data S1). We also provide a second 'candidate' list of 321 proteins that either (i) have the potential to be Sfps based on expression data, but for which we lack evidence of transfer to females; (ii) fall into predicted functional categories that suggest intracellular 'housekeeping' functions (i.e. they are involved in cytoskeletal structure, transcription, translation and cellular trafficking); or (iii) were defined putatively as transferred Sfps but are also in the sperm proteome [28] (E Whittington, A Singh, S Pitnick, MF Wolfner, S Dorus 2020, unpublished data). More work is required to establish whether or not proteins in this candidate list are Sfps. A full explanation of our categorizations is given in electronic supplementary material, table S1.

The 292 high-confidence Sfps fall into predicted functional classes described by previous studies. The largest categories include proteases, protease inhibitors, redox-related proteins, immunity-related proteins and lipid metabolism-related proteins (functional classes predicted by FlyBase; electronic supplementary material, figure S2), though 115 Sfps have as-yet-unknown molecular functions. High-confidence Sfps are significantly enriched in gene ontology (GO) terms associated with these molecular functional classes, and—as expected—with biological processes such as reproduction, mating behaviour and regulation of female receptivity (determined using ClusterProfiler [29]; electronic supplementary material, figure S3). Candidate Sfps have a very different set of enriched GO terms, consistent with the idea that they are mostly not Sfps, and instead represent proteins that have other functions within the male reproductive tract (electronic supplementary material, figure S4). Although most high-confidence Sfps for whom the site of synthesis is known are made in the male accessory glands, the site of synthesis is not known for most Sfps [15,16] (electronic supplementary material, figure S5). Of the high-confidence Sfps, 95% contain predicted signal peptides, suggesting that a minority rely on alternative mechanisms of secretion, or may be transferred to females in exosomes or as a consequence of whole-cell delamination from the accessory gland [30,31].

(b) Comparisons between the *D. melanogaster* and human seminal proteome

Even when relatively few Sfps were known, striking similarities in Sfp functional classes between *D. melanogaster* and human were apparent [32]. However, given the rapid evolution of many Sfp primary sequences in *Drosophila* [20,33,34] and more broadly across taxa [10,35,36], we might expect relatively little similarity between specific Sfp genes of distant animal taxa. Now that we have more comprehensive seminal proteomes for these species (and other taxa [10]), we can revisit the comparison between *D. melanogaster* and human Sfps. We used DIOPT v. 8 (http://www.flyrnai.org/diopt; [37]) to examine overall amino acid similarity between *D. melanogaster* Sfps and all human proteins. This approach applies a voting score derived from 18 orthology-finding tools to determine which *D. melanogaster* proteins best match human proteins. We then determined which proteins were in the human seminal plasma proteome, using a recent database consisting of 2146 proteins [38] (out of a full all-tissue human proteome of 20 050 proteins [39]).

One hundred and sixty-three (57%) *D. melanogaster* Sfps show at least some evidence of similarity (high-, moderate- or low-ranked by DIOPT) to 444 human proteins, while 135 *D. melanogaster* Sfps were 'high' or 'moderate' hits to 279 human proteins (electronic supplementary material, data S2). Note that among these hits are duplicates, whereby multiple *D. melanogaster* Sfps match to a single human protein, or vice versa. We focused on the high- and moderate-ranked hits for further analyses, because these represent higher confidence matches. We found that 89 *D. melanogaster* Sfps had high/moderate hits to 110 known human Sfps. This represents a strong over-representation of human Sfps among the *D. melanogaster* hits to the human proteome, almost three times higher than expected based on chance (proportion test; $\chi^2_1 = 244.2$, $p < 0.001$; electronic supplementary material, table S2). An additional 10 hits (from 16 *D. melanogaster* Sfps) represent human proteins that are not currently listed in the human seminal plasma, but have known or likely roles in

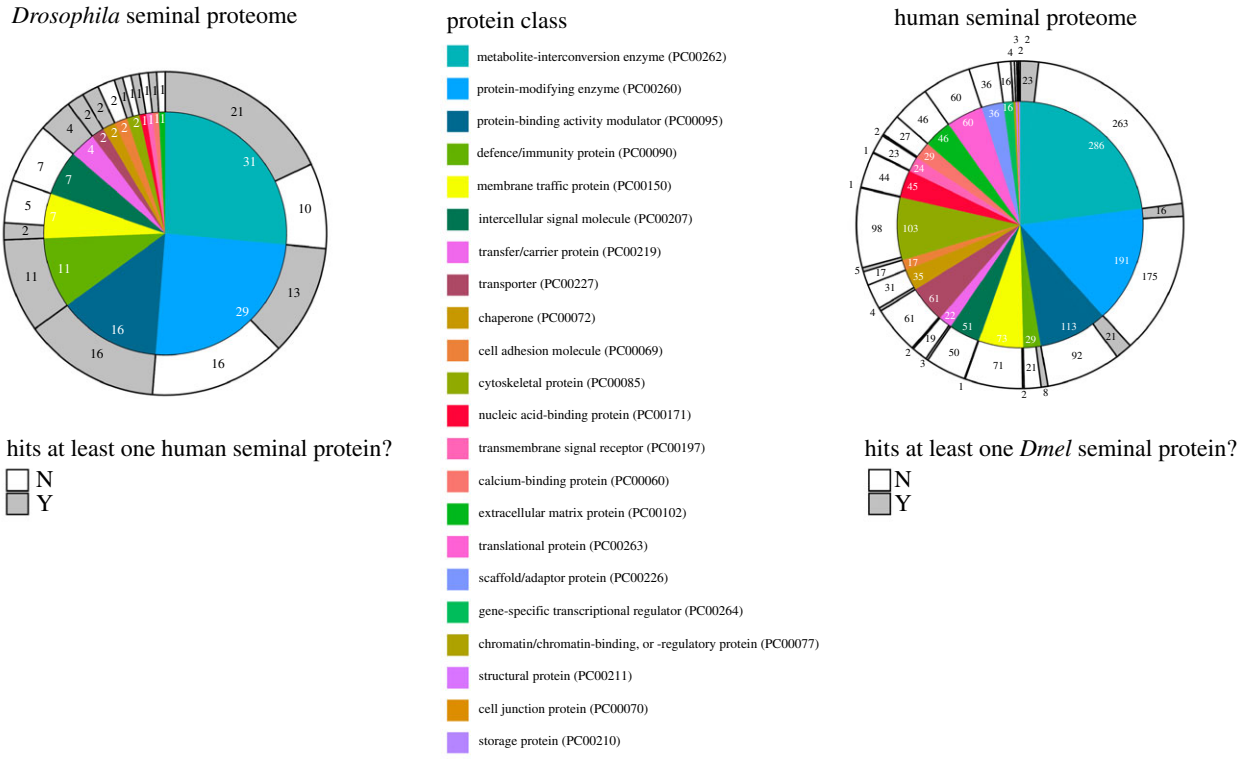

Figure 1. Protein class categorization of *D. melanogaster* and human seminal proteomes using PANTHER [41]. Classes in the pie charts are ordered the same as in the legend, clockwise from the top. Values in each segment indicate the number of Sfps classed in that category. The grey and white outer donut indicates the number of Sfps that have high or moderate DIOPT hits, respectively [37] to the other species.

reproduction or reproductive tissues, based on Uniprot functional information [40].

Therefore, 30% of *D. melanogaster* Sfps show high or moderate similarity to human Sfps. This could indicate cases of orthology or paralogy, or perhaps rare cases of convergent evolution, between *D. melanogaster* and human Sfps. However, distinguishing between these possibilities is beyond the scope of this paper. The representation and enrichment of protein classes is similar between *D. melanogaster* and human (using PANTHER [41], figure 1; using ClusterProfiler. Ten of 14 significantly over- or under-represented *D. melanogaster* Sfp classes show the same significant differences in human Sfps, and 13 of 14 show at least trends in the same direction (using PANTHER [41]; table 1). This supports previous findings of shared functional requirements of Sfps in species spanning insects to mammals [32]. Thus, a deeper understanding of *Drosophila* Sfps could provide insights that are broadly relevant to other species, including mammals.

3. The role of *D. melanogaster* seminal fluid proteins in postcopulatory sexual selection

Two features of *D. melanogaster* Sfps indicate their likely involvement in PCSS. First, female responses to the receipt of Sfps often support the male's competitive paternity success: Sfp receipt regulates storage and release of sperm, females show decreased interest in mating with other males, and females increase egg production and ovulation (reviewed extensively in [7,11,42]). Second, many Sfps, both in *Drosophila* and other taxa, also show considerable interspecific diversity, many evolve rapidly, driven by positive selection, and they show high turnover between species. These are all features expected of genes under strong sexual selection [34–36,43–46].

Laboratory assays of differential paternity outcomes between males are straightforward in *D. melanogaster*, simplified by the availability of visible markers that permit the determination of the relative paternity by males of different phenotypes that mate with a single female [47,48]. Furthermore, reciprocal matings allow the determination of P1 and P2 of the same male genotype, which are measures of first male paternity (sperm 'defence') and second male paternity (sperm 'offence'), respectively [49]. The availability of *D. melanogaster* lines with GFP- or RFP-labelled sperm also allows the unravelling of PCSS mechanisms via direct quantification of rival male sperm storage and sperm utilization within the female reproductive tract [13,50]. These methods and tools have allowed researchers to uncover roles of Sfps in PCSS and to dissect the relative roles of males and females [51,52]. The main approaches to ascertaining the role of Sfps in paternity share or sperm dynamics have been (i) association studies, which examine correlations between the traits of interest and natural variation in Sfp alleles, and (ii) functional genetic studies, involving the genetic manipulation of Sfps, or the cells and tissues that produce them. Note that these approaches have distinct strengths and caveats: association studies do not definitively prove that a particular Sfp directly influences PCSS, because a correlated third factor could be the cause. Meanwhile, functional genetic studies do not establish whether variation among males in the Sfp being studied alters PCSS outcomes. As such, the approaches provide complementary information about the potential role of Sfps in PCSS, and the evidence should be taken together. Moreover, it is important to note that our knowledge is based heavily on laboratory studies. While frequent remating

Table 1. Significantly over- and under-represented *D. melanogaster* Sfp classes, and matched human Sfp classes, determined using PANTHER [41]. *p*-values are FDR (false discovery rate)-corrected and values <0.05 are italicised. We have indicated whether the direction and significance of enrichment matches between the species.

PANTHER protein classes	*D. melanogaster* over/under	*D. melanogaster* fold enrichment	*D. melanogaster* FDR-corrected *p*-value	human direction same? yes/no	human significance same? yes/no	human over/under	human fold enrichment	human FDR-corrected *p*-value
protease inhibitor (PC00191)	+	11.33	*0.000*	yes	yes	+	4.5	*0.000*
defence/immunity protein (PC00090)	+	16.8	*0.000*	no	no	–	0.73	0.217
protease (PC00190)	+	2.99	*0.000*	yes	yes	+	2.48	*0.000*
lipase (PC00143)	+	8.02	*0.000*	yes	no	+	1.49	0.493
serine protease (PC00203)	+	4.13	*0.000*	yes	yes	+	2.23	*0.000*
hydrolase (PC00121)	+	3.17	*0.000*	yes	yes	+	2.65	*0.000*
protein-binding activity modulator (PC00095)	+	2.81	*0.006*	yes	yes	+	1.88	*0.000*
protein-modifying enzyme (PC00260)	+	1.81	*0.038*	yes	yes	+	1.71	*0.000*
apolipoprotein (PC00052)	+	13.33	*0.042*	yes	no	+	1.68	0.523
gene-specific transcriptional regulator (PC00264)	–	<0.01	*0.002*	yes	yes	–	0.15	*0.000*
nucleic acid-binding protein (PC00171)	–	0.09	*0.004*	yes	yes	–	0.52	*0.000*
DNA-binding transcription factor (PC00218)	–	<0.01	*0.004*	yes	yes	–	0.14	*0.000*
RNA-binding protein (PC00031)	–	0.11	*0.041*	yes	yes	–	0.36	*0.000*
transporter (PC00227)	–	0.19	*0.045*	yes	no	–	0.88	0.616

and mixed paternity has been ascertained from wild females [12,53], our understanding of PCSS processes under natural conditions remains limited.

(a) Population genetic associations between seminal fluid proteins and paternity share

An initial study demonstrated associations between variation in paternity share among wild-derived *D. melanogaster* lines and alleles of several accessory gland-derived Sfps: *ovulin* (*Acp26Aa*), *Acp29AB*, *Acp36DE* and *Acp53Ea* [49]. This suggested that natural populations contain variation for Sfp-mediated PCSS processes, upon which selection could potentially act. Subsequent studies using broadly similar approaches identified additional Sfps (e.g. *Acp33A*, *Acp62F*, *CG6168*, *CG14560*, *CG8137* and *sex peptide* (*Acp70A* or *SP*)) whose variation is associated with P1 or P2 [54–57]. (Note that knockout studies have failed to find an effect of *ovulin* on P1 or P2 ([58]; MA White and MF Wolfner 2020, unpublished data), suggesting that its association with paternity share [49,54] may result from linkage disequilibrium with a gene that influences PCSS such as the closely linked gene *Acp26Ab*, or perhaps with natural variants that are gain of function.)

A pressing question is why such variation exists at all; one might expect alleles that associate with high paternity to eliminate less successful ones. However, the complexities both within and between animals mean that there may be several optima. There is non-transitivity in genotypic effects on paternity share due to at least two conditions. First, the paternity success of a given male genotype can depend on the genotype of his mate, i.e. male A outcompetes male B when they mate with female X, but A loses to B when they mate with female Y [59]. For example, specific variants of the male Sfp *SP* and its receptor in females (*SPR*) strongly interact to mediate P1 and female remating rates [56]. Second, the relative success of a male genotype can depend on the genetic background of the rival males with which he is competing: i.e. male A outcompetes male B, and male B outcompetes male C, but male C outcompetes male A, analogous to a 'rock–paper–scissors' game [60,61]. Three additional factors add to the complexity: (i) apparent epistatic interactions among some Sfp alleles on paternity share (e.g. between *Acp62F* and *Acp76A*) contribute to the considerable complexity [55]; (ii) pleiotropic effects on paternity share of some Sfp alleles that affect other post-mating responses, such as female refractoriness and fecundity (described in the next section); and (iii) in the wild, there may be myriad environmental factors that interact with Sfps in mediating sperm success, so that relative successes of competitors become highly condition-dependent. These considerations suggest that there may be no single 'best' allele (or allelic combination) across every combination of male and female types, thus maintaining Sfp variation within populations.

(b) Functional genetics of postcopulatory sexual selection

Genetic disruption of accessory gland development results in a near or complete failure of those male's mates to produce offspring, primarily due to deficiencies in the transport of sperm into, or release of sperm from, female storage organs [62–64]. Genetic removal of some individual Sfps can similarly cause considerable reductions in sperm storage, and thus fertilization success, even in the absence of competition [65,66]. Deficiencies like these would generally be expected to place males at a severe disadvantage in PCSS.

An example of an Sfp that is required for both non-competitive fertility and paternity share under competition is Acp36DE. This is a glycoprotein in seminal fluid and the mating plug that had previously been linked to paternity outcomes via genotype association [49] and is required for proper sperm entry into storage in mated females [65,66]. Males null for the *Acp36DE* gene display reduced P1 and P2, presumably because the poor initial storage of their sperm results in a smaller paternity share relative to rivals [67].

It is possible that other Sfps required for non-competitive fertility could also lose paternity share under PCSS. For example, knockdown of *PEBme*, an Sfp that is derived from the ejaculatory bulb and contributes to the mating plug, also results in fewer sperm than normal getting stored in mated females [66]. Based on the results described above for Acp36DE, we would predict that PEBme-lacking males should suffer low P1 and P2, although this has not been tested to date. The Sfp lectin Acp29AB, which was previously associated with paternity outcomes [49], mediates efficient retention of sperm in storage. Thus, males lacking this protein have a low P1, presumably because their mates retained fewer sperm to compete with the second male's sperm [68]. However, consistent with Acp29AB's role in sperm retention, males that lack it have normal P2, presumably reflecting that normal numbers of sperm from the mutant males can enter storage, and displace a prior male's sperm.

Some genetic manipulations that remove Sfps, or otherwise alter the composition of the seminal proteome, counterintuitively improve male P1. For example, males null for *SP* (SP^0) display higher P1 [64,69]. This can be explained by SP's function in facilitating the release of sperm from storage: mates of SP^0 males lay and fertilize fewer eggs, and more sperm are retained than normal [64]. Having more sperm present in the female's storage organs should, all else being equal, give the first male a competitive boost in defence against incoming rival sperm [13]. However, the reduced egg laying of their mates lowers SP^0 male reproductive success prior to female remating. Moreover, females mated to SP^0 males remate more readily [70,71], meaning that the male's sperm encounters competition sooner. These effects typically offset the reduction in P1, meaning that the normal transfer of SP is net beneficial to males under most conditions [69,72,73].

Removal of several other Sfps, including some that modulate SP activity (CG9997, lectin-46Ca (also known as CG1656), lectin-46Cb (also known as CG1652) and CG17575; [74–76]), or disruption to the secondary cells that make some Sfps [77,78] also increases P1 [79] (but see also [80] which found the opposite pattern for CG9997). Again, these effects are due to the over-retention of the manipulated male's sperm and are accompanied by a decreased rate of oviposition following a single mating, an effect that would likely hamper male fitness under normal conditions. Curiously, adult-specific inhibition of BMP signalling in male accessory gland secondary cells [30] alters the seminal proteome and boosts P1, but does not alter oviposition rates [30,81]. In this case, females mated to these genetically manipulated males appear to release fewer sperm per fertilized egg, again resulting in the over-retention of sperm [81]. However, mates of these males remate much more quickly [30,81], meaning that the net effect of the

manipulation would again likely harm male fitness. Removal of the Sfp protease inhibitor Acp62F from males has a similar P1 effect, but the mechanism for this is unknown [82].

(c) Quantitative variation: evolved and plastic allocation of seminal fluid proteins

So far, we have focused on variation in Sfp gene sequences and the presence/absence of Sfps, but variation can also be quantitative: the amount of Sfps males make and transfer can vary between species, populations and individuals, or even within individuals across different contexts, and can have important consequences for male and female reproduction [10,83,84]. For example, males that mate repeatedly in quick succession become depleted of Sfps, which can lead to male infertility even while sperm continue to be transferred. This suggests that, in the short term, Sfp supplies can be limiting in *D. melanogaster* [85] (a pattern also seen in bed bugs [86]).

Laboratory evolution studies represent a powerful approach to exploring Sfp quantity variation and PCSS. For example, artificial selection for large accessory glands led to significantly increased levels of the Sfp SP but not of ovulin, revealing separable, selectable variation for Sfp quantity [87]. Furthermore, experimental evolution under enforced random monogamy—which removes all aspects of sexual selection, including PCSS—led to reductions in male competitive paternity share, and reduced RNA expression levels of many Sfp genes, including some with known PCSS roles (*Acp29AB*, *Acp36DE*, *SP* and *Acp62F*) [88]. This suggests that PCSS favours higher expression of many Sfp genes relative to mating systems that lack PCSS.

In PCSS situations, males also display plastic changes in the production and/or transfer of Sfps (as well as sperm). Strategic allocation of sperm in response to the risk of PCSS is well established both in theory and empirical studies across a range of taxa [89,90]. Sperm production is predicted to be costly [91], so males of many polyandrous species, including *D. melanogaster*, can adjust sperm numbers in response to their local social environment, boosting sperm numbers when there is a high risk of PCSS [92,93]. Similar principles could apply to Sfps, which might also be energetically demanding to make [10]. Several studies have shown that male *D. melanogaster* can alter Sfp production and transfer in response to their social environment [87,93–96]. For example, quantitative proteomics revealed that Sfp production and transfer peaked when males encountered many rival males, relative to 1 or 0 rivals, which were associated with improved oviposition stimulation in their mates [93]. Sfp gene expression can also change: reduced RNA levels of *ovulin* and *Acp62F* have been found in males exposed to rivals [94]. The latter data are difficult to reconcile with the results of studies that quantify Sfps at the protein level, but may reflect the existence of important post-transcriptional regulation [97–99], or they may simply reflect strain differences, or differences in experimental design. In any case, males display modulation of Sfp production and transfer in response to perceived PCSS, which may represent an adaptive strategy to maximize reproductive returns from costly ejaculate investment and/or result from constraints imposed by resource limitation [100].

A further potential influence on Sfp allocation is the exploitation of a previous males' ejaculate [101]. If a single dose of an Sfp causes strong long-lasting effects that extend beyond the time when a female remates, then subsequent males do not need to transfer this Sfp (or as much of it); they can save their resources for future matings, or investment into other traits. In support of this idea, when mating with previously mated females, males transfer less ovulin, but normal amounts of SP [95]. The receipt of ovulin boosts egg production after mating by modulating neural connections in females in what appears to be a long-lasting way [102,103]. Although the SP receipt also has long-term effects on females, such as increasing refractoriness to remating [22,70,104,105], a second dose of SP from a subsequent male boosts female refractoriness [95]. The data suggest that second males may exploit the first male's ovulin investment, strategically saving their ovulin supplies for future matings, but still transferring normal amounts of SP. Consistent with the idea that one male's Sfps can help another, seminal fluid can boost the offspring production of rivals [106]. For example, the female receipt of Acp36DE from a subsequent male can improve the offspring production of an Acp36DE-deficient male [67], and SP can bind to a previous rival male's sperm and provide SP function to those sperm [107].

(d) Role of seminal fluid proteins in sexual conflict and female-mediated postcopulatory sexual selection

Sfps are central to conflicts between the sexes in *D. melanogaster* [7,42]. First, some Sfps can reduce female lifespan and fitness under certain conditions in the laboratory [72,108–113] (although whether this effect occurs in the wild is unclear). Moreover, male genotypes that have higher sperm defence (P1) tend to generate higher female mortality [114]. These findings are consistent with the idea that Sfps can harm females as a side-effect of functions that promote male reproductive success [42]. Females are expected to evolve resistance to male harm, which has potential to spark an evolutionary arms race between the sexes. If female resistance reduces the benefits to males of their trait (such as Sfps that boost paternity share), then this may, in turn, select for males with higher levels of their trait, resulting in increased harm to females, and so on [115].

PCSS also presents the opportunity for females to exert CFC between the sperm of rival males [3]. PCSS simultaneously creates an inevitable and insoluble postcopulatory conflict between the sexes, because one or more males will lose paternity after mating [116]. Unequivocally identifying CFC and its underlying mechanisms is experimentally challenging, but evidence is accumulating for a number of species [7,51,117]. A potential CFC mechanism in *D. melanogaster* is regulation by the female of the timing of ejaculate ejection. Ejection of the ejaculate (approx. 1–2 h post mating) terminates sperm storage and—if the female contains sperm from previous mates—sperm displacement. It can therefore affect relative paternity outcomes, such that delayed ejection benefits the current male's sperm [118]. The sperm mass also contains Sfps (such as Acp36DE; [119]), whose removal might also give the female more control over Sfp-mediated sperm dynamics. Neuronal pathways that control sperm ejection [120] or other aspects of sperm use, storage and retention [51] might represent targets for interference by Sfps to promote success of that male's sperm. Consistent with this idea, males whose secondary cell function has been manipulated transfer an altered seminal proteome, and when these males are the first of two males, their mates are slower to eject the sperm of second males [81].

Females could also exert CFC by altering how they process Sfps or the sensitivity of their receptors to them [7,9]. SP, ovulin

and Acp36DE undergo processing within the female reproductive tract after mating, for which other Sfps and unknown female factors can be required [27,105,121–125]. In some cases, Sfp processing is important for the Sfp's function (e.g. Acp36DE; [124]), but whether Sfp processing by the female can alter Sfp activity to benefit the female remains unknown. Nor do we know whether there is variation in these processes among individual females or populations, or whether it correlates with variation in paternity shares. Similarly, females could modulate Sfp receptor levels, production or activity. For example, oviduct expression of the *SPR* evolved in the *melanogaster* species group, coinciding with these species showing post-mating responses to SP [126,127]. It is unknown whether natural variation or plasticity in the SPR could allow females to alter their response to SP in order to bias paternity.

In summary, there is clearly great potential for *D. melanogaster* females to use molecular interactions with male Sfps in their exertion of CFC [7]. To date, there are no studies that unequivocally differentiate between Sfp-mediated CFC and sperm competition in this species. However, it is important to note that the criteria that are generally accepted for demonstrating CFC tend to be more stringent than those applied to claims of sperm competition. Nonetheless, the combination of sperm competition, CFC and an ongoing antagonistic arms races with females, may ultimately be responsible for the rapid evolution and turnover of many Sfps in *D. melanogaster* and other taxa [10,35,128].

To test this idea—that PCSS drives evolutionary novelty in Sfps—we conducted further analysis of sequence similarity between *D. melanogaster* Sfps and human genes. We examined the set of *D. melanogaster* Sfps for which there is evidence of a role in PCSS from association studies or functional genetic approaches (and where there are reporting inconsistencies, we preferentially used the findings from functional genetic studies, e.g. ovulin, which shows associations with paternity share [49,54], was excluded due to negative results from functional genetic experiments ([58]; MA White and MF Wolfner 2020, unpublished data)). We tested what proportion of these PCSS Sfps, relative to the remaining *D. melanogaster* Sfps, shows sequence similarity to human genes from the DIOPT analysis. However, we found no evidence that PCSS Sfps differ from what would be expected by chance in their representation among the *D. melanogaster* Sfps that show similarity to human genes. This was true either when comparing across all hits (low-, moderate- or high-ranking), or in analyses restricted to high and moderate hits, or to hits specifically against human seminal plasma proteins (electronic supplementary material, data S2; all $\chi^2_1 < 2.3$, $p > 0.12$). Thus, our analysis does not suggest that Sfps which function in PCSS are any less likely to show sequence similarity to human genes in general, or to human Sfps. One possible explanation for this apparent lack of evolutionary novelty among PCSS Sfps is that many of the Sfps we labelled as playing a role in PCSS have additional functions. Thus, additional factors beyond PCSS likely shape their evolution and may, in some cases, result in conservation.

4. Conclusion and future research prospects

In conclusion, we now have a good understanding of the makeup of the *D. melanogaster* seminal proteome and the evolutionary dynamics of many Sfps. Seminal proteomes appear to retain protein class representation across species, pointing to some degree of shared functional requirements. There is an accumulating body of evidence demonstrating how *D. melanogaster* Sfps are involved in PCSS, derived from association studies, functional genetics and analysis of protein levels. Here, we suggest some important questions in the field to address in future research.

(a) Are there more seminal fluid proteins that impact postcopulatory sexual selection?

To date, we know 14 Sfps that have been implicated in competitive paternity outcomes, via genetic association studies, or through genetic manipulation, out of our best-estimate seminal proteome of 292 Sfps (electronic supplementary material, data S2). This small proportion might be because not many Sfps are involved in PCSS, though we suspect more likely it simply reflects the fact that we have a good understanding of the function of, at best 30 Sfps. As more population and functional genetic studies of Sfps take place in the future, we would expect to find more that have roles in PCSS. In particular, we might predict that recently evolved proteins, or those differentially expressed across species, represent prime candidates, due to sperm competition and CFC driving evolutionary novelty [33,35,129]. One further potential route to identifying good candidates is to apply evolutionary rate covariation approaches to already-known PCSS-mediating Sfps, a method employed to successfully target and identify novel SP-network Sfps [76].

(b) Can seminal fluid proteins target and harm rival sperm?

Currently, the individual Sfps understood to link to competitive paternity outcomes are thought primarily to influence the movement of the sperm transferred with them in or out of the sperm storage organs. But could Sfps influence PCSS by interacting with, and harming, rival male sperm directly? In social insects, there is evidence that sperm survival suffers in the seminal fluid of non-self males, suggesting that in those species Sfps might directly bias against rival sperm [130]: but there is no compelling evidence to date for the same in *D. melanogaster*. By contrast, SP can associate with rival sperm and restore its function, potentially benefitting the rival male [107]. There is also evidence that seminal fluid can promote the survival of self or rival sperm equally [131] (but see also [132]). A greater understanding of interactions between the Sfps of one male and the sperm and Sfps of rivals is sorely needed for a clearer picture of cooperation and conflict between rival ejaculates.

(c) Do qualitative changes in seminal fluid proteins play a role?

Our focus in this review was on the genetics and quantity of Sfps. However, Sfps could potentially vary in qualitative ways. For example, as males age some Sfps show evidence of qualitative changes associated with poor competitive fertilization success that might reflect post-translational modifications (PTMs), degradation or aggregation [132]. More broadly, inappropriate PTMs are of human biomedical interest as potential markers of infertility [38,133]. However, in *D. melanogaster*, we know almost nothing about the overall prevalence or function of Sfp PTMs. One hypothetical possibility is that males might be capable of strategically altering Sfp function via

PTMs in response to PCSS cues, in order to better compete against rival ejaculates when PCSS is high, or alternatively to minimize harm to females when competition is low, and male and female interests coincide. Another possibility is that varying PTMs might allow males to target Sfp effects to self or rival sperm.

(d) How do males tailor ejaculates to postcopulatory sexual selection?

Males appear capable of quite sophisticated alterations to the composition of their sperm and seminal proteome in response to the social environment, including cues of PCSS [87,92,93,95,96,100,134]. It appears that males use a mix of sensory cues to assess the presence of rival males, which ultimately result in altered reproductive investment [135–139]. At the other end, the diversity of male secretory cells and tissues [6] may provide ample opportunity for males to 'fine tune' the composition of their seminal proteome. However, the process by which males translate sensory information into changes in Sfp production and/or transfer is unclear. Possible mechanisms include the actions of neurons that control reproductive tissue activity [140], and/or steroid signalling. For example, ecdysteroids show quantitative responsiveness to social stimuli [141], and they are involved in secretory tissue, cell growth and Sfp expression [142–144]. They therefore represent a key candidate for mediating signals between the male sensory and reproductive organs.

(e) What female proteins interact with seminal fluid proteins, and how do they function?

Symptomatic of the sexual selection field more widely, we have a much better understanding of the roles of males than of females in PCSS. However, mating and reproduction are intricate processes that involve a carefully choreographed 'dance' between males and females both physically and at the molecular level. As long as our knowledge is biased towards one sex, it will be incomplete. To date, we only know one direct male–female molecular interaction: the male *SP* and its receptor in females, *SPR*, which can influence female remating rates and paternity outcomes [56,145]. We also know that *SP* can enter the female haemolymph when it can be cleaved by a trypsin [146]. A key challenge for the future is to identify other female receptors to male Sfps, and female reproductive proteins that modify or process them. Once we know what the female proteins are, we can use genetic approaches to assess their function, determine whether they influence sperm competition or CFC, and determine whether they participate in conflict or cooperation between the sexes by analysing their impact on male and female fitness.

Data accessibility. All data are included in the electronic supplementary material.

Authors' contributions. N.C.B., J.L.S., A.G.C. and M.F.W. compiled the *Drosophila* seminal fluid protein database. S.W. and I.S. conducted the fly–human seminal proteome comparison. All authors wrote the paper.

Competing interests. We declare we have no competing interests.

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