The Sexually Selected Ejaculate

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Abstract

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The ejaculate is composed of many different parts: proteins, lipids, and much else travels alongside sperm. With so many elements comes the problem of composition. Does the mix matter for male reproductive success? To what extent is the composition fixed? What happens when males lose control over the composition? It is with these questions that this thesis is principally concerned.

I start by testing whether males alter ejaculate composition in relation to the intensity of male-male competition. I find that divergent allocation rules govern the transfer of sperm and seminal fluid proteins ('SFPs'). While the allocation of both responds to competition, only SFP allocation responds to its intensity. I further show that variation between SFPs in their responsiveness leads to differentially-composed seminal fluid. The resulting ejaculate compositions are each accompanied by distinct costs and benefits for male reproductive performance, some of which appear specific to seminal fluid.

I next demonstrate that loss of BMP-regulated secretions from a seminal fluid-contributing cell-type ('secondary cells') imparts a syndrome of dysregulation on male and female reproductive performance. Within this, males lose the ability to reduce female receptivity to remating, but gain an advantage in defensive sperm competition. Through a systematic dissection of different episodes influencing sperm competition outcome, I find that loss of these secretions influences sperm entry into storage and potentially enhances their resistance to displacement.

In the following two chapters I show that loss of BMP-regulated secondary cell secretions and the keystone SFP sex peptide affects seminal fluid transfer to females. In both cases, I find clear signals of between-SFP dependencies in the regulation of SFP transfer, which collectively highlight novel mechanisms of seminal fluid organisation. I argue that these organising mechanisms may be used to a male's advantage to exercise fine-scale, real-time control over the transfer of ready-made seminal fluid.

Now, thin fruit flies like thunderstorms,
And thin farm boys like farm girls narrow;
And tax firm men like fat tax forms –
But time flies like an arrow.

When tax forms tax all firm men's souls,
While farm girls slim their boyfriends' flanks;
That's when the murd'rous thunder rolls –
And thins the fruit flies ranks.

Like tossed bananas in the skies,
The thin fruit flies like common yarrow;
Then's the time to time the time flies –
Like the time flies like an arrow.

Edison B. Schroeder

'This is a matter of how we prioritise the money that we spend… And where does a lot of that earmark money end up? It goes to projects having little or nothing to do with the public good – things like fruit fly research…'

Sarah Palin

'Die for adultery? No. The wren goes to 't, and the small gilded fly does lecher in my sight. Let copulation thrive.'

King Lear (Act 4, Scene 6)

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Declaration and author contributions

The work presented in this thesis is my own with the following acknowledgements.

Chapter 2 – Divergent allocation of sperm and the seminal proteome along a competition gradient in *Drosophila*

I conceived and designed the experiments along with Stuart Wigby and Irem Sepil. Sally Le Page, Jen Perry, Ellie Bath, Stuart Wigby, Irem Sepil, Thomas Miller, and James Craig helped monitor experimental matings. James Craig additionally aided with offspring counts in the reproductive decline experiment. Thomas Miller helped with male dissections as part of the accessory gland size change experiment. Marie-Laëtitia-Thézénas helped with the proteomic sample preparation, undertook the mass spectrometry, and supervised the quantitation. All analysis and experimental work other than that recorded above was undertaken by myself.

Chapter 3 – Seminal-fluid mediated sperm competition mechanisms revealed via manipulation of secondary cell BMP-signalling

I conceived and designed the experiments along with Stuart Wigby and Irem Sepil. Stuart Wigby, Irem Sepil, Jen Perry, Sally Le Page, James Craig, and Thomas Miller helped monitor matings. Thomas Miller helped with female dissections and performed the sperm counts for the first *UAS-Dad* mating experiment. All analysis and experimental work other than that recorded above was undertaken by myself.

Chapter 4 - Quantitative proteomics reveals division of labour between reproductive gland cell-types in Drosophila melanogaster

I conceived and designed the experiment along with Stuart Wigby and Irem Sepil. Sarah Bonham performed the proteomic sample preparation, mass spectrometry, and supervised the quantitation. All analysis and experimental work other than that recorded above was undertaken by myself.

Chapter 5 - Loss of *Sex Peptide* stimulates seminal fluid protein production and distorts the seminal fluid proteome in *Drosophila melanogaster*

I conceived and designed the experiments along with Stuart Wigby, Irem Sepil, and Clive Wilson. Stuart Wigby, Ellie Bath, and Irem Sepil helped monitor matings. Marie-Laëtitia-Thézénas helped with the proteomic sample preparation, undertook the mass spectrometry, and supervised the quantitation. Philip Charles performed the additional *Peaks* search and produced the individual peptide quantitation plot given in Supplementary Figure 2. All analysis and experimental work other than that recorded above was undertaken by myself.

Note on references

The referencing style from the journal *Evolution* is used consistently throughout this thesis. A single reference list is given at the end to save space and avoid duplication

Chapter 1

General Introduction

N.B. Sections included in this chapter are excerpts from two published pieces of work:

1. **Hopkins BR**, Avila FW, Wolfner MF (2018) Insect Male Reproductive Glands and Their Products. *Encyclopedia of Reproduction* (Elsevier), pp 137–144.
2. **Hopkins BR**, Sepil I, Wigby S (2017) Seminal fluid. *Curr Biol* 27(11): R404–R405.

DESIGNING AN EJACULATE

Irrespective of whether sperm are expelled into an aqueous medium in which they meet and fuse with ova ('external fertilisation'), or passed directly from one individual to another ('internal fertilisation'), we can refer to an 'ejaculate' - a sperm-bearing fluid, the molecular composition, structure, and functions of which vary across taxa. The existence of this variation and, more importantly, the correlation it often holds with features of organismal reproductive landscapes, raises the problem of how the ejaculate is designed (*sensu* Gardner 2009): how does selection, both natural and sexual, act to optimise the ejaculate?

The study of ejaculate design has historically followed one of two approaches. The first has used the framework of sperm competition, where sperm from rival males compete for access to ova, to develop and test theory relating to how males should allocate finite resources to ejaculate production as opposed to self-maintenance (reviewed in Parker and Pizzari 2010). The second has utilised molecular and genetic techniques to identify and functionally annotate components of the non-sperm containing portion of the ejaculate, the 'seminal fluid' (reviewed in Avila et al. 2011; Perry et al. 2013; Hopkins et al. 2017, 2018).

Although there are some exceptions, integrated treatments of how the ejaculate as a collection of interdependent parts evolves is lacking. And this lack of integration is

significant. Underdeveloped connections between these two bodies of work leads to conceptual deficiencies in each: when we focus exclusively on sperm, we neglect the pivotal contributions made by seminal fluid to fertilisation success (Cameron et al. 2007) and offspring fitness (Bromfield et al. 2014; Watkins et al. 2018). Conversely, we lack for seminal fluid composition an evolutionary framework that has anything near the explanatory value of that developed to explain the evolution of sperm number and ejaculate investment more broadly.

In this introduction, I explore the two distinct research streams in the wider context of a set of design considerations for the ejaculate. I outline the predictions and successes that have come from treating ejaculate components individually, such as how many sperm an ejaculate should contain and what those sperm should look like. As we move into discussion of the seminal fluid, I emphasise that reductive approaches to understanding the role of individual ejaculate components are inadequate due both to the interdependence of seminal fluid proteins ('SFPs') and the mediating effect of seminal fluid on sperm competition outcome. A complete picture of ejaculate evolution must, therefore, appreciate the importance of composition at its broadest.

To this end, I follow my treatment of ejaculate design considerations with an overview of work that has pushed towards a more holistic treatment of ejaculate evolution. Here, I include work that has tested theory developed for sperm allocation on non-sperm ejaculate components, such as SFPs (*e.g.* Wigby *et al.*, 2009; Fedorka, Winterhalter and Ware, 2011; Sirot, Wolfner and Wigby, 2011; Ramm *et al.*, 2015); work that has identified or proposed evolutionary forces acting on seminal fluid products (*e.g.* Wigby and Chapman 2005; Sirot et al. 2017; Chapman 2018); and work that has explored co-evolution between sperm and non-sperm components of the ejaculate (e.g. Cameron et al. 2007; Dhole and Servedio 2014).

DESIGN CONSIDERATION 1: EJACULATE INVESTMENT AND SPERM NUMBER

The framework of sperm competition is constructed on the notion that where females mate multiply (*i.e.* exhibit polyandry), ejaculates can overlap in time and space, thus extending the opportunity for sexual selection to operate beyond the act of mating (Parker 1970). Nearly half a century on from its original formulation, the theory has proven invaluable in explaining myriad elements of reproductive physiology, anatomy, and behaviour: peculiar genital morphologies (Waage 1979; Simmons and Jones 2007; Eberhard 2009), unconventional insemination techniques (Tatarnic et al. 2014), and substantial testes (Harcourt et al. 1981), to name but a few. The centrality of sperm competition to the diversification of life is suggested to have been so great that it may have contributed to transitions towards behavioural complexity and mobility (Parker 2014).

Much of the work on sperm competition has operated under the principle of ejaculate economics, which describes the economisation of ejaculate expenditure in the face of sperm competition (Parker and Pizzari 2010). In its focus on optimal investment, ejaculate economics is firmly rooted in the fundamental tenets of life-history theory: resources are finite and investment in one area comes at the expense of investment elsewhere (Stearns, 1992). Although the idea of ejaculate economics has matured over the past decade or two, its essence is captured in the early writings of Donald Dewsbury (Dewsbury 1982). Dewsbury contravened the prevailing view at the time that the costs of ejaculate production were trivial. To illustrate the perspective held by some of his contemporaries, consider Richard Dawkins' claim that 'the word excess has no meaning for a male' (Dawkins 1976 p. 176). There now exists considerable empirical work

demonstrating both the costs of spermatogenesis and the very real prospect of sperm depletion (Birkhead 1991; Preston et al. 2001).

The process of ejaculate economisation can be considered over two timescales. The first, evolutionary, wherein selection favours the level of expenditure that best fits the average reproductive and competitive context a male finds himself in (Parker and Pizzari 2010). The second acts within the lifetime of an organism. Where the reproductive context a male inhabits is variable - perhaps predictably so - males should gain by partitioning resources across mating events, prudently allocating when the return on investment is greatest (Wedell et al. 2002).

For much of the history of sperm competition research, ejaculate investment has been treated as synonymous with sperm number (Parker and Pizzari 2010), or, rarely, as an allocation budget to be split between sperm number and size (Parker et al. 1972, 2010). Accordingly, tests of prudent ejaculate allocation have often focused on the differential allocation of sperm across matings ('sophisticated sperm allocation') in relation to the reproductive context experienced by a male – a phenomenon now known to be taxonomically widespread (Wedell et al. 2002; Kelly and Jennions 2011). While its mechanistic basis is poorly understood, its existence demonstrates that males are able to control either or both of the rate at which sperm are produced (Moatt et al. 2014) and the number that are transferred in a given ejaculate (Garbaczewska et al. 2013).

The complement of conditions that influence male post-copulatory reproductive success (the 'reproductive context') are varied and many. The most general prediction made by sperm competition theory, however, is that males transferring greater numbers of sperm enjoy a numerical advantage over rivals (Parker et al. 1972). Empirical support for this prediction comes from intra-specific tests of competitive success in populations with known variation in sperm number (Gage and Morrow 2003) and comparative studies

of relative gonad size in relation to sperm competition proxies, such as mating system (Harcourt et al. 1981) or social group size (Hosken 1997).

However, the extent to which increased sperm number leads to increased success depends upon the process by which sperm from a pool of competitors are drawn for fertilisations. This consideration is captured in the language of the raffle. Under a 'fair raffle' sperm are randomly selected from a pool of sperm of mixed origin (Parker 1990). Accordingly, fertilisation success is directly proportional to the number of transferred sperm – the more tickets purchased, the higher one's chance of winning – and inversely proportional to the number transferred by competitors. Where the raffle is 'loaded', the relationship between sperm number and fertilisation success is weakened. Loading can occur if, say, there exists between-male variation in sperm fertilising ability (Parker and Pizzari 2010).

Simple models of sperm competition raffles consider how sperm of mixed origin are drawn from a stable pool, such as would be contained in a sperm storage organ (Parker and Pizzari 2010). However, the stability of this pool can vary and feed back to influence the reproductive context. To explain this, we can return to the language of the raffle. Imagine that the individual overseeing the raffle (the female) starts removing tickets, either at random or specifically targeting those purchased by particular individuals. Alternatively, other ticket holders (rival males) might start removing the tickets purchased by competitors. In these cases, the overseer may ultimately draw fairly (or unfairly) from the raffle, but only after the tickets have been manipulated following purchase. The physical structure of the environment in which sperm compete can influence this. When sperm are large and the female reproductive tract small (as in many invertebrates; Immler et al. 2011) space is constrained. In order to take in new sperm, those that are in residence may need to be dumped (Snook and Hosken 2004; Lee et al.

2015), used, or physically displaced by incoming sperm (Manier et al. 2010). Consequently, ejaculates should be selected to have both defensive and offensive capabilities.

Where there is displacement of resident sperm, male fertilisation success can be rendered predictably dependent upon mating order. Under such conditions, males fall into a 'favoured' or 'disfavoured' role (Simmons 2001; Cameron et al. 2007). In many insects and birds, the favoured role is occupied by the last male to mate, who consequently sires the majority of offspring. This feature is referred to as 'last male sperm precedence' (Laturney et al. 2018). If males can detect female mating status, they have access to more complete information regarding the reproductive context they face and should gain by adjusting their ejaculate transfer accordingly. For example, a male mating with a mated female faces a probability of encountering sperm completion that approximates to 1. Males should therefore increase their transfer of sperm when mating with a mated female, as has been shown to occur in *Drosophila melanogaster* (Lüpold et al. 2011).

So far, we have established that males face taxonomically and temporally variable reproductive contexts. These contexts dictate the optimal level of ejaculate investment, which, historically, has referred to the number or size of sperm produced by males, without considering the mediating effect of seminal fluid products. The context is itself dependent upon a suite of factors including (a) raffle fairness (*i.e.* the strength of the relationship between sperm number and competitive fertilisation success), (b) the stability of the selectable sperm pool (*i.e.* the extent to which third-parties may interfere with the sperm a male transfers), and (c) how competitive fertilisation success is distributed with respect to mating order. Two further features also bear influence on the reproductive context. Firstly, the 'risk' that sperm competition is encountered, which is defined as the probability that a female double mates (Parker et al. 1997). Under a fair raffle model,

ejaculate investment is predicted to increase in direct proportion to risk. However, the slope of this relationship decreases as the unfairness of the raffle increases (Parker et al. 1997). Across taxa, a general trend exists for males to plastically respond to cues that correlate with the risk of sperm competition, such as the presence of a rival male competitor (Kelly and Jennions 2011). These responses to risk can be accompanied by adaptive benefits in competitive matings (Bretman et al. 2009). A second feature is sperm competition 'intensity', which is reflected in the number of ejaculates simultaneously competing (Parker et al. 1996). At an inter-specific level, an increased number of competitors is predicted to lead to greater ejaculate investment (Parker et al. 1996). Within-species, males are predicted to show peak ejaculate investment in the presence of a single competitor, with investment subsequently declining with the introduction of additional competitors (Parker et al. 1996). The logic here is that, under a fair raffle situation, a male's fertilisation success is inversely proportional to the number of competing sperm present. Thus, at high competition, the marginal gains of elevating ejaculate investment are heavily depreciated and conservation of resources represents the optimal strategy.

DESIGN CONSIDERATION 2: SPERM MORPHOLOGY

Alongside the decision of how many sperm to transfer, an additional design consideration is the appearance of those sperm. Across taxa, sperm morphology is highly variable ranging from the immotile disk of *Eosentonon transitorium* (Simmons 2001), through the amoeboid form of *Caenorhabditis elegans* (Lüpold and Pitnick 2018), to the gigantic, but familiar, tadpole-like form of *Drosophila bifurca* (Bjork and Pitnick 2006). Some species, such as many in the order *Lepidoptera*, produce multiple morphologically and proteomically distinct sperm forms within a single ejaculate (Whittington et al. 2015).

The evolutionary forces driving the considerable diversification of sperm morphology are poorly resolved and evidence for a causative role of sexual selection is scant (Lüpold and Pitnick 2018). There are, however, exceptions. For instance, sperm competition is thought to have driven the evolution of hook-structures on the heads of North American deer mice (*Peromyscus maniculatus*) sperm, which allow for the formation of motile aggregates. Aggregation boosts the velocity of individual sperm and thus provide an advantage in sperm competition (Fisher and Hoekstra 2010). Apical sperm hooks are widespread among murine rodents and the degree of hook reflection correlates with the predicted intensity of sperm competition (Immler et al. 2007).

A further example of how sperm morphology is shaped comes from work on sperm size. Males face a decision as to how they should divide resources between sperm number and size (Parker et al. 1972). Models generally predict the evolution of numerous, tiny sperm (see preceding section) operating on the assumption that the benefits of increased sperm size can only manifest through (ultimately negligible) increases in zygote provisioning, which boost zygote viability (Parker et al. 1972). However, theory predicts that the allocation split can favour investment in size when the female reproductive tract is small relative to sperm size (Parker et al. 2010), a result supported by empirical studies of birds and *Drosophila* species (Immler et al. 2011). The benefits of large sperm in a small female reproductive tract have been further revealed through studies in *Drosophila.* Here, the length of the female primary sperm storage organ, the seminal receptacle (SR), sets a preference for particular sperm sizes: a lengthened SR elevates the advantage held by longer sperm in displacing resident sperm from storage (Lüpold et al. 2012). Such work illustrates how in internal fertilisers the female makes profound contributions to the design parameters driving ejaculate evolution.

DESIGN CONSIDERATION 3: SEMINAL FLUID COMPOSITION, STRUCTURE, AND PRODUCTION

Despite the explanatory success of sperm competition theory, it has for the most part made little attempt to integrate the wealth of mechanistic detail uncovered for the actions and design of seminal fluid. This is a major omission given that sperm seldom, if ever, travel alone. In addition to water, seminal fluid may encompass a rich diversity of lipids, proteins, carbohydrates, nucleic acids, free amino acids, water, hormones, vesicles, mucus, vitamins, microbes and, in some species, glandular cells (Gillott 2003; Poiani 2006; Avila et al. 2011; Perry et al. 2013; Hopkins et al. 2017). At a higher level, the overall structure of the seminal fluid is taxonomically variable. Firstly, the relative proportions of sperm to non-sperm differs across species. While boar and stallion ejaculates contain large volumes of seminal fluid, the sperm in bull ejaculates is at very high concentrations (Mcgraw et al. 2015). Secondly, the overall conformation of the ejaculate varies. For example, sperm may be free-swimming within a seminal fluid medium, as in humans and *D. melanogaster*, or transferred within a spermatophore, as in butterflies and springtails, where both sperm and non-sperm components are encased within a proteinaceous capsule (Zizzari et al. 2014; Meslin et al. 2017). Spermatophores may adopt complex, multi-layered conformations as in the rove beetle *Aleochara cutula* (Gack and Peschke 1994). In this species, the male secretes a 'tube-like structure' into the female sperm storage organ, which serves to guide the elongation of a secondary tube that will eventually inflate and burst to release sperm.

The task of producing seminal fluid falls to a collection of specialised accessory reproductive tissues (Chen 1984; Hopkins et al. 2018). Between species, accessory glands are highly variable in number, size, and identity. For example, whereas the seminal fluid of dogs is composed of secretions from the prostate, ampullary glands, and epididymis,

bulls and humans further draw upon contributions from their bulbourethral glands and seminal vesicles (Mcgraw et al. 2015). These glands can also display peculiar traits — the lifelong growth of the human prostate being the most familiar (Wilson et al. 2017). The diversity of insect accessory glands is even greater than that exhibited by mammals, ranging from Drosophila's two lobes to the house cricket's (*Acheta domesticus*) tangled mass of several hundred tubules (Chen 1984). Even within a single family, such as *Diptera*, there is considerable morphological diversity in the accessory glands (Throckmorton 1962), the adaptive significance of which is unclear.

Different reproductive glands, as well as different cell-types in these glands, work interdependently to produce the seminal fluid. For example, spermatophores in beetles, crickets, and moths are composed of layers derived from the secretions of different reproductive glands (Grimnes et al. 1986; Meslin et al. 2017). Among species within the suborder *Ensifera*, the 'rough glands' produce the larger spermatophylax component of the spermatophore, while the 'smooth glands' produce the ampulla, which bears the sperm (Lewis and South 2012). In the cabbage white butterfly (*Pieris rapae*), both the accessory glands and the distal section of the mating duct contribute to the soft inner matrix of the spermatophore, whereas the proximal region contributes most to the tough outer envelope (Meslin et al. 2017). In another case, the *D. melanogaster* mating plug includes contributions from at least the ejaculatory bulb and accessory glands (Lung and Wolfner 2001; Bretman et al. 2010b; Avila et al. 2015), with the accessory gland secretion itself being composed of products from two biochemically distinct cell-types, the 'main cells' and 'secondary cells' (Monsma et al. 1990; Bertram et al. 1992; Leiblich et al. 2012; Gligorov et al. 2013; Corrigan et al. 2014; Redhai et al. 2016). If and how these cells coordinate their activities to build an ejaculate is currently poorly understood.

DESIGN CONSIDERATION 4: SEMINAL FLUID PROTEIN FUNCTION

The protein component of the seminal fluid has been shown to be particularly diverse in all species studied so far. To date, nearly 100 SFPs have been identified as being transferred to females during mating in the yellow fever vector mosquito *Aedes aegypti* (Sirot et al. 2011a). In *D. melanogaster*, over 200 SFPs are known (Findlay et al. 2014; Sepil et al. 2018). The seminal fluid proteome of vertebrates is more diverse with some 1141 detected in the red junglefowl *(Gallus gallus)*(Borziak et al. 2016) and 2425 unique proteins described in human seminal plasma (Rolland et al. 2013). These SFPs encompass a range of molecular classes from antioxidants, lipases, and lectins to odorant-binding proteins, proteases and protease inhibitors (Gillott 2003; Ram and Wolfner 2007a; Findlay et al. 2008; Avila et al. 2011, 2015; Mcgraw et al. 2015); proteases are particularly diverse in both insect and mammalian seminal fluid (Mueller et al. 2004; Laflamme and Wolfner 2013). In *Drosophila* (and likely in other insects), SFPs' amino acid diversity is further supplemented by a variety of post-translational modifications, such as glycosylation, that may further influence their functions (Gligorov et al. 2013).

Roles of SFPs in females can be broadly categorized into those affecting behaviour, physiology, or anatomy. Behavioural modifications include changes in aggression levels (Bath *et al.*, 2017), feeding and dietary preferences (Carvalho *et al.*, 2006; Ribeiro and Dickson, 2010; Vargas *et al.*, 2010; Tsukamoto *et al.*, 2014; Walker, Corrales-Carvajal and Ribeiro, 2015), altered sleep patterns (Isaac *et al.*, 2010), and reduced receptivity to re-mating. This latter change may be short-term, just a few days in the Mediterranean fruit fly *Ceratitis capitate* (Miyatake et al. 1999), or permanent, as suggested by some studies of the dengue vector mosquito *Ae. Aegypti* (Craig 1967). In *D. melanogaster* females, a ~2-week reduction in receptivity post-mating is controlled by the SFP sex peptide (SP), which manifests in a set of specific rejection behaviours

(Connolly and Cook 1973; Bussell et al. 2014). Modifications of physiology by SFPs include induction of an inflammatory response (Robertson and Sharkey 2016), stimulation of oviposition (Perry and Rowe 2008), egg maturation (Jin and Gong 2001), neuromuscular stimulation (Rubinstein and Wolfner, 2013; Mcgraw, Suarez and Wolfner, 2015), broad-scale changes in gene expression (Gioti et al. 2012), and immunomodulation (Peng, Zipperlen and Kubli, 2005; Schwenke and Lazzaro, 2017). Transfer of SP to *D. melanogaster* females also induces endocrinal change (Moshitzky et al. 1996; Carvalho et al. 2006), which is in turn is associated with diminished female sex pheromone production (Bontonou et al. 2015) and vitellogenic oocyte progression (Soller et al. 1999). Anatomical changes include remodelling of the midgut (Cognigni et al. 2011; Apger-McGlaughon and Wolfner 2013; Lemaitre and Miguel-Aliaga 2013; Reiff et al. 2015) and conformational changes to the uterus and oviduct in *D. melanogaster* (Avila and Wolfner 2009; Mattei et al. 2015).

Seminal fluid components can further form novel structures within the female reproductive tract (Parker 1970; Meslin et al. 2017). These 'mating plugs' provide a physical barrier to the entry of sperm from rival males, and have been implicated in reducing female receptivity to remating (reviewed in Avila *et al.*, 2011). In some butterfly species, the mating plug includes an external component that covers the female copulatory opening. In contrast, the *D. melanogaster* mating plug sits within the posterior uterus at the distal end of the reproductive tract (Lung and Wolfner 2001). In this species, the mating plug is bipartite: the posterior portion is composed predominantly of ejaculatory bulb derived proteins, such as PEBme (a protein with some similarity to homopolymer-forming proteins in spider-silk), PEBII, and PEBIII. The posterior plug forms within the first 5-minutes of mating (Lung and Wolfner 2001; Bretman et al. 2010b; Avila et al. 2011, 2015), while the anterior section of the plug forms around 20-minutes

after the start of mating and is primarily composed of accessory gland-derived proteins such as Acp36DE (Bertram et al. 1996). Mating plugs also function in promoting sperm storage and retention. The seminal fluid of *An. gambiae,* for example, coagulates to form a mating plug in females and is required for the initial entry of sperm into the female storage organs (Giglioli and Mason 1966; Rogers et al. 2009).

DEISGN CONSIDERATION 5: EJACULATE INTERDEPENDENCE

While some SFPs have clear and distinct phenotypic effects in females, others seem to occupy a supporting role within broader networks. Work in *D. melanogaster* has revealed that these network proteins can, for example, function by processing other SFPs as they pass through the ejaculatory duct *en route* to the female (LaFlamme et al. 2012; Laflamme and Wolfner 2013; LaFlamme et al. 2014). Considerable research effort has been directed towards uncovering the actions of proteins that support the activities of SP within the female reproductive tract. While SP is able to induce physiological change in females on its own, its effects are extended in the presence of sperm (Manning 1962; David 1963; Manning 1967). This so-called 'sperm effect' arises through the binding of SP to both the head and tail of sperm via the N-terminus region of the peptide (Peng et al. 2005a), a process that depends upon the action of an interdependent network of SFPs (Ram and Wolfner 2007b, 2009; Findlay et al. 2014; Sitnik et al. 2016). These SFPs, which include CG1652, CG1656, CG9997, CG17575, antares, and aquarius, are variously required to promote the transfer, stabilisation, and processing of one another, and ultimately catalyse SP-binding (Ram and Wolfner 2009; Findlay et al. 2014; Singh et al. 2018). As well as some SFPs functioning in tandem, others show bear signatures of co-regulation via the expression of shared micro-RNA binding sites (Mohorianu et al. 2018). Collectively,

these relationships between ejaculate parts render the specific composition of seminal fluid important – loss of one component could have wide-ranging effects on others.

HOLISTIC EJACULATE EVOLUTION

A picture of ejaculate evolution that fails to account for the broad functions and interdependencies of seminal fluid components is conceptually incomplete. Holistic attempts to better characterise how the ejaculate evolves fall into one of three categories. The first takes theory developed for ejaculate investment or sperm number and tests it on non-sperm ejaculate components. Like with sperm, males have been shown to become depleted of SFPs with repeated matings and replenishment can take non-trivial amounts of time. In *D. melanogaster,* males transfer successively lower abundances of both SP and ovulin over consecutive matings (Sirot et al. 2009), which aligns with phenotypic data demonstrating the diminished post-mating responses of females mated to multiply-mated males (Linklater *et al.*, 2007). Accordingly, Wigby *et al.* (2009) used *D. melanogaster* to test whether the same theory that predicts prudent allocation of sperm applies to SFPs. Their ELISA-based methods detected significantly elevated transfer of two SFPs, SP and ovulin, in response to the presence of a rival male, a suggested predictor of sperm competition risk.

In addition to the sensitivity of SFP transfer to sperm competition risk, follow-up work demonstrated that males are sensitive to mating order (Sirot et al. 2011b). In this study, males were shown to transfer equivalent SP, but decreased ovulin, when mating with a mated rather than a virgin female. This study proved particularly significant for three reasons. Firstly, it demonstrated that males can alter the composition of their ejaculate in real-time in relation to the sociosexual context. Previous work left open the possibility that ejaculate plasticity simply operated through differential protein

production over longer time periods (Wigby et al. 2009; Fedorka et al. 2011). Secondly, it provided empirical support for a novel ejaculate design consideration that could not apply to sperm: ejaculate exploitation, wherein a second mating male can reduce his own investment in fitness-enhancing SFPs and parasitize the contributions of a female's previous mate (Hodgson and Hosken 2006). Thirdly, it revealed that males don't simply adjust the volume of seminal fluid they transfer (Wigby et al. 2009), but rather they can tailor the relative abundances of SFPs to drive compositional changes, a result consistent with SFP gene expression data (Fedorka et al. 2011). In keeping with this finding, recent proteomics work has revealed that exposure to cues of sperm competition risk induces broad-scale compositional change in the mouse seminal vesicle proteome (Ramm et al. 2015). Whether this translates into ejaculate compositional change remains untested.

A second approach to understanding holistic ejaculate evolution has sought to identify broader evolutionary forces outside of the ejaculate economics framework that may be acting on the non-sperm portion of the ejaculate. SFPs are among the fastest evolving genes in the *Drosophila* genome (Haerty et al. 2007) and individual SFPs show signatures of being acted upon by divergent evolutionary forces (Aguadé 1998). Sexual conflict, where male and female fitness optima diverge (Parker 2006), is thought to be a potent driver of seminal fluid evolution (Sirot et al. 2017). A number of studies in *D. melanogaster* have demonstrated toxic, life-shortening effects of individual SFPs on females (Lung et al. 2002; Wigby and Chapman 2005). The deleterious effects experienced by females are thought to represent collateral damage from adaptations that promote male reproductive success, particularly under competitive conditions. Indeed, experimental evolution work has demonstrated that polyandry leads to the evolution of seminal fluid that improves male competitive success, but that negatively impacts females (Rice 1996). The deleterious effect of seminal fluid on females is ameliorated when

females are allowed to co-evolve with males, demonstrating the multi-party coevolution between rival males and females (Rice 1996). Where lifetime monogamy is enforced, the fitness optima of male and female align. Accordingly, both male harm and female resistance to harm are reduced (Holland and Rice 1999). But even in polyandrous mating systems, ejaculate-female interactions will include cooperative elements, as evidenced by the combined molecular contributions made by male and female in the processing of ovulin (Park and Wolfner 1995) or the purported nutritional benefits of the edible portion of the spermatophores (Gwynne 1984). Furthermore, the level of conflict associated with SFP receipt may vary in time and space. For example, SP receipt by females oscillates between being antagonistic and beneficial in relation to the nutritional environment (Fricke et al. 2010).

Through sexual conflict theory, we can make predictions about how seminal fluid should evolve. For example, arms races between rival males and with females may lead to general changes in the expression levels of existing SFPs, the protein sequences of SFPs, or even diversification of the number of SFPs (Sirot et al. 2017). Indeed, it has been suggested that the functional redundancy observed among SFPs (*e.g.* 15 different trypsin-class proteases in *D. melanogaster* seminal fluid) may be a vestige of historical sexually antagonistic co-evolutionary arms races where females have evolved resistance to male adaptations (Sirot et al. 2017). More recent theory has drawn on the general phenomenon of the evolution of resistance (particularly in relation to antimicrobials, vaccines, and insecticides) to make predictions about how SFPs might evolve (Chapman 2018). While some SFPs seem to exert fairly specific effects (*e.g.* SFP cleavage by Semp1, LaFlamme *et al.*, 2014; Avila and Wolfner, 2017), others, notably SP, seem to act as 'master regulators' of post-mating change in females (Gioti et al. 2012; Chapman 2018). It has

been suggested that by simultaneously targeting many female responses, SFPs such as SP can slow the evolution of female resistance (Chapman 2018).

The final cluster of studies that mark an attempt to more holistically describe ejaculate evolution do so by exploring how different parts of the ejaculate co-evolve or co-vary in typical sperm competition games. Theory developed by Cameron *et al.* (2007) has shown that the rules governing sperm and seminal fluid allocation should diverge from one another in relation to mating role. In systems where the second-mating male is preferred and seminal fluid components boost female fecundity (as in *D. melanogaster,* Avila et al. 2011; Laturney et al. 2018), first mating males maximise fitness by transferring smaller ejaculates, with less seminal fluid, but more sperm. Second-mating males do best by transferring larger ejaculates with greater quantities of seminal fluid and fewer sperm. The logic is that fecundity-stimulating seminal fluid contributions from the first male disproportionately benefit a second male, and thus a first-mating male should retain resources for future matings. The predictions change when ejaculate components, either sperm or seminal fluid, influence the degree of bias in sperm competition outcome (Cameron et al. 2007). In a separate study, Dhole *et al.* allowed for seminal fluid to maintain multiple simultaneous functions and derived the optimal allocation split of resources between these distinct functions (Dhole and Servedio 2014). Their model recognises that the defensive and offensive ability of sperm can be separately mediated by distinct clusters of SFPs, as can fecundity. They found that the optimal ejaculate composition is set by the relative efficiencies of each product in carrying out its function and the relative costs associated with the production of each.

THESIS OVERVIEW

Having identified design principles influencing the evolution of ejaculate composition and argued for the importance of considering the ejaculate as a system of interconnected parts, the subsequent chapters are concerned with efforts to test the consequences and extent of compositional change in the ejaculate. In Chapter 2, I test for congruence in the rules governing plastic sperm and seminal fluid allocation in response to varying intensities of sperm competition. In Chapter 3, I detail a syndrome of phenotypic dysregulation that affects the reproductive performance of a male and his partner following the loss of a set of reproductive gland secretions. In Chapter 4, I take a quantitative proteomics approach to test how cellular dysregulation in a primary site of seminal fluid production affects ejaculate composition. In Chapter 5, I expose the interconnectedness of seminal fluid components by testing how the removal of a seminal fluid protein disorders the transfer of many others. Finally, I use Chapter 6 both to summarise the findings outlined elsewhere in this thesis and to highlight avenues for further work.

STUDY SYSTEM: DROSOPHILA MELANOGASTER

The model organism *D. melanogaster* provides an ideal system in which to test the importance of ejaculate composition. Firstly, due to its well-documented suitability to lab studies: rapid reproduction, short lifespan, and the low costs and relative ease associated with its husbandry. Secondly, a wealth of widely-used genetic tools are readily available, such as individual gene knockouts and toolkits for controlling the expression of specific genes in specific tissues (*e.g.* the UAS-GAL4 system). Thirdly, the seminal fluid proteome of *D. melanogaster* is relatively well-characterised in terms of its breadth (Wolfner et al. 1997; Findlay et al. 2008, 2014; Sepil et al. 2018) and the functions of its

constitutive elements (reviewed in Ram and Wolfner 2007a; Avila et al. 2011; Laflamme and Wolfner 2013; Hopkins et al. 2018). Fourthly, it has emerged as a model system for exploring plastic responses to rivals at the behavioural and ejaculate levels (see references in Bretman et al. 2011a; Perry et al. 2013). Fifthly, we have a good knowledge of the operation of sperm competition in this species (Gilchrist and Partridge 2000; Manier et al. 2010; Lupold et al. 2013). Sixthly, the broader action of sexual conflict and sexual selection within this species is well-studied (Bateman 1948; Rice 1996; Holland and Rice 1999; Wigby and Chapman 2005; Fricke et al. 2010). Finally, the existence of parallels in the fundamental biology of the fly accessory glands and human prostate offers the potential for broader applicability of any findings (Leiblich et al. 2012; Corrigan et al. 2014; Wilson et al. 2017).

Chapter 2

Divergent allocation of sperm and the seminal proteome along a competition gradient in Drosophila

TITLE

Divergent allocation of sperm and the seminal proteome along a competition gradient in *Drosophila*

ABSTRACT

Across taxa, males have been shown to strategically allocate sperm and some seminal fluid components to females in response to the presence of rival males. However, it is currently unclear whether sperm and seminal fluid show parallel responses or whether males exercise independent control over different parts of the ejaculate and thus alter its composition. To address this, we performed a comprehensive, integrated test of the allocation of different ejaculate components along a gradient of male-male competition intensities in *Drosophila melanogaster*. Using quantitative proteomics, we identify that males show significantly greater production and transfer to females of many seminal fluid proteins at high levels of competition. Moreover, we identify that SFPs differ in the directionality and sensitivity of their response, which leads to compositional change in the seminal fluid proteome. In contrast, we find using fluorescent-labelling that sperm allocation conforms to a distinct, intensity-independent allocation rule. By tracking the rate of male reproductive decline, we provide evidence that compositional changes in the ejaculate represent an adaptive response to sperm competition, but one that comes at a cost to future mating performance. Collectively, our work reveals a previously unknown divergence in ejaculate component allocation rules, highlights complex among-protein variation in competition sensitivity, and exposes downstream costs of elevated seminal fluid protein investment.

INTRODUCTION

Through repeated mating, males become depleted of sperm and seminal fluid (Lefevre and Jonsson 1962; Linklater et al. 2007; Sirot et al. 2009; Reinhardt et al. 2011). Depleted ejaculates are associated with reduced fertilisation success, particularly where males encounter sperm competition, the process of competition between rival male sperm for access to fertilisations (Preston et al. 2001). To ameliorate the negative effects of depletion, males are predicted to plastically partition ejaculate products across matings, strategically transferring them when the return on investment is greatest (Parker and Pizzari 2010). This phenomenon of 'strategic ejaculate allocation' has received cross-taxa support in empirical studies (Wedell et al. 2002; Kelly and Jennions 2011) - even human males produce higher-quality ejaculates in response to sperm competition cues (Kilgallon and Simmons 2005). Understanding when and how males alter ejaculate composition, both at the sperm and non-sperm level, must be a research priority given the current male fertility crisis (Levine et al. 2018), and now that it is apparent that non-sperm seminal fluid components influence fertility outcome (Rodríguez-Martínez et al. 2011; Mcgraw et al. 2015), female reproductive tract health (Robertson and Sharkey 2016), and offspring disease risk (Bromfield et al. 2014; Chen et al. 2016; Sharma et al. 2016; Watkins et al. 2018).

Historically, work on strategic ejaculate allocation has taken a narrow approach in four key respects. Firstly, both theoretical and empirical work has generally considered either how the number of sperm transferred changes or how investment in the ejaculate as a whole changes (Parker and Pizzari 2010). This approach neglects that ejaculates are composites of many functionally-important elements: lipids, nucleic acids, glandular cells, amino acids, extracellular vesicles, and proteins ('seminal fluid proteins'; 'SFPs') may be transferred to the female alongside sperm (Poiani 2006; Perry et al. 2013; Hopkins

et al. 2017). Whether different ejaculate components generally co-vary in their response is unclear, but some theory predicts not; instead sperm and seminal fluid should be subject to independent allocation rules (Cameron et al. 2007). Secondly, while studies often explore how the binary presence or absence of competition (sperm competition 'risk') affects some measure of the ejaculate, integrated tests of how investment changes along a gradient of cues are rarer. Such tests are important: theory predicts males should lower ejaculate investment with increasing competition (sperm competition 'intensity') as the marginal gains of elevated ejaculate investment decrease (Parker et al. 1996). However, empirical studies have provided mixed support for this (Kelly and Jennions 2011). Thirdly, although work has uncovered costs and benefits associated with responding to rivals (Bretman et al. 2009, 2013b), how these reflect patterns of compositional change in the ejaculate is unclear. Knowledge of the relative costs and benefits associated with individual portions of the ejaculate will be key to understanding how and when ejaculate plasticity evolves (Perry et al. 2013; Dhole and Servedio 2014). Fourthly, proxies for plastic ejaculate allocation, such as mating duration, are often employed when testing male responses to competition (e.g. Bretman et al. 2010a). While mating duration shows robust and clear co-variation with sperm competition cues, it is unclear if and how this actually translates into changes to the ejaculate components produced by males and transferred to females (Gilchrist and Partridge 2000; Lüpold et al. 2011; Bretman et al. 2012; Crickmore and Vosshall 2013).

Here, we take a multi-level approach, from protein to multi-mating reproductive success, to test these deficiencies in sophisticated ejaculate allocation theory in the fruit fly *Drosophila melanogaster*. We begin by using lines transformed to express fluorescently-labelled sperm to test for differences in how males allocate sperm along a spectrum of competition intensities. Next, we test whether the allocation pattern identified

for sperm also applies to seminal fluid proteins ('SFPs'). SFPs belong to a diverse range of molecular classes, make varied and male fitness-enhancing contributions to reproductive outcome, and are among the fastest evolving genes in the *D. melanogaster* genome (reviewed in Avila *et al.*, 2011; Hopkins, Avila and Wolfner, 2018). We test SFP allocation by performing quantitative proteomics on the accessory glands of virgin and mated males exposed to varying degrees of male-male competition. The accessory glands are the primary site of production for almost all of the >200 SFPs known to be transferred by males (Findlay et al. 2008, 2014; Sepil et al. 2018) and exhibit similarities to the human prostate (Leiblich et al. 2012; Wilson et al. 2017). Comparison of virgin and mated gland proteomes provides a deep and direct analysis of how males change both the production and transfer of SFPs (Sepil et al. 2018). Consequently, this approach pushes us beyond studies investigating changes in SFP gene expression (Fedorka et al. 2011; Simmons and Lovegrove 2017; Sloan et al. 2018), individual protein levels (Wigby et al. 2009; Sirot et al. 2011b) or those that rely on the assumption that what is produced translates into what is transferred (Ramm et al. 2015). Finally, we test whether differences in ejaculate composition manifest in distinct, detectable costs by measuring the rate of reproductive decline experienced by males over successive matings in relation to the intensity of male-male competition.

Our predictions follow the general trends revealed by meta-analysis (Kelly and Jennions 2011): (a) males exposed to competition will elevate sperm transfer relative to isolated males; (b) males at high competition will show no further increase in transfer. We predict that SFPs will follow the same response. Accordingly, males exposed to low and high competition intensities will experience the same set of costs and benefits associated with responding to competition.

RESULTS AND DISCUSSION

Sperm transfer responds to competition risk, but not intensity

We began by testing how males allocate sperm to females along a gradient of competition intensities. Specifically, males were held alone, in pairs, or in a group of eight. These groupings simulate no, low, and high competition, respectively. *D. melanogaster* males are known to transfer more sperm in response to the presence of competition (Garbaczewska et al. 2013), but there have been no previous tests of allocation along a competition gradient. By counting the number of sperm in newly-mated females, we found an overall effect of competition on sperm transfer ($F_{2,149}$=3.43, p=0.035; Fig. 1). Tukey-corrected post-hoc comparisons revealed that the difference between no and low competition males was significant (t_{149}=2.57, p=0.011), and that there was no significant difference between those held at low and high competition (t_{149}=0.786, p=0.712). Thus, males appear to respond to the binary presence/absence of competition when allocating sperm, supporting our initial prediction. Mechanistically, how elevated sperm transfer is achieved is unclear, but male *D. melanogaster* are known to produce greater sperm numbers in response to prolonged exposure to competitors (Moatt et al. 2014). However, greater production is unlikely to operate alone given that males are able to adjust sperm numbers if exposed to rivals only at the point of mating (Garbaczewska et al. 2013).

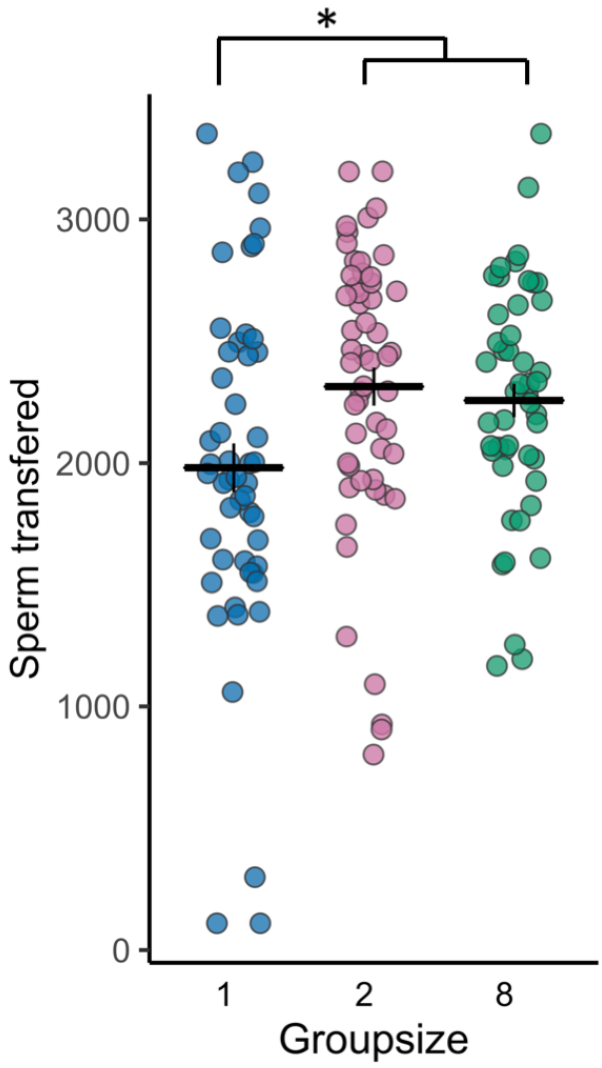


Figure 1. Sperm transfer is sensitive to the presence, but not intensity, of competition

The number of sperm in the reproductive tract of females flash-frozen 25-minutes after the start of mating to males held in groups of 1,2, or 8. Horizontal lines give the treatment mean with vertical lines giving ±1 SE of the mean. Sample sizes: N_1=51, N_2=54, N_8=48 pooled from two replicates. Asterisk denotes an overall significant effect of group size at p<0.05 level. The comparison between group size 2 and 8 is non-significant.

Intensity-sensitive, non-linear responses of the seminal fluid proteome to competition

To test whether the allocation rule we identified for sperm extends to other ejaculate components, we again grouped males into 1s, 2s, or 8s before applying quantitative proteomics to the accessory glands of males retained as virgins or frozen after mating. After excluding proteins with fewer than 2 unique peptides (as in Borziak *et al.*, 2016; Sepil *et al.*, 2018), we detected 1277 proteins in the accessory glands of which 118 were SFPs known to be transferred to females (see Methods). We began by performing a hierarchical clustering analysis to identify distinct patterns of abundance change within the SFP proteome in relation to both mating and competition intensity. We further included structural proteins to act as a protein outgroup. Three distinct higher-order clusters were identified (Fig. 2a). Cluster 3 was highly enriched for structural proteins (7 of 8 included), and showed no clear signal of virgin-mated difference. We therefore omitted it form further analysis.

In Cluster 1 and 2, the degree of difference in protein abundance between virgin and mated glands, which represents the quantity lost during mating, varied significantly with group size (LME, Mating x Group size: Cluster 1: $F_{2,350}$= 36.316, p<0.0001; Cluster 2: $F_{2,170}$=7.227, p=0.001; Fig. 2b). To our surprise, the pattern of change was for SFPs to be generally transferred in greater abundances at high, but not low, competition. Thus, the allocation of SFPs is dependent upon the intensity of competition, unlike the pattern identified for sperm, which was dependent upon the presence, but not intensity, of competition.

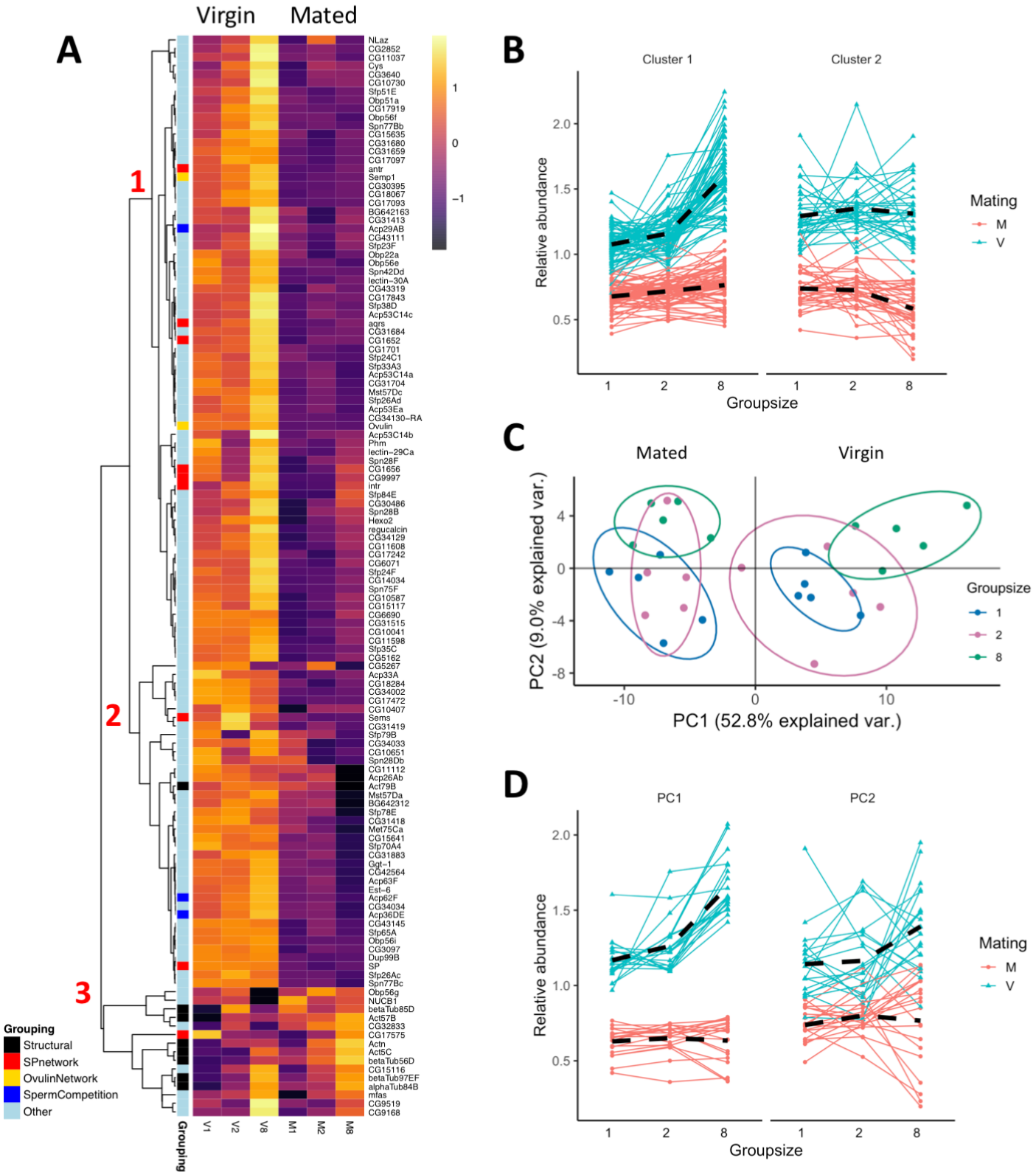


Figure 2. Intensity-specific responses to competition in the seminal fluid protein proteome

(Text overleaf)

Figure 2. Intensity-specific responses to competition in the seminal fluid protein proteome

(A) A heatmap showing the abundance patterns of all 118 seminal fluid proteins (SFPs) and 8 structural proteins detected in a quantitative proteomics analysis of male accessory glands. All values are $\log_2$ transformed. Each cell gives the mean across five replicates for that protein in a particular treatment combination. Starting from the left-hand side of the plot, the first three columns are from virgins and the latter three are from mated males. Within each trio of columns are plotted, from left to right, glands from males held in a group of 1, 2, or 8. Row annotations highlight particular protein functional groupings as well as proteins with established roles in sperm competition. (B) The average SFP response to competition in each of the two major clusters given in (A). Each of the 118 detected SFPs are plotted separately for mated (red) and virgin (blue) glands for each group size treatment. The mean response is given by a dashed line. The abundance values are relativised by dividing the abundance of each SFP in a sample by the mean across all samples for that protein. A treatment mean across the five replicates is then taken for each SFP and plotted. (C) Output of a PCA analysis conducted on the normalised, $\log_2$ abundances of the 118 detected SFPs. PC1 is given on the *x* axis and PC2 on the *y*. Points represent each of the 30 samples coloured according to the group size males were reared in (blue = 1, pink =2, green=8). Mated glands fall on the left of the plot, virgin glands on the right. Ellipses denote 80% normal probability. (D) The abundance pattern of the top 20 contributing SFPs to each of PC1 (left) and PC2 (right) calculated as part of the PCA analysis referred to in (C) and following the approach for (B).

Elevated transfer of SFPs can be achieved by two mechanisms: (i) producing more, or (ii) depleting more. Interestingly, our hierarchical clustering analysis revealed that SFPs separate according to these two mechanisms. In Cluster 1, SFPs are only produced in significantly greater quantities by virgin males in response to high competition (Table 1; Fig. 2b). After mating, the magnitude of difference is substantially lower between groups (Table 1; Fig. 2b). In contrast, Cluster 2 captures proteins that show no significant differences across competition intensities in virgin glands, but which show significantly greater post-mating depletion in high competition males (Table 1; Fig. 2b).

Collectively, these analyses reveal that (a) unlike sperm, the seminal fluid proteome responds to the intensity of competition, and (b) that the seminal fluid proteome is split according to how greater SFP transfer is achieved: elevated production or greater depletion. Considered alongside our sperm transfer results, it is clear that different components of the ejaculate can vary independently of one another. Divergence in allocation rules for different ejaculate components implies the existence of independent mechanisms controlling their transfer.

Cluster	*Mating*	*Group size*	*Estimate*	*SE*	*DF*	*T ratio*	*p value*
1	M	1-2	-0.065	0.038	350	-1.689	0.2109
1	M	1-8	-0.148	0.038	350	-3.878	0.0004
1	M	2-8	-0.084	0.038	350	-2.189	0.0744
1	V	1-2	-0.058	0.038	350	-1.507	0.2888
1	V	1-8	-0.544	0.038	350	-14.224	<0.0001
1	V	2-8	-0.486	0.038	350	-12.717	<0.0001
2	M	1-2	0.046	0.083	170	0.548	0.8477
2	M	1-8	0.413	0.083	170	4.952	<0.0001
2	M	2-8	0.367	0.083	170	4.404	0.0001
2	V	1-2	0.029	0.083	170	0.346	0.9363
2	V	1-8	0.017	0.083	170	0.198	0.9786
2	V	2-8	-0.012	0.083	170	-0.148	0.9881

Table 1. Cluster-specific responses of SFPs to mating and group size

Summary statistics from least-square means post-hoc comparisons between group sizes (1,2, or 8 males) within mating treatments (M=mated, V=virgin). Conducted separately for Clusters 1 and 2, which are described in Figure 2a. All *p*-values are Tukey-corrected. Significant values at the $p<0.05$ level are given in red. Estimates are on a $\log_2$ scale.

The composition of the SFP proteome changes in response to competition intensity

Having identified that sperm and seminal fluid allocation operate independently, we next asked whether SFPs vary in their sensitivity to competition. Variation between SFPs in their responsiveness would generate compositional change in seminal fluid. To test this we used a PCA. PC1 explained over half of the variation in the data (52.9%; eigenvalue = 62.5; Fig. 2c; Supp. Table 1) and was significantly associated with an interaction between mating and competition intensity ($F_{2,20}$=4.9315, p=0.018; Supp. Table 1). A significant interaction means that the degree of difference between virgin and mated glands depends on the level of competition males are exposed to. It therefore captures change in the abundance of SFPs transferred to females in the ejaculate during mating. The abundance pattern captured in the top contributing proteins to PC1 accords with the results of our hierarchical clustering analysis: males produce and transfer higher abundances of SFPs at high competition, with no clear response at low competition (Fig. 2d). Nearly all loadings in PC1 were positive (114/118) demonstrating high alignment between SFPs in their responses, which suggests that elevation in SFP production and transfer at high competition is widespread within the seminal fluid.

PC2, which explained 8.9% of the variance (eigenvalue = 10.5), represents an axis of variation solely associated with competition intensity ($F_{2,24}$=12.8178, p=0.0002; Supp. Table 1). PC2's loadings were split in the direction of their correlation (59/118 positive), indicating a more variable intra-proteome response to competition than in PC1. Visual inspection of the abundance pattern of the top 20 contributing proteins reveals that PC2 captures variation in the relative responsiveness, direction of change, and degree of post-mating retention of SFPs (Fig. 2d). Consequently, this axis of variation provides a clear indication that change in the seminal fluid proteome at high competition is compositional, rather than just a uniform elevation in SFP production and transfer.

High-confidence, intensity-specific upregulation of functionally-important sperm competition SFPs

Our preceding analyses tested for generic axes of variation associated with compositional change in the seminal fluid. To better understand the responses of individual SFPs, we used a differential expression analysis to identify high-confidence competition-sensitive SFPs. We detected 44 SFPs that showed a significant response to competition intensity (Supp. Table 2; Fig. 3). This list included a number of functionally-annotated SFPs. We identify 5 'sex peptide network' proteins as being differentially expressed at high competition (antr, aqrs, CG1652, CG1656, and CG9997). These proteins all contribute to the binding of sex peptide to sperm – a requirement for the long-term persistence of female post-mating responses (Ram and Wolfner 2007b, 2009; Findlay et al. 2014). The regulation of sex peptide storage and cleavage has direct consequences for patterns of female sperm use and sperm competition outcome (Avila et al. 2010; Gligorov et al. 2013). Four of the detected differentially expressed sex peptide network proteins are known to bind to the sperm itself and enter into the female sperm storage organs (Singh et al. 2018). Another of the differentially expressed SFPs, Acp29AB, has previously been linked to sperm competition performance (Clark et al. 1995; Fiumera et al. 2005), is required for sperm storage and, enters into the storage organs (Wong et al. 2008). The 44 differentially expressed proteins, showed no significant associations with gene ontology terms in a DAVID search (Huang et al. 2009a,b) or four recently-proposed regulatory miRNA hubs (Mohorianu et al. 2018)(Supp. Analysis 1). That traditional bioinformatics tools fail to detect associations with broad categories of SFP function supports previous findings that SFPs belonging to disparate functional classes can act in concert (Singh et al. 2018).

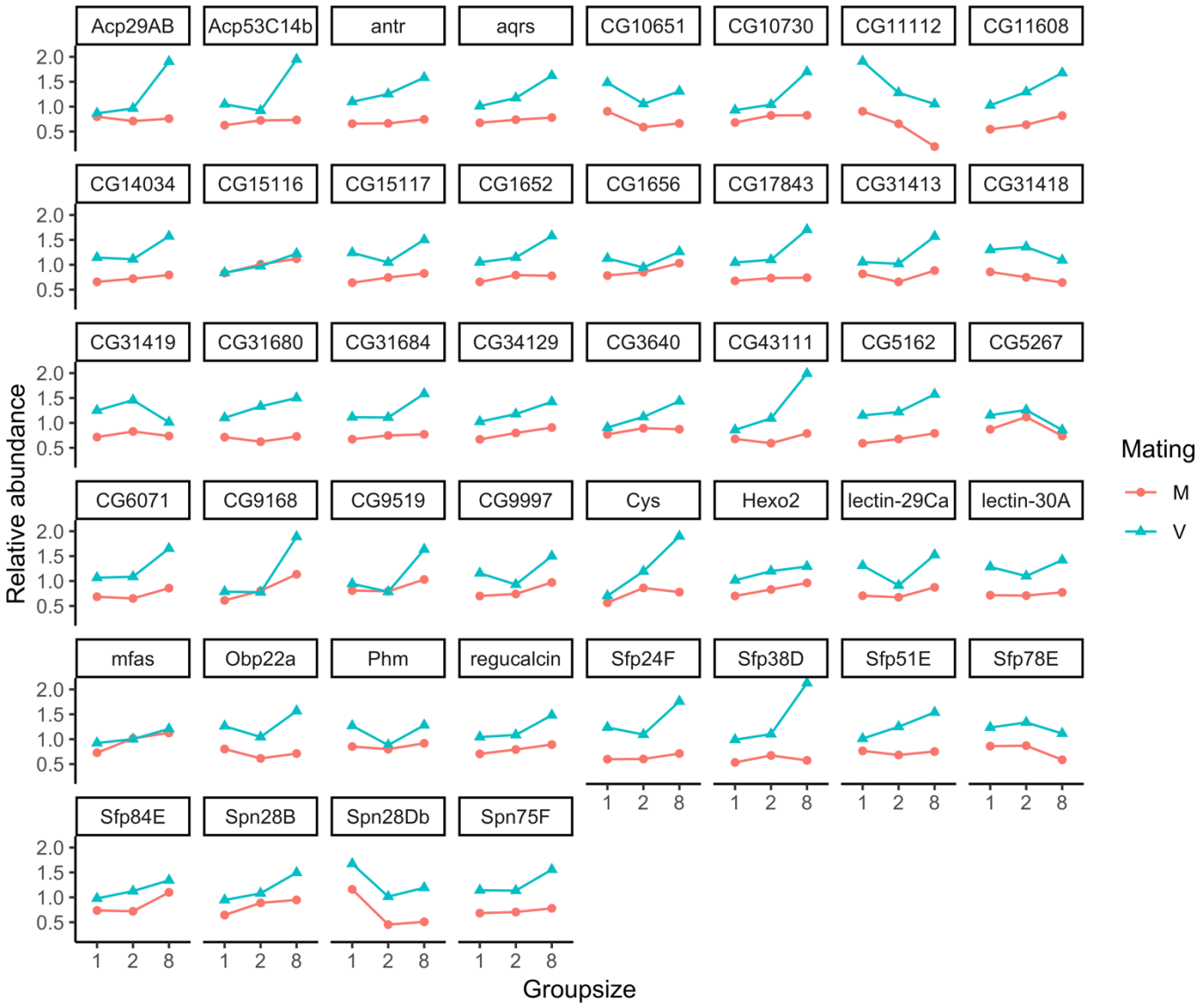


Figure 3. Abundance profiles of SFPs identified as differentially-expressed in relation to competition intensity

Average abundance patterns for each differentially-expressed SFP plotted in relation to group size (FDR $p<0.05$) and separately for mated (red) and virgin (blue) glands. Relative abundances are calculated by dividing the normalised values by the mean across all samples for each protein and then taking a treatment mean.

Identification of highly-dynamic, putative sperm competition SFPs

We might predict that the SFPs showing the greatest response to competition would be those involved most directly in sperm competition. Within our 44 differentially expressed SFPs, we find that 7 are twice as abundant in high competition males compared to no and/or low competition males. One of these is the aforementioned Acp29AB. The others include a cysteine protease inhibitor (Cys, Laflamme and Wolfner, 2013), an oxidoreductase (CG9519), and an alkaline-phosphatase like enzyme (CG9168). The remainder (Acp53C14b, CG43111, and Sfp38D) have no molecular information associated with their FlyBase entry. Indeed, CG43111 and CG9519 are newly-discovered SFPs (Sepil et al. 2018). Curiously, we also find one differentially expressed protein (CG11112) at half the abundance in high competition relative to no competition male virgin glands. This cluster of especially dynamic SFPs contains prime candidates for proteins that play key roles in post-mating male competitiveness.

Greater SFP production in high competition males correlates with a steeper rate of reproductive decline

We have identified two divergent allocation rules influencing ejaculate composition. These rules intersect to produce competition-specific ejaculates: (i) no competition: low sperm, low SFPs; (ii) low competition: high sperm, low SFPs; (iii) high competition: high sperm, high SFPs (Fig. 4). That males respond differently to low and high competition suggests that the two sperm competition scenarios are different enough to warrant alternative responses. This difference may arise through an increased probability of female double mating or via conditions associated with >2 matings (*e.g.* Laturney, van Eijk and Billeter, 2018). But if males refrain from further elevating their sperm transfer at high competition intensities, why would they transfer greater quantities of SFPs?

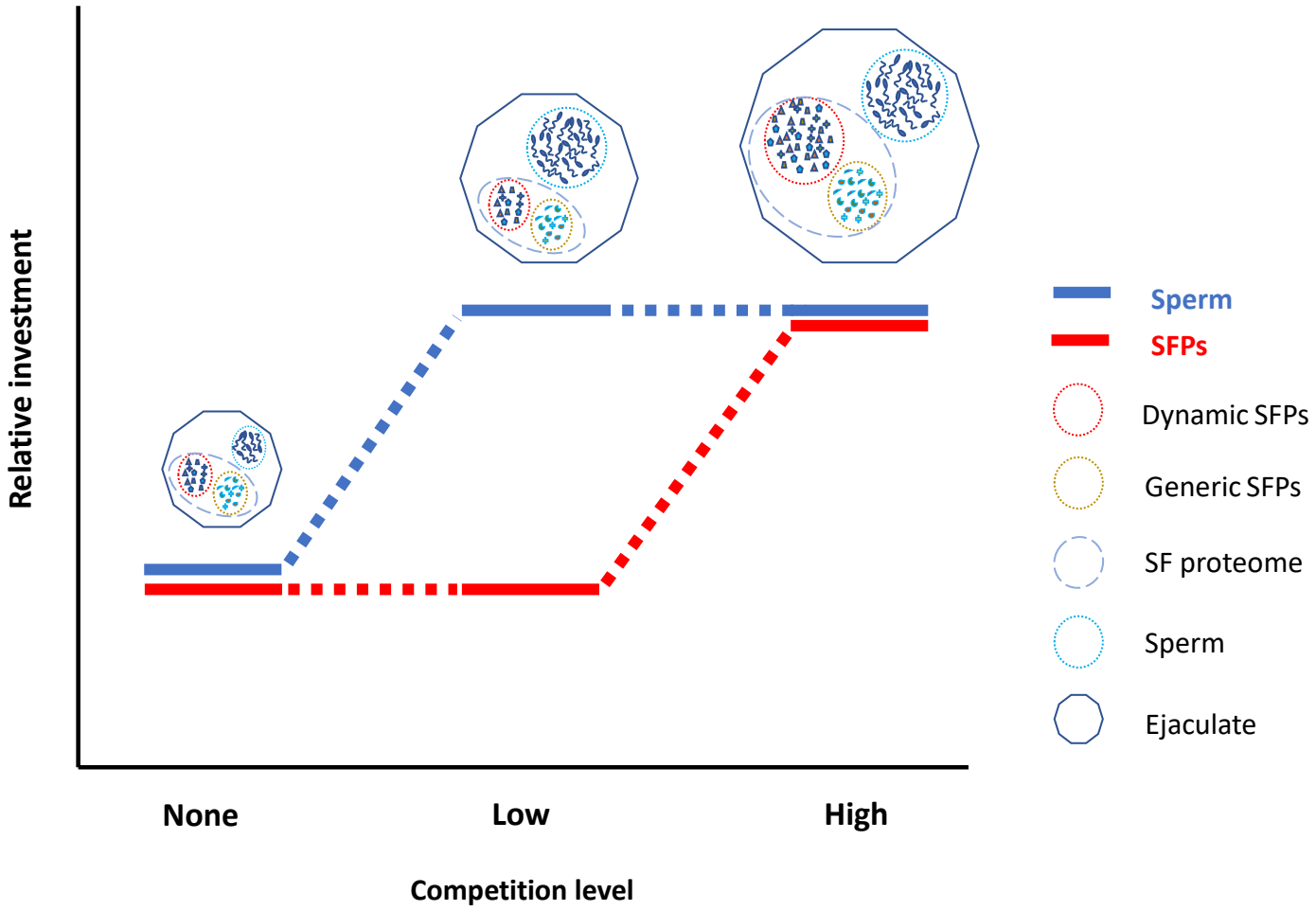


Figure 4. The operation of plasticity in the *D. melanogaster* ejaculate

A schematic summarising the ejaculate allocation patterns revealed in this study. Males increase the transfer of sperm (blue line; dotted turquoise circle) in accordance with the binary presence/absence ('risk') of competition. Conversely, significant increase in seminal fluid protein transfer (red line) occurs only at high ('intense') competition. Consequently, total ejaculate investment increases stepwise with increased competition (denoted by the size of the blue decagon). The seminal fluid proteome (dashed ellipse) can be divided into a 'generic' portion, which shows a general upregulation in response to high competition (dotted yellow circle), and a 'dynamic' portion that shows high sensitivity to competition (dotted red circle).

Theory predicts that the energetic cost of producing individual ejaculate components exerts a powerful designing force on the evolution of ejaculate composition (Dhole and Servedio 2014). That males will elevate their sperm transfer before that of SFPs may indicate the higher costs associated with SFP transfer compared to sperm. These greater transfer costs for SFPs may manifest through slower replenishment compared to sperm, a higher energetic cost of replenishment, or higher costs associated with being SFP-depleted in future matings. To test for specific costs associated with elevating SFP transfer, we explored the rate of reproductive decline across 5 consecutive matings by males held in groups of 1,2, or 8. That group size 2 and 8 males transfer equivalent sperm numbers means that differences between them at the ejaculate-level are attributable to differential SFP transfer.

We first tested whether group size influences the speed with which males start mating. This may represent a proxy for ability to compete for matings. We failed to find any difference between competition intensities across the first three matings (Coxph: Mating 1, LRT=1.1532, p=0.562; Mating 2, LRT=2.743, p=0.254; Mating 3, LRT=4.131, p=0.127; Fig. 5a). However, by the fourth mating, high competition males were significantly slower to mate (Coxph: LRT=11.180, p=0.004; Fig. 5a). This effect was larger again in the fifth (Coxph: LRT=22.696, p<0.0001; Fig. 5a). Overall, significantly fewer high competition males completed 5 matings (GLM: Dev=11.79, df=2, p=0.003; Fig. 5b). The comparison between no and low competition males was non-significant (z-ratio = 1.834, p=0.159), but significantly fewer high competition males completed 5 matings than low competition males (z-ratio =-2.706, p=0.019). This decline in mating speed may therefore represent a specific cost of elevated SFP production. Alternatively, it may represent a hangover of increased energy expenditure in the pre-copulatory social environment (*e.g.* higher rates of male-male contests).

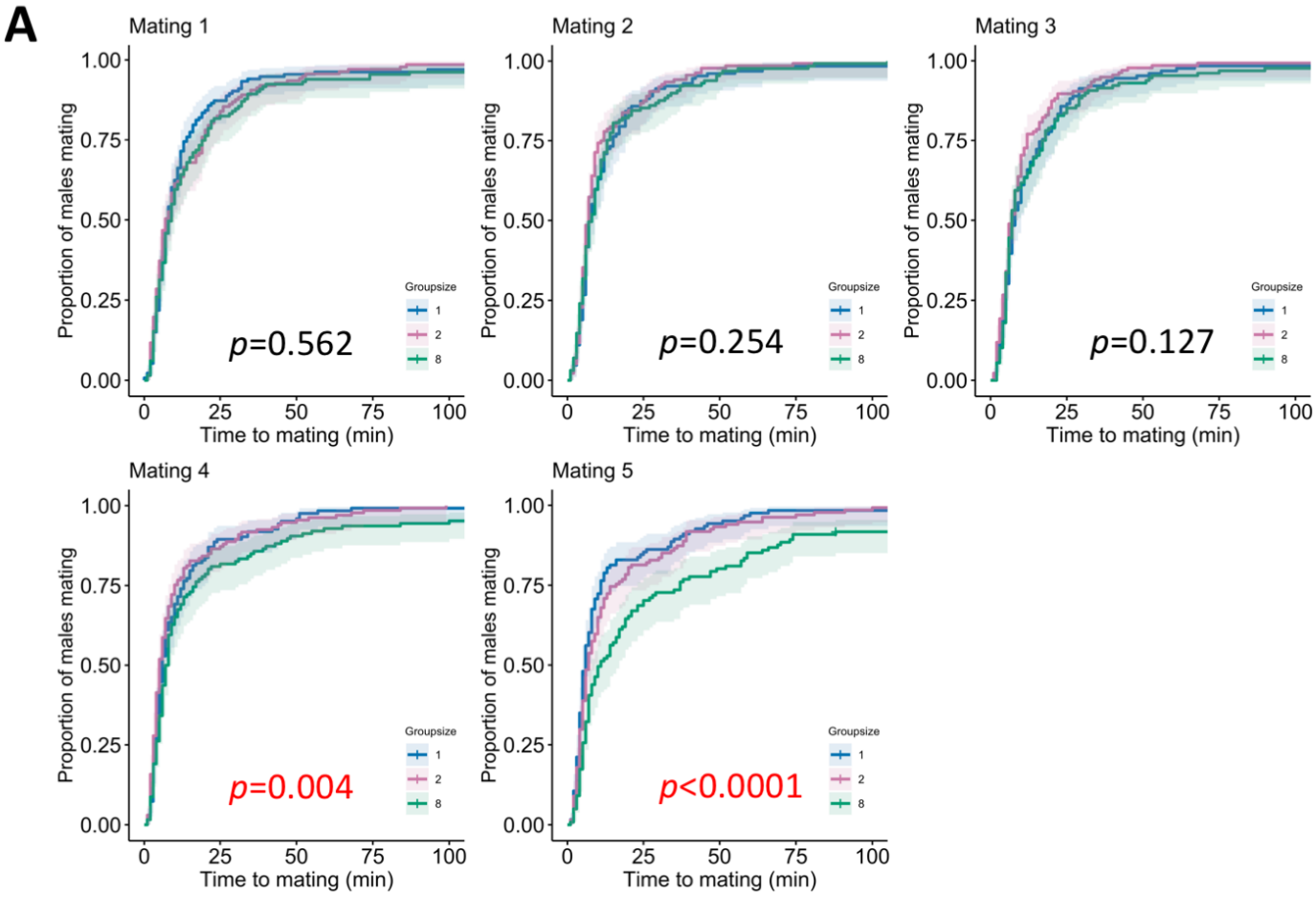
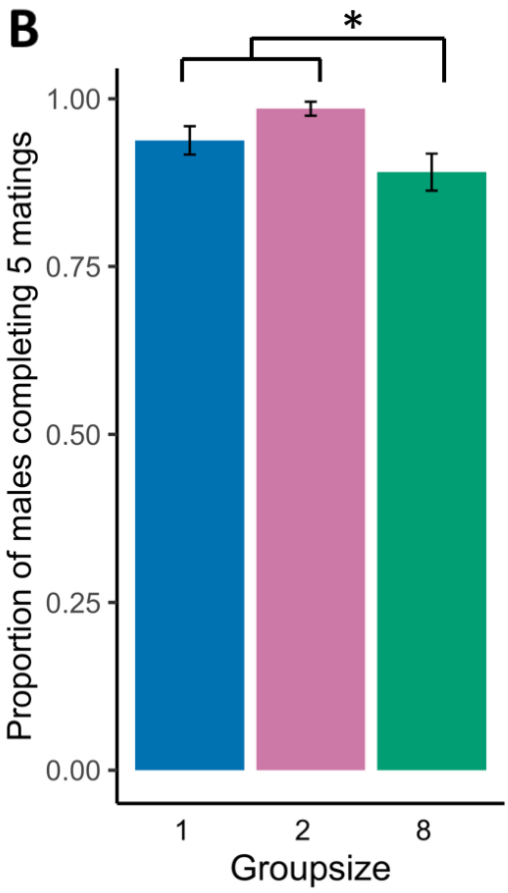


Figure 5. High competition males decline in mating ability over successive matings

(A) The time to mating for males depending on their group rearing conditions (blue, held individually; pink, held in a pair; green, held in a group of 8). Matings 1 to 5 are plotted in separate panels. *p*-values are taken from Cox proportional hazard models. Data are censored according to whether the male mated and pooled from two replicates. Confidence intervals are at 95%. Starting sample sizes: N_1=115, N_2=130, N_8=109.

(B) The proportion of males completing 5 matings. Sample sizes as in (A). Asterisk denotes significance at the p<0.05 level. Error bars are ± 1 standard error of a proportion.



Rather than looking at ejaculate costs in terms of male behaviour, we next looked at declines in offspring production over successive matings. We hypothesised that a larger, high-competition ejaculate would lead to relatively more offspring in a first mating, but relatively fewer in a fifth as a result of ejaculate depletion (*e.g.* Linklater *et al.*, 2007; Sirot *et al.*, 2009). Consistent with this prediction, we detected a significant interaction between group size and mating number on the number of offspring females produced over 3-days following mating (LME: square-transformation, $F_{2,315}$=3.740, p=0.025; Fig. 6a). High competition males produced more offspring in their first mating compared to both low (Tukey: t=-2.629, p=0.024) and no competition (Tukey: t=-1.543, p=0.271) males, albeit not significantly so in the latter. The average difference in first mating offspring production between low and high competition males amounted to 12 (mean±SE; Group 1: 184±5; Group 2: 180±4; Group 8: 192±4).

In an alternative analysis, we subtracted the number of fifth mating offspring from the number of first mating offspring for each male to generate a differential of reproductive decline. This analysis revealed a significant effect of group size (LM: $F_{2,315}$=3.066, p=0.048; Fig. 6b). Post-hoc comparison revealed the only significant difference was between no and high competition males (Tukey: t=-2.476, p=0.037), with high competition males showing a stronger reproductive differential. Low competition males showed an intermediate reproductive differential.

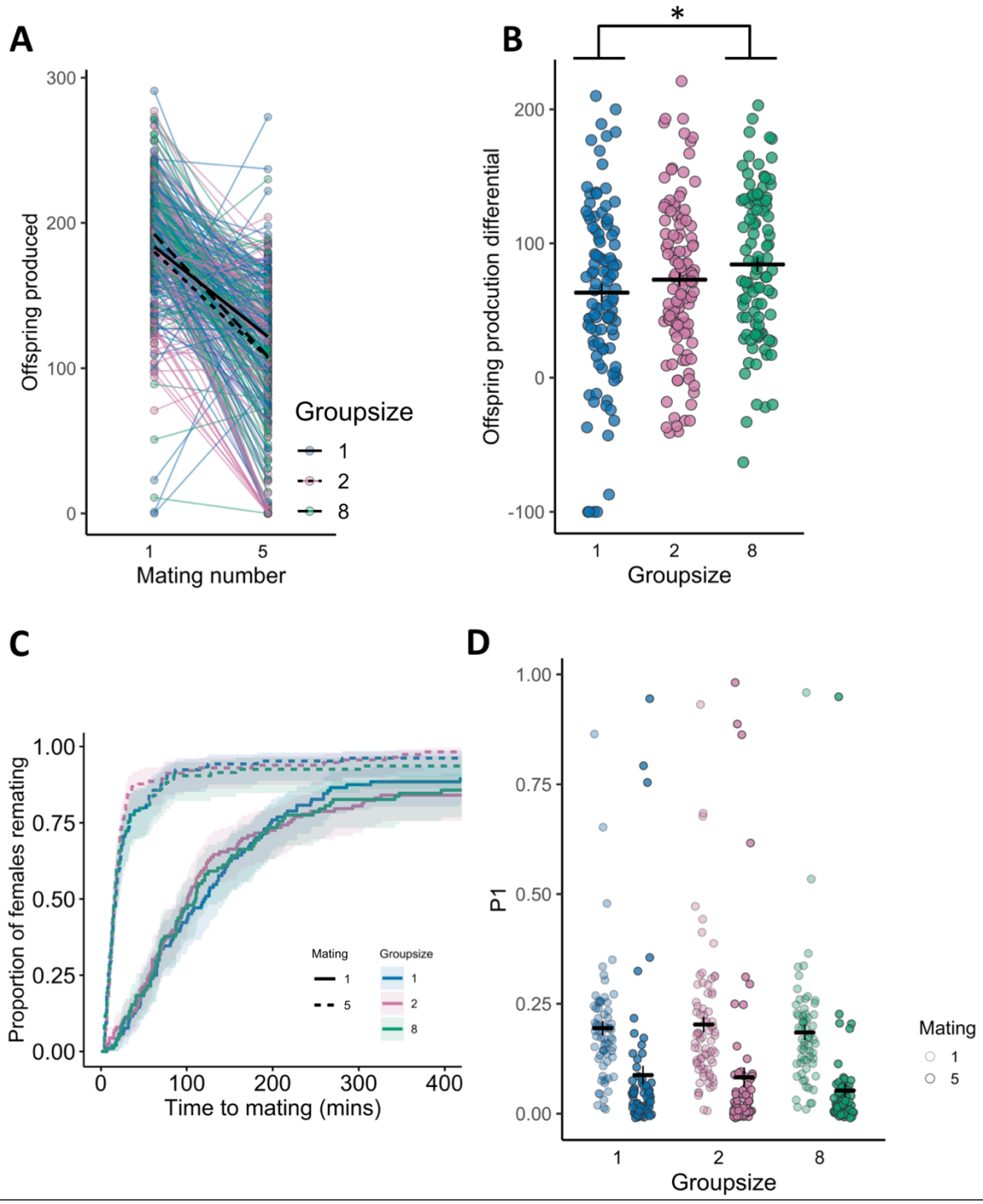


Figure 6. Effects of competition and multiple mating on male reproductive performance

(A) Offspring produced in a 3-day period by females mated to males held in groups of 1,2, or 8. Each line links an individual male's offspring total from his first and fifth mating. The black lines give the mean reaction norm for each group size: 1, unbroken line; 2, short dashes; 8, long dashes. Sample sizes: N_1=101, N_2=106, N_8=95 pooled across two replicates. (B) Difference in offspring production between each male's first and fifth mating. Asterisk denotes p<0.05. (C) Survival plot showing the proportion of females remating 3-days after mating to a male from one of the three group size treatments on either his first (unbroken lines) or fifth (dashed lines) mating. Confidence intervals are at 95%. (D) The proportion of offspring sired by the first mating male in relation to his group size and number of matings. Mating 1 (faded circles), mating 5 (filled circles). Sample sizes N_1=68, N_2=75, N_8=63.

Surprisingly, given their strong associations with SFPs, we failed to find an effect of competition intensity on either female receptivity to remating (Coxph: Group size x Mating, χ^2_2=1.056, p=0.59; Group size, χ^2_2=0.280, p=0.869; Fig. 6c) or defensive sperm competition performance (GLM: Group size x Mating, df=2, $Dev.$=5066.8, p=0.366; Group size, df=2, $Dev.$=9845.6, p=0.654; Fig. 6d). Although both were significantly diminished after multiple matings (Latency: Coxph: χ^2_2=212.92, p<0.0001; P1: GLM: Df=1, $Dev.$=9845.6, p<0.0001). For the most part, males retained fertility into a fifth mating, contrasting with previous claims that males are infertile after three consecutive matings (Tayler et al. 2012). Finally, we found no change in the rate at which accessory gland size increased after five successive matings (Supp. Analysis 2). We had hypothesised this would reflect the rate of SFP replenishment and would be diminished in high competition males having previously invested in greater SFP production.

Collectively, these data show a clear separation in the reproductive performance of low and high competition males. High competition males enjoy higher offspring production in a first mating than low competition males, but suffer a steeper reduction in subsequent offspring production, latency to mating, and ability to complete matings. Given that males from these two groups transfer equivalent sperm numbers, some of these costs to high competition males are likely attributable to the specific cost of elevated SFP transfer. Our data support the finding that exposure to rivals leads to increased offspring production without affecting paternity share after the female remates, and that this benefit is lost after several matings (Bretman *et al.*, 2013; but see Bretman, Fricke and Chapman, 2009). However, our data go further to suggest that this benefit is not associated with the mere exposure to competition, nor the elevation in sperm number, but rather the intensity-specific elevation in SFP transfer.

Elevated mating duration in response to competition is lost through successive mating and aligns poorly with ejaculate transfer

D. melanogaster males are known to increase their mating duration in response to the binary presence/absence of competition, and that this response is insensitive to competition intensity (Bretman et al. 2009, 2010a, 2011b). Our 5-mating experiment supports that males significantly elevate mating duration in response to the binary presence/absence of competition (LME: χ^2_2=5.460, p=0.005; Fig. 7; Supp. Table 3a). This pattern of change matches the response we find for sperm number, but not seminal fluid. Therefore, responses at the level of mating duration do not capture underlying compositional changes. Moreover, it lends support for the finding from neurogenetic work that the mechanisms controlling mating duration and ejaculate transfer are distinct (Crickmore and Vosshall 2013).

We further find that the competition effect on mating duration declines with successive matings (LME: χ^2_8 = 14.83, p=0.062; Fig. 7; Supp. Table 3a). A group size effect is apparent across the first two matings, although weaker in the second (Supp. Table 3c), and is lost from the third mating onwards. Males have previously been shown to retain elevated mating durations if held with rivals throughout their life and mating only every few days (Bretman et al. 2013b). However, our data suggests that this elevation is not maintained across successive matings within a short-time period.

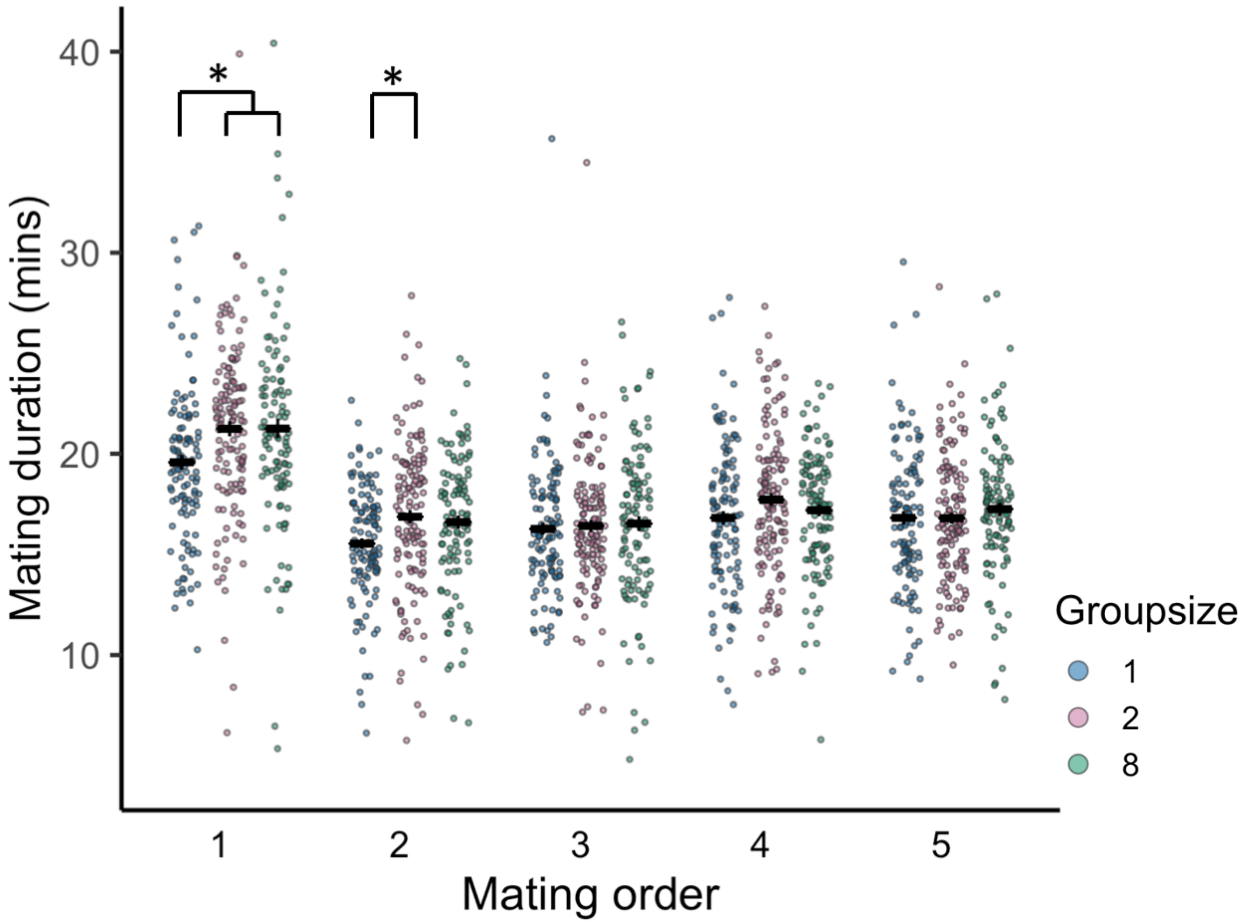


Figure 7. Elevated mating duration in response to competition is lost through multiple mating

Mating duration (measured in minutes) across 5 consecutive matings. Males were held either individually (blue circles), in pairs (pink circles) or in groups of 8 (green circles) for between 3 and 4 days prior to mating. Sample sizes: N_1=115, N_2=130, N_8=109. Data pooled across two replicates. Asterisks denote p<0.05 in post-hoc Tukey-corrected comparisons using least-square means from a linear mixed effects model.

Inter-mating interval modulates the benefits of changes to ejaculate composition

Our data suggest that exposure to rivals does not lead to improved paternity shares when a female remates. This matches the results of one previous study (Bretman et al. 2013b), but contrasts with those of another (Bretman et al. 2009). We hypothesised that our failure to detect a group size effect may relate to the length of time separating a female's first and second mating. While a 3-day inter-mating interval is commonplace in many sperm competition assays (Laturney et al. 2018), competition will be more intense with shorter inter-mating intervals where fewer sperm have been used. To test this hypothesis, we repeated the previous experiment, this time providing males held in the same three competition groups with only a single mating and leaving a 1-day gap between a female's first and second mating. Pooling data across 5 independent replicates, we again found no significant effect of competition intensity on the key SFP-mediated traits of defensive sperm competition performance (GLM: df=2 Dev=10775, p=0.79; Fig. 8a) or female receptivity to remating (Coxph: $\chi^2_2 = 0.45$, p=0.80; Fig. 8b). Moreover, we found no significant difference in the number of offspring produced during the 24-hours following mating (LM: $F_{2,836}$=0.290, p=0.75; Fig. 8c), despite a significant response at the level of mating duration (LM: $F_{2,836}$=46.185, p<0.0001; Fig. 8d). That we find greater offspring production from high competition ejaculates only over longer time periods indicates that the benefits of altered ejaculate composition depend on female remating behaviour. Moreover, it suggests that longer-term acting SFPs may underlie these benefits. This aligns with our finding that many sex peptide network proteins are transferred in greater abundances at high competition. These proteins govern, amongst other things, long-term rates of female sperm use via their regulation of sex peptide within the female reproductive tract (Avila et al. 2010; Gligorov et al. 2013).

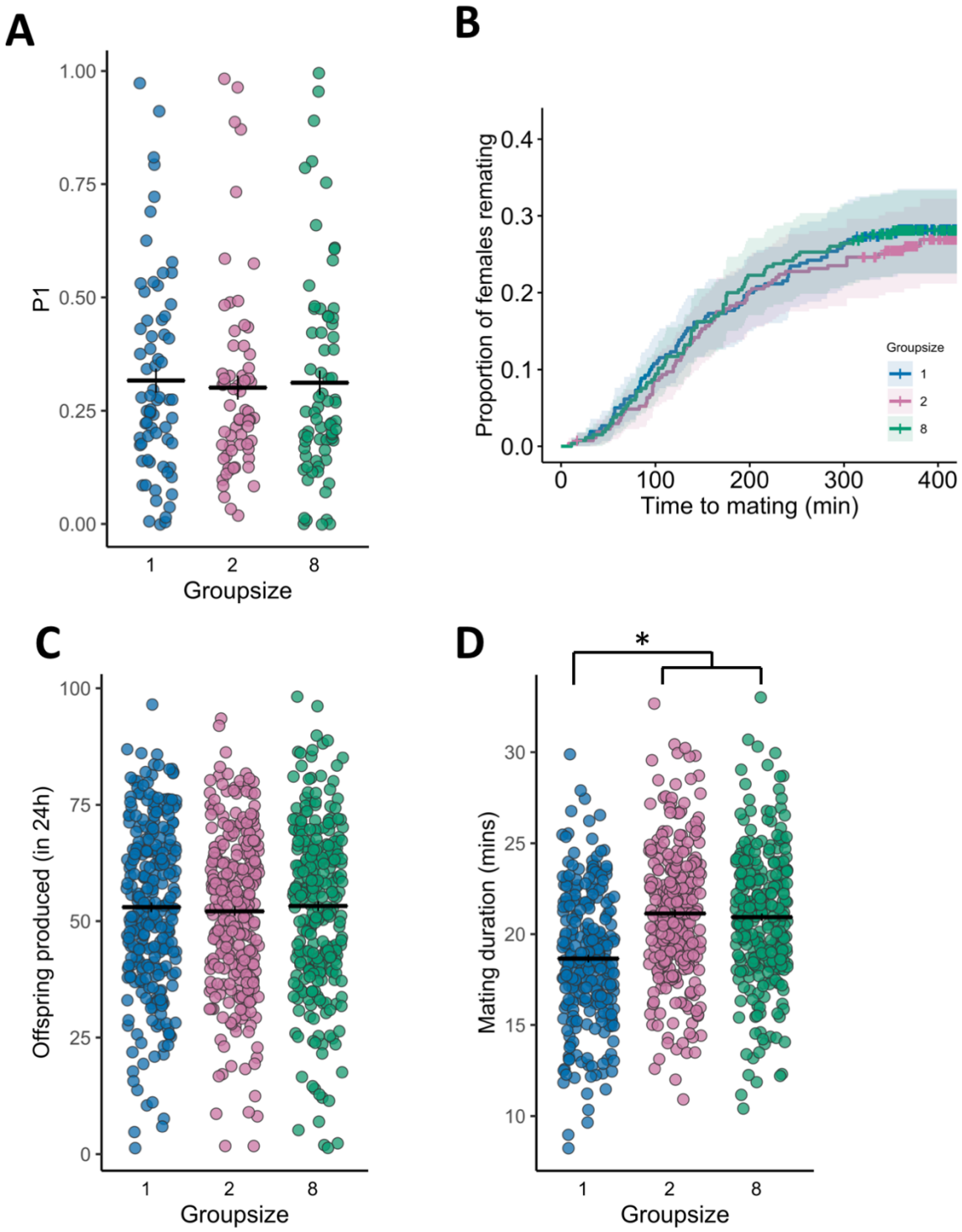


Figure 8. Benefits of plastic ejaculate composition do not manifest with short inter-mating intervals

(A) Proportion of offspring sired when males held in different group sizes (1,2, or 8) mate to a virgin female who remates 24-hours later. Sample sizes: N_1=70, N_2=65, N_8=72. (B) Time to remating (in minutes) and the proportion of females that remated in relation to the group size their previous partner was reared in. Non-maters are censored. Confidence intervals are at 95%. (C) Offspring produced in the 24-hours following mating. (D) Mating duration (measured in minutes). In all plots, group size 1 males are in blue, group size 2 males in pink, and group size 8 males in green. In plots A,C, and D, horizontal bars represent the treatment mean while vertical lines give ±1 SE of the mean. Sample sizes in B,C, and D: N_1=279, N_2=281, N_8=283, pooled across 5 replicates. Asterisk denotes p<0.05.

CONCLUSION

Here, we have identified for the first time that males can exercise independent control over the transfer of sperm and SFPs to females. Further, we show that each of these two parts of the ejaculate responds differently to the intensity of competition and that SFPs vary in the sensitivity and directionality of their response to competition. Consequently, males deploy compositional change both between and within portions of the ejaculate (Fig. 4). We find that compositional change in the seminal fluid portion of the ejaculate is associated with increased offspring production – elevated sperm transfer alone appears insufficient. Increased offspring production in females mated to males from high competition backgrounds is therefore likely attributable to elevated transfer of SFPs that stimulate female sperm use. This would include the sex peptide network proteins that we find upregulated at high competition. That these proteins act to maintain long-term post-mating responses may explain why a longer female inter-mating period is required for the benefits of a larger ejaculate to be detected. The differing rates of reproductive decline associated with low and high competition males suggest that the transfer of SFPs is costlier than the transfer of sperm, a factor likely to structure how and when males employ plasticity.

Collectively, our data highlights unprecedented sensitivity of ejaculate composition to the social environment, and points to the existence of highly-sophisticated and as-of-yet unknown mechanisms by which males translate social stimulation into differentially composed ejaculates. If this level of sensitivity is taxonomically widespread then its dysregulation may represent an underappreciated driver of fertility problems in humans. Similarly, understanding how males allocate non-sperm components of the seminal fluid may offer novel routes of therapeutic intervention to modify their effects both on the female reproductive tract and offspring development.

METHODS

Fly stocks and husbandry

All of the flies used were from a lab-adapted, outbred Dahomey wild-type stock. Originally collected in West Africa in 1970, the stock has since been kept in large population cages with overlapping generations. This same line has been used in much of the work previously conducted on male *D. melanogaster* reproductive plasticity (Bretman et al. 2009; Wigby et al. 2009; Sirot et al. 2011b; Bretman et al. 2013b,a; Mohorianu et al. 2017). For sperm competition assays, females and competitor males were taken from the same Dahomey genetic background into which the recessive *sparkling*^{poliert} (*spa*^{pol}) mutation (Fu and Noll 1997) was previously backcrossed for four generations. This allows for the visual assessment of offspring paternity (Fiumera et al. 2005; Fricke et al. 2009; Wigby et al. 2009; Edward and Chapman 2012). To generate a line with fluorescent sperm, we backcrossed *GFP-ProtB* (courtesy of Stefan Lüpold, University of Zürich) into the Dahomey background for 6 generations. This construct fluorescently labels sperm heads (Manier et al. 2010). All flies were kept in a non-humidified room at 25°C on a 12:12 L:D cycle. Experimental flies were fed on Lewis medium (Lewis 1960) and reared at standardised larval densities by transferring approximately 200 eggs (via methods described in Clancy and Kennington 2001) to 250mL bottles containing 50mL of food. All rival exposure males were collected as virgins (*i.e.* within 7h of eclosion) under ice anaesthesia and randomly placed either individually, in pairs, or in a group of 8 in 36mL Lewis medium-containing plastic vials. Here, they were aged for 3-4 days to allow for continued reproductive maturation (Ruhmann et al. 2016) and to maximise the magnitude of responses shown to rivals (Bretman et al. 2010a). We collected the competitor males used in sperm competition assays and all females as virgins and held them in vials in groups of 10-12. Females were between 3 and 4 days old at time of first mating while

homozygous *spa*^{pol} males were between 4 and 5 days old. All experimental flies were supplemented with *ad libitum* live yeast for at least 24-hours before experiments.

Sperm counts

We aspirated GFP-tagged sperm males form the three group size treatments into yeasted vials containing individually isolated *spa*^{pol} females. We flash froze the females 25-minutes after the start of mating before moving them to -80°C. Females were dissected in ice-cold PBS under a light microscope, with the region between the vulva and common oviduct being retained. We sealed the cover slip in place with rubber cement (Fixogum, Marabu) and stored slides at 5°C. These were later imaged using a Zeiss 880 confocal microscope. We used an average intensity Z-projection in the Fiji distribution of ImageJ (Schindelin et al. 2012) to condense Z-stacks into a single image for easier counting. Sperm counts were performed manually using the multi-point feature in Fiji. All matings, dissections, and sperm counts were performed blind with respect to group size treatment. This experiment was performed in two experimental replicates.

Proteomics experiment:

(a) Fly work

The evening before experimental matings, we transferred 105 single virgin Dahomey females into yeasted vials under ice anaesthesia. Each of these vials was paired with a vacant, yeasted vial. The following morning, we individually aspirated 35 males from each of the three different group size treatments into the female-containing vials. At the same time, 35 males from each of the three group sizes were individually introduced into a vacant vial to be retained as virgins. We varied whether the first or second male selected from a grouped-male vial was moved to a female-containing vial or vacant vial so as to

minimise biases that might arise from non-random selection. The time at which we introduced the male was recorded, as was the start and end of mating. 25-minutes after the start of mating, we aspirated the male into a cryovial, which we immediately flash-froze in liquid nitrogen. We simultaneously froze the virgin male in the partner vial. By taking this approach, we could ensure that the distribution of freezing times among virgin and mated males was equivalent. Furthermore, it ensured that both mated and virgin males had experienced an equivalent period of separation from male competitors before being frozen. Finally, by introducing each male into the female-containing arena, rather than females into the male arena, we avoid a potential problem with previous work on ejaculate allocation where it is hard to exclude the possibility that enhanced reproductive performance of paired males is actually an artefact of a better-male-gets-the-mating rule (Wigby et al. 2009). Our males were then stored at -80°C until dissection. We ran this experiment five times to produce five independent biological replicates.

(b) Sample preparation

A sample for each of our 6 treatment levels (group size = 1, mated; group size = 1, virgin; group size = 2, mated; group size = 2, virgin; group size = 8, mated; group size = 8, virgin) was composed of 20 pairs of accessory glands pooled in 25µl of PBS. We performed the dissections on ice in PBS buffer, severing the ejaculatory duct at the distal end. Factoring in the 5 replicates, we had 30 samples in total, which we subsequently held at -80°C.

To prepare the sample for proteomic analysis, we used a clean pestle to macerate the pooled glands in each sample for 1-minute, washing the residue from the pestle back into the sample with 25µl of Pierce RIPA Buffer. Our samples were digested in accordance with the gel-aided sample preparation (GASP) protocol outlined in detail elsewhere (Fischer and Kessler 2015; Sepil et al. 2018). Briefly, 50mM of the reducing

agent DTT was added to the sample, incubating for 10 to 20 minutes. To the resulting lysate, we added an equal volume of 40% acrylamide/Bis solution (37.5:1 National Diagnostics), which was subsequently left for 30 minutes at room temperature to promote the alkylation of cysteine to propionamide. To induce acrylamide polymerization and form a gel plug, we next added 5µl of 10% APS and an equivalent quantity of TEMED. The gel plug was then shredded via centrifugation through a membrane-less Spin-X filter insert (CLS9301, Sigma/Corning). The resulting gel fragments were then fixed in 5% acetic acid/40%ethanol prior to two consecutive episodes of buffer exchange with 50mM ammonium bicarbonate, 1.5M Urea, and 0.5M Thiourea. These were subsequently removed with acetonitrile. Digestion of the immobilised proteins was carried out overnight through the action of trypsin (Promega). The resulting peptides were extracted via two repeated acetonitrile replacements, dried, desalted in Sola SPE columns (Thermo), and then suspended in 0.1% FA, 2% ACN before LC-MS/MS (liquid chromatography- mass spectrometry/mass spectrometry) analysis.

(c) LC-MS/MS

Our peptides were analysed using a LC-MS/MS platform composed of a Dionex Ultimate 3000 and a Q-Exactive mass spectrometer (Thermo). Peptides were loaded on a trap column (PepMAP C18, 300µm x 5m, 5µm particle, Thermo) in a solution of 0.1% TFA in 2% ACN and then separated on an easy spray column (PepMAP C18, 75µm x 500m, 2µm particle, Thermo) with a gradient 2% ACN to 35% ACN in 0.1% FA in 5% DMSO. MS spectra were collected at a resolution of 70,000 in profile mode on the Q-Exactive (ion target = $3x10^6$). The 15 most intense features were selected for subsequent MS/MS analysis at a resolution of 17,500. The following parameters were set: dynamic exclusion

= 27 seconds; AGC target = $1x10^5$; isolation width = 1.6 m/z; and maximum acquisition time = 128ms.

(d) MS data processing

The MS data processing pipeline we used has previously been described by Sepil *et al.* (Sepil et al. 2018). We imported the RAW data into Progenesis QIP (version 3.0.6039.34628), exporting spectra as MGF files using the 200 most intense peaks without deconvolution for searching. For peptide identification, we used the *Drosophila melanogaster* UniProt reference proteome as a search target, with database retrieval conducted on 15/02/2017 (23302 sequences) in Mascot 2.5.1. Our search parameters incorporated the following: Oxidation (M), Propionamide (K), and Deamidation (N,Q) as variable modifications; Propionamide (C) as a fixed modification; two missed cleavage sites; 0.05 Da fragment mass accuracy; 10ppm precursor accuracy. Prior to importing the search results into Progenesis for quantification via the Top3 method, we applied a peptide-level 1% FDR alongside an additional Mascot ion score cut-off of 20. The resulting protein abundance data was subsequently normalised using the internal Progenesis algorithm to all proteins.

Rates of reproductive decline

On 'day 1', we introduced males from the three group size treatments into yeasted vials containing individually isolated *spa*^{pol} females and recorded the time into the mating arena, the time the pair started mating, and the time they finished (hereafter: 'mating metrics'). Following the end of mating, the female was moved to a fresh, yeasted vial and a new female introduced into the male-containing vial. This process was repeated such that each male received five matings. We retained only the first- and fifth-mating females

for assessment of post-mating responses. To test for differences in defensive sperm competition ability, we then transferred the first and fifth-mating females into fresh yeasted vials on days 2 and 3. On day 4, we aspirated the first and fifth-mated females into yeasted vials containing a pair of spa^{pol} males. We recorded mating metrics before dispensing with the males and transferring the female into new vials on day 5,6,7 discarding them on day 8. We froze vials upon the eclosion of the offspring, where they remained until counting and phenotyping. This experiment was undertaken in two experimental replicates.

As part of this same experiment, we flash-froze males at different time points after mating in order to investigate differences in the rate of post-mating accessory gland size change in response to rival exposure. We predicted that this would reflect changes in the rate of SFP replenishment. We froze males 25-minutes after the start of their fifth mating, and ~12, ~24, ~26, and ~60 hours afterwards. We selected these time points to capture the period over which males are known to full replenish SFPs, which is complete by 72 hours (Sirot et al. 2009). Males were randomly allocated to a freezing treatment in advance of their first mating. We performed dissections as in our proteomics study and photographed the glands using a Chromyx HD camera under bright-field microscopy (Motic plus imaging) at 10x magnification. We then traced the outline of each gland using the polygon selection tool and measured the internal area (summed across the two lobes) in the Fiji distribution of ImageJ (Schindelin et al. 2012). Images where one gland was punctured were omitted from analysis.

Sperm competition and female post-mating responses with a shorter inter-mating interval

We mated males from the three group size treatments to spa^{pol} females individually isolated the evening before. We recorded the time into the mating arena, the time the pair started mating, and the time they finished, from which both the latency and duration of mating was calculated. Immediately after copulation the male was discarded. 24-hours later, we individually aspirated the mated females into yeasted vials containing paired spa^{pol} males. Mating metrics were recorded as on the previous day. Following the end of mating, the two sparkling males were removed. The female was then transferred into fresh, yeasted vials every 24-hours for 4-days. Once the offspring from each vial had eclosed we froze the vials. We then counted and phenotyped the offspring. This experiment was undertaken in five experimental replicates.

Statistics

All statistical analyses were performed with R statistical software (version 3.5.1)(Team 2013) in RStudio (version 1.1.456)(RStudio Team 2015). We assessed model fit by visual inspection of diagnostic plots, as recommended (Zuur et al. 2010). Mixed effects models were used when we had random effects with 6 or more levels (Bolker et al. 2009), such as protein identity in cluster analyses and male/female ID when analysing rates of reproductive decline. Therefore, where we had multiple experimental replicates (either 5 or 2 depending on the experiment) it was included as a fixed effect. The significance of factors was assessed by dropping individual terms from the full model using the 'drop1' function. *p*-values from linear mixed effects models were calculated using Satterthwaite's method. Post-hoc pairwise comparisons were performed using the *lsmeans* package with a Tukey correction (Lenth 2016).

Mating latency data, either for males or females, was analysed through Cox proportional hazard models using the *survival* package (Therneau and Grambsch 2000; Therneau 2015) and graphed using 'ggsurvplot' in the *survminer* package (Kassambara and Kosinski 2018). Data were censored according to whether the male/female mated. Proportional data was analysed by GLM with a quasibinomial distribution to account for over or under-dispersion, inferred from mismatches between residual deviance and the degrees of freedom. For sperm competition analyses, we included data only from individuals that produced at least one offspring from each male at some point after the first mating. This ensures that all females received sperm from both males. Similarly, for all analyses of post-mating effects we included only males that showed fertility in at least one of their matings. We further removed a small number of individuals that mated for less than 5 or greater than 40 minutes in any of their matings as these fall well outside the normal range.

Data were transformed to match the assumptions of ANOVA in the following: we log-transformed accessory gland area, and squared total sperm numbers and offspring production over 3-days post-mating. In all but the first case we opted to plot the untransformed data as we believe the raw data will be of interest. We winsorized data in two instances. Firstly, each of an extremely high and low outlier in our sperm count data, which held disproportionate influence in our models. Secondly, 4 extremely low (<-100) outliers in the offspring differential analysis. These points all fall in the no competition treatment and acted to exaggerate the magnitude of the effect we found. Winsorizing here thus represents a conservative decision.

We conducted all of our proteomics analysis on $\log_2$ transformed normalised abundances. Proteins were described as SFPs if they are known to be transferred to females based on a reference list kindly provided by Mariana Wolfner (Cornell

University, NY) and Geoff Findlay (College of the Holy Cross, MA) and updated to include the high confidence SFPs from Sepil *et al.* (Sepil et al. 2018). PCAs were conducted using the prncomp function in *stats*. Variables were scaled to have unit variance and shifted to be zero-centred. PCA plots were produced using *ggbiplot* (Vu 2011). Ellipses denote 80% normal probability, raised from the default 67%. We extracted scores for the first 3 PCs from the PCA dataframe to which we then fitted a linear model. Contributions to each PC were extracted using the 'fviz_contrib' function in the package *factoextra* (Kassambara and Mundt 2017). We produced heatmaps using an average across 5 replicates for each protein in the 6 treatment combinations. Heatmaps were generated using the *pheatmap* package (Kolde 2018). For visualisation of relative abundance patterns we divide each protein's normalised abundance value by the mean across all 30 samples for that protein. This allows for comparison between different SFPs across the dynamic range of SFP abundances. For our differential expression analysis, we iterated an ANOVA over all detected proteins across the 30 samples, controlling for group size, replicate, and mating status. We used a tail-based false discovery rate correction from the 'fdrtool' package (Strimmer 2008). Pairwise $\log_2$ fold changes use the mean across replicates for each individual SFP within a treatment combination. Fold changes are calculated according to $\chi_{i,j} = \chi_j - \chi_i$ where χ = virgin or mated and i and j = the group sizes being compared.

SUPPLEMENTARY MATERIAL

	PC1	*PC2*	*PC3*	*PC4*
Variance explained (%)	52.93	8.89	8.37	5.97
Eigenvalue	62.46	10.48	9.88	7.05
No. positive loadings	114	58	68	61
No. negative loadings	4	60	50	57

PC1 *Effect*	*Df*	*Sum of sq*	*RSS*	*F*	*P*
*Groupsize*Mating*	2	60.482	13.619	4.9315	0.0182
Groupsize	2	80.45	9.170	4.8322	0.0146
Mating	1	1491.76	1674.89	179.2129	<0.0001
Replicate	4	55.95	239.08	1.6804	0.1904

PC2 *Effect*	*Df*	*Sum of sq*	*RSS*	*F*	*P*
*Groupsize*Mating*	2	4.8592	118.34	0.4282	0.6575
Groupsize	2	137.893	256.23	12.8178	0.0002
Mating	1	4.868	123.21	0.9051	0.3518
Replicate	4	42.947	161.28	1.9960	0.1304

PC3 *Effect*	*Df*	*Sum of sq*	*RSS*	*F*	*P*
*Groupsize*Mating*	2	30.455	206.15	1.7335	0.2022
Groupsize	2	4.191	210.34	0.2236	0.8014
Mating	1	2.004	208.15	0.2138	0.6483
Replicate	4	74.114	280.26	1.9774	0.1333

Supplementary Table 1. Summary statistics from a PCA conducted on 118 detected SFPs

The first table gives the variance, eigenvalue, and loadings associated with the first four principal components (PCs). The next three tables give the output from linear models fitted to each of the first three PCs, using the measured variables of group size (1,2, or 8 males), mating status (mated or virgin), and replicate (5 in total). Significant associations at the $p<0.05$ levels are given in red.

	qval		Virgin fold change			Mated fold change				
Protein	Groupsize	Mating	V18	V28	V12	M18	M28	M12	Functional class	Mating-related process
Acp29AB	0.0280	0.0088	1.0247	0.9525	0.0722	-0.1013	0.1385	-0.2397	C-type lectin	Sperm competition
Acp53C14b	0.0422	0.0129	0.8605	1.3471	-0.4866	0.2556	0.0793	0.1763	Peptide/Prohormone	
antr	0.0030	0.0001	0.5307	0.3408	0.1900	0.1695	0.1584	0.0112	CRISP	SPN
aqrs	0.0350	0.0002	0.5992	0.4379	0.1613	0.2008	0.1301	0.0707	Serine protease	SPN
CG10651	0.0468	0.0139	-0.0270	0.6496	-0.6766	-0.4742	0.1953	-0.6695	CRISP	
CG10730	0.0081	0.0005	0.7607	0.6395	0.1212	0.2794	0.0303	0.2491	Alkaline-phosphatase	
CG11112	0.0015	0.0006	-1.1334	-0.5745	-0.5590	-2.9134	-2.4236	-0.4898	Peptide/Prohormone	
CG11608	0.0091	0.0001	0.6902	0.4270	0.2632	0.5759	0.4127	0.1632	Triglycerol lipase	
CG14034	0.0427	0.0002	0.5093	0.5329	-0.0237	0.2684	0.2113	0.0571	Phospholipase	
CG15116	0.0179	0.5027	0.5075	0.2713	0.2362	0.4173	0.1608	0.2565	Glutathione peroxidase	
CG15117	0.0195	0.0001	0.2742	0.5127	-0.2385	0.3695	0.2043	0.1652	Glycosyl hydrolases	
CG1652	0.0375	0.0002	0.5909	0.4788	0.1120	0.2432	-0.0123	0.2555	C-type lectin	SPN
CG1656	0.0034	0.0011	0.1702	0.4268	-0.2567	0.3956	0.2817	0.1139	C-type lectin	SPN
CG17843	0.0170	0.0001	0.7120	0.6895	0.0225	0.1280	0.0213	0.1067	Thioredoxin	
CG31413	0.0018	0.0001	0.5484	0.6114	-0.0629	0.1038	0.4179	-0.3141		
CG31418	0.0058	0.0001	-0.2645	-0.3228	0.0583	-0.4350	-0.2215	-0.2134	Peptide/Prohormone	
CG31419	0.0468	0.0005	-0.2888	-0.5869	0.2980	0.0245	-0.1950	0.2195		
CG31680	0.0288	0.0001	0.4513	0.1763	0.2750	0.0179	0.2209	-0.2030	Peptide/Prohormone	
CG31684	0.0126	0.0001	0.4958	0.5113	-0.0155	0.1907	0.0400	0.1507		
CG34129	0.0054	0.0001	0.4464	0.2718	0.1745	0.4391	0.2150	0.2241	Serine protease	
CG3640	0.0346	0.0125	0.5862	0.3709	0.2153	0.1954	-0.0176	0.2129	CRISP	
CG43111	0.0046	0.0002	1.1353	0.8502	0.2851	0.1947	0.4254	-0.2307		
CG5162	0.0052	0.0001	0.4184	0.3286	0.0898	0.4096	0.2451	0.1645	Lipase	
CG5267	0.0208	0.1638	-0.4204	-0.4382	0.0178	-0.2502	-0.5942	0.3440	Trypsin protease inhibitor	
CG6071	0.0008	0.0001	0.5891	0.5921	-0.0030	0.3134	0.3984	-0.0851	Metalloprotease	
CG9168	0.0004	0.0726	1.3438	1.3373	0.0065	0.9947	0.5916	0.4031	Phosphatase	
CG9519	0.0123	0.1779	0.7676	1.0329	-0.2652	0.3264	0.4545	-0.1281		
CG9997	0.0027	0.0003	0.3798	0.7352	-0.3553	0.4648	0.4333	0.0315	Serine protease	SPN
Cys	0.0083	0.0083	1.2176	0.5088	0.7088	0.4991	-0.1245	0.6236		
Hexo2	0.0015	0.0001	0.3545	0.1130	0.2416	0.4555	0.1985	0.2570		
lectin-29Ca	0.0020	0.0001	0.2151	0.8266	-0.6115	0.3025	0.3679	-0.0655	Lectin	
lectin-30A	0.0487	0.0001	0.1434	0.4122	-0.2688	0.1192	0.1250	-0.0058	Lectin	
mfas	0.0073	0.1730	0.3881	0.2934	0.0948	0.6320	0.1703	0.4617	Fasciclin	
Obp22a	0.0103	0.0001	0.3011	0.5881	-0.2871	-0.1908	0.2383	-0.4291	Oderant binding	
Phm	0.0106	0.0015	0.0138	0.5568	-0.5430	0.1236	0.1958	-0.0722		
regucalcin	0.0016	0.0001	0.5104	0.4427	0.0677	0.3378	0.1837	0.1541		
Sfp24F	0.0103	0.0001	0.4791	0.6907	-0.2116	0.2408	0.2234	0.0173	C-type lectin	
Sfp38D	0.0345	0.0001	1.0045	0.8296	0.1749	0.1022	-0.1665	0.2687		
Sfp51E	0.0484	0.0001	0.6289	0.3316	0.2974	-0.0015	0.1345	-0.1360		
Sfp78E	0.0048	0.0001	-0.1553	-0.2445	0.0892	-0.5872	-0.5704	-0.0168		
Sfp84E	0.0237	0.0172	0.4167	0.1967	0.2200	0.5778	0.5773	0.0004		
Spn28B	0.0057	0.0027	0.6312	0.4895	0.1418	0.5664	0.1483	0.4182	Serpin	
Spn28Db	0.0004	0.0001	-0.4854	0.3506	-0.8361	-1.2096	0.2132	-1.4228		
Spn75F	0.0031	0.0001	0.4453	0.4879	-0.0427	0.1969	0.1421	0.0548	Serpin	

Supplementary Table 2. SFPs detected as significantly differentially-expressed in response to competition intensity

44 SFPs all showing an FDR-corrected *p*-value of <0.05 in their association with competition intensity. q-values are calculated by applying a tail-based FDR correction to *p*-values obtained from a linear model iterated over each detected protein. q-values are given both for the effect of mating status (mated/virgin) and the group size males were reared in (1,2, or 8). Fold changes are given on a $\log_2$ scale and calculated for each group size comparison within a mating status. Fold changes are calculated according to $\chi_{i,j} = \chi_j - \chi_i$ where χ = virgin or mated and *i* and *j* are the first and second integers in the column header, respectively. Functional information associated with each protein's FlyBase entry is provided, along with whether a protein has a known role in sperm competition or in the sex peptide network ('SPNs').

A

Effect	*NumDf*	*Sum of sq*	*Mean sq*	*F*	*P*
*Groupsize*Mating number*	8	163.310	20.414	1.8477	0.0645
Groupsize	2	121.2	60.62	5.4604	0.0046
Mating number	4	4640.7	1160.18	104.5072	<0.0001
Replicate	1	27.4	27.35	2.4640	0.1174

B

	Estimate	*Std. Error*	*df*	*t value*	*Pr(>|t|)*
(Intercept)	19.6996	0.3486	1491	56.514	<0.0001
Groupsize 2	1.6700	0.4658	1580	3.586	0.0003
Groupsize 8	1.6637	0.4864	1404	3.421	0.0006
Mating 2	-4.0348	0.4383	1404	-9.205	<0.0001
Mating 3	-3.3043	0.4383	1404	-7.538	<0.0001
Mating 4	-2.7565	0.4383	1404	-6.288	<0.0001
Mating 5	-2.7565	0.4383	1404	-6.288	<0.0001
Run2	-0.3706	0.2361	350	-1.570	0.1174
*GS2*M2*	-0.3344	0.6018	1404	-0.556	0.5785
*GS8*M2*	-0.6074	0.6284	1404	-0.967	0.3339
*GS2*M3*	-1.5187	0.6018	1404	-2.524	0.0117
*GS8*M3*	-1.4112	0.6284	1404	-2.246	0.0249
*GS2*M4*	-0.7589	0.6018	1404	-1.261	0.2075
*GS8*M4*	-1.2985	0.6284	1404	-2.066	0.0390
*GS2*M5*	-1.6819	0.6018	1404	-2.795	0.0053
*GS8*M5*	-1.2343	0.6284	1404	-1.964	0.0497

Supplementary Table 3. Elevated mating duration in response to the presence of competition is lost after two matings

(Continues overleaf)

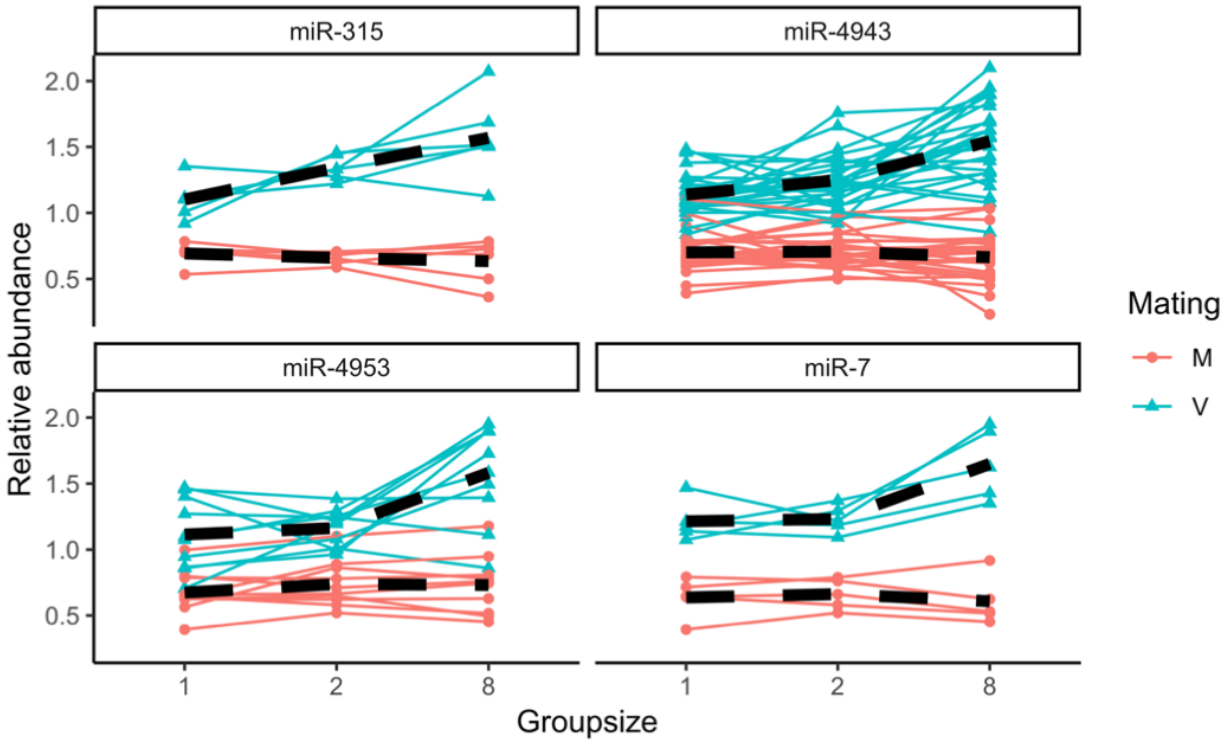
C

GROUPSIZE	*MATING*	*ESTIMATE*	*SE*	*DF*	*T.RATIO*	*P.VALUE*
1-2	1	-1.6700	0.4658	1580	-3.586	0.0010
1-8	1	-1.6637	0.4864	1580	-3.421	0.0019
2-8	1	0.0063	0.4726	1579	0.013	0.9999
1-2	2	-1.3356	0.4658	1580	-2.868	0.0117
1-8	2	-1.0563	0.4864	1580	-2.172	0.0764
2-8	2	0.2793	0.4726	1579	0.591	0.8250
1-2	3	-0.1513	0.4658	1580	-0.325	0.9435
1-8	3	-0.2525	0.4864	1580	-0.519	0.8621
2-8	3	-0.1012	0.4726	1579	-0.214	0.9750
1-2	4	-0.9111	0.4658	1580	-1.956	0.1236
1-8	4	-0.3652	0.4864	1580	-0.751	0.7332
2-8	4	0.5460	0.4726	1579	1.155	0.4803
1-2	5	0.0119	0.4658	1580	0.026	0.9996
1-8	5	-0.4294	0.4864	1580	-0.883	0.6512
2-8	5	-0.4413	0.4726	1579	-0.934	0.6189

Supplementary Table 3. Elevated mating duration in response to the presence of competition is lost after two matings

Mating duration was measured for each male over the course of five successive matings. Males had previously been held in groups of either 1,2, or 8. (A) Contribution and significance of fixed effects in a linear mixed effects model fitted to mating duration data. Male identity was included as a random effect. (B) Summary of the model given in (A). (C) Tukey-corrected post-hoc pairwise comparisons between each group size treatment within a particular mating. Significant values at the $p<0.05$ level are given in red. Those <0.1 are given in orange.

A



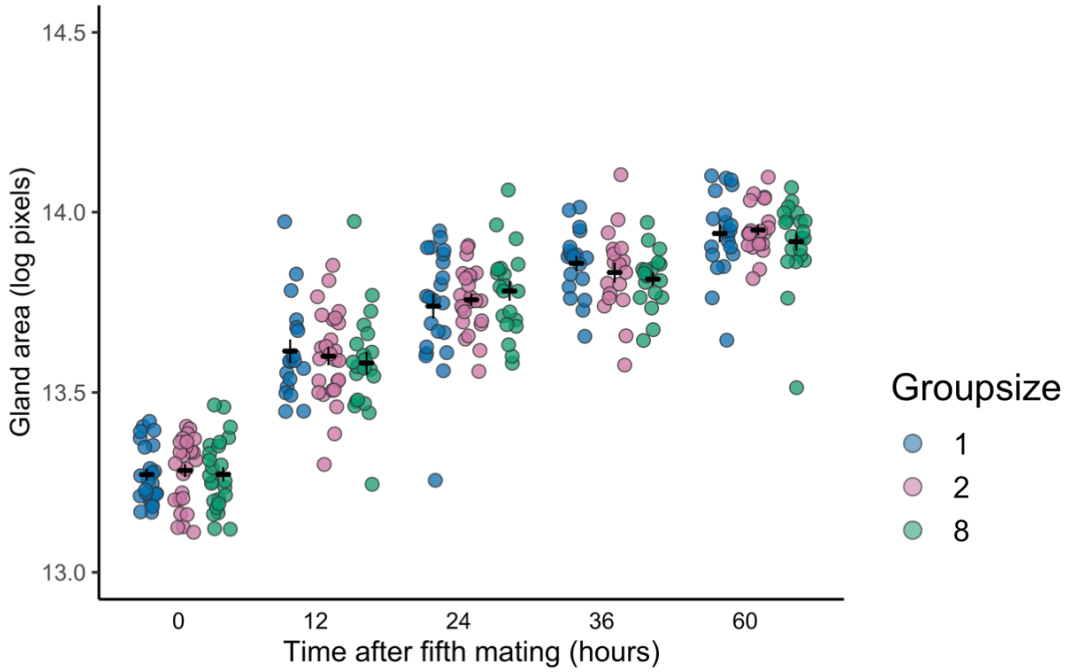
B

Effect	*NumDf*	*Sum of sq*	*Mean sq*	*F*	*P*
*Groupsize*Mating*miRNA*	6	1.0595	0.1766	0.5993	0.7312
*Groupsize*miRNA*	6	0.6227	0.1038	0.3528	0.9085
*Mating*miRNA*	3	0.6796	0.6796	2.3171	0.0740
miRNA	3	0.0023	0.0023	0.0077	0.9991
Replicate	4	3.8397	3.8397	13.0818	<0.0001
*Groupsize*Mating*	2	9.0465	9.0465	30.8211	<0.0001

Supplementary Analysis 1. No association between differentially-expressed SFPs and miRNA regulators

(A) Average abundance patterns for each of the detected SFPs for which a putative miRNA regulator has been proposed (Mohorianu et al. 2018) plotted in relation to group size, the miRNA suggested to regulate it, and separately for mated (red) and virgin (blue) glands. Relative abundances are produced by dividing the normalised values by the mean across all samples for each protein. An average response is given in dashed black lines. Where a SFP has multiple putative miRNA regulators it is plotted separately for each. (B) Output of the effects of measured variables from a linear mixed effects analysis. Significant *p*-values at the <0.05 levels are given in red, those below <0.1 are given in orange.

A



B

Effect	*Df*	*Sum of sq*	*RSS*	*AIC*	*F*	*P*
*Groupsize*Timepoint*	2	0.0038	6.8112	-1241.3	0.0884	0.9155
Groupsize	2	0.0144	6.8526	-1244.66	0.3364	0.71459
Timepoint	1	15.5517	22.3629	-858.16	728.3585	<0.0001
Replicate	1	0.0839	6.8951	-1239.38	3.9282	0.04834

Supplementary Analysis 2. Experience of competition does not affect the rate of post-mating accessory gland growth

(A) Accessory gland area (log pixels) of males held in different group sizes (1, blue; 2, pink; 8, green) and frozen at different time points following the end of five matings. Sample sizes, pooled across two replicates: 0 hours, N_1=24, N_2=26, N_8=25; 12 hours, N_1=18, N_2=26, N_8=21; 24 hours, N_1=22, N_2=24, N_8=20; 36 hours, N_1=19, N_2=18, N_8=18; 60 hours, N_1=21, N_2=21, N_8=21. (B) Output of a linear model giving the significance associated with each effect. Red denotes significance at the p<0.05 level.

Chapter 3

Seminal-fluid mediated sperm competition mechanisms revealed via manipulation of secondary cell BMP-signalling

TITLE

Seminal-fluid mediated sperm competition mechanisms revealed via manipulation of secondary cell BMP-signalling

ABSTRACT

Competition between ejaculates for fertilisations is thought to exert strong selection on their design. As well as acting on the morphology and number of sperm, we expect it to also influence the evolution of the non-sperm portion of the ejaculate, the seminal fluid. In *Drosophila melanogaster*, the ultimate outcome of sperm competition is dependent upon performance in a number of distinct post-copulatory episodes, beginning with the initial entry of sperm into storage through to the termination of displacement by a second male ejaculate. To what extent components in the seminal fluid can exert influence at these many stages is poorly-known. To this end, we test whether loss of BMP-dependent secretory activity in a seminal fluid contributing cell-type affects male sperm competition performance. As in previous work, we find a curious decoupling of key post-mating responses in females mated to males with disrupted secondary cell activity: normally stimulated offspring production, but high receptivity to remating. We further show that the loss of secondary cell secretions enhances defensive (P1), but not offensive (P2), sperm competition performance. This effect ultimately arises through a syndrome of dysregulation affecting both that male's sperm and how the female treats a second partner and his ejaculate. Our work represents an important step in exposing the varying degrees of interconnectedness and independence that exist both within ejaculates and the female post-mating neural circuitry.

INTRODUCTION

Where sperm from multiple males overlap in the female reproductive tract, the opportunity for male-male competition continues beyond mating (Parker 1970). Accordingly, ejaculates should be 'designed' (*sensu* Gardner, 2009) to promote the fertilisation success of a male under competitive conditions. Theory and empirical work alike supports the action of selection on both the number and morphology of sperm in response to competition (Parker et al. 1996, 2010; Gage and Morrow 2003; Immler et al. 2007, 2011; Fisher and Hoekstra 2010; Parker and Pizzari 2010; Lupold et al. 2016). But ejaculates are more than sperm, being further composed of a complex cocktail of proteins, lipids, vesicles, and much else besides (Poiani 2006; Hopkins et al. 2017). How these different portions of the ejaculate work together to collectively promote male fertilisation success is less well-understood.

In the fruit fly *Drosophila melanogaster*, the outcome of sperm competition is determined by performance across several post-copulatory episodes. Firstly, the number of sperm that enter into the female sperm storage organs - reduced sperm entry being associated with lower sperm competition performance (Chapman et al. 2000). Secondly, the rate the female uses sperm prior to re-mating (*e.g.* Chapter 2), which determines the number of sperm that will experience competition, or whether she remates at all. Thirdly, the degree of displacement by an incoming ejaculate, a process known to occur in two distinct phases, one dependent upon sperm the other acting independently (Manier et al. 2010), and ultimately terminated by the female ejecting the residual ejaculate (Lupold et al. 2013; Lee et al. 2015). A mechanistic role for seminal fluid proteins ('SFPs') in mediating this latter episode has yet to be demonstrated, but involvement of SFPs in the first two processes is known: Acp36DE is required to corral sperm into storage (Bloch Qazi and Wolfner 2003; Avila and Wolfner 2017), while sex peptide reduces female

receptivity to remating and stimulates the release of sperm from storage for use in fertilisations (Chapman et al. 2003; Liu and Kubli 2003; Avila et al. 2010; Gligorov et al. 2013).

Where other SFPs have been identified that affect sperm competition outcome, their mechanism of action generally operates through sex peptide. The long-term persistence of sex peptide mediated phenotypes depends on the entry of the protein into the female storage organs, its binding to sperm, and subsequent gradual cleavage from sperm (Manning 1962; Peng et al. 2005a). This process is coordinated by at least eight 'sex peptide network' proteins (Ram and Wolfner 2007b, 2009; Findlay et al. 2014; Singh et al. 2018). Loss of these proteins or abnormalities in their post-translational modification is associated with dramatic shortening or loss of sex peptide induced phenotypes, including defects in the release of sperm from storage and lowered offspring production (Ram and Wolfner 2009; Gligorov et al. 2013). Consequently, and as in sex peptide nulls, their loss leads to improved sperm competition performance, largely because females have used fewer sperm between matings (Avila et al. 2010). A rare example of an SFP that affects sperm competition performance independently of changes in offspring production is Acp62F, but its mechanism of action is unknown (Mueller et al. 2008). SFPs falling into this category are likely to be of particular interest as they may specifically modulate the capability of sperm to displace those in residence or the capacity to themselves resist displacement.

In *D. melanogaster*, the accessory glands produce the majority of the 200 seminal fluid proteins ('SFPs') transferred to females. Disruption to the secretory activity of the minority gland cell-type (the 'secondary cells') in adults does not affect the fecundity of a male's mates (Leiblich et al. 2012). Surprisingly, however, they still show the typical post-mating reduction in receptivity to remating – a curious decoupling of sex peptide

mediated phenotypes (see also Fricke, Bretman and Chapman, 2010; Haussmann *et al.*, 2013; Fricke *et al.*, 2014). Conversely, developmental abnormalities in these cells are associated with more severely compromised post-mating phenotypes, including low offspring production (Minami et al. 2012; Gligorov et al. 2013). In at least one developmental mutant this is known to be due to aberrant processing of sex peptide network proteins (Gligorov et al. 2013). The driver of phenotypic discordance between developmental and secretory defects in the secondary cells is unclear, but it may relate to the former disrupting channels of communication between the gland's constituent cell-types, leading to broader glandular abnormalities (Chapter 4), or the latter only affecting a subset of secretory pathways used by the cells, specifically those that are driven by BMP-signalling. Some of these BMP-dependent secretory pathways are non-conventional, encapsulating material within extracellular vesicles called exosomes that fuse with sperm, associate with the female reproductive tract, are required to reduce female receptivity to remating, but play no part in driving female fecundity (Corrigan et al. 2014). Ejaculate components packaged within exosomes may be particularly likely to function in sperm competition as packaging could promote their targeting to reaction sites or protect sexually-antagonistic molecules from degradation by females or the ejaculates of other males.

To test whether BMP-regulated secondary cell secretions contribute to sperm competition, we expressed a BMP-antagonist (*Daughters-against-Decapentaplegic, 'Dad'*) specifically in adult secondary cells (Leiblich et al. 2012; Corrigan et al. 2014; Redhai et al. 2016). We find that sperm competition performance is increased following disruption to the secondary cells, without differences in offspring production rate, suggesting a degree of independence from the actions of sex peptide. To assess what underlies this improved sperm competition performance, we undertake a multi-stage

dissection of the contributions made by these cells to individual determinants of sperm competition outcome. Specifically, we test the hypotheses that loss of secondary cell products influence sperm competition outcome via:

(1) Enhanced resistance of sperm to displacement by a second male ejaculate, which may manifest through
    - resistance to the displacing effects of second male seminal fluid
    - premature termination of displacement by a second male ejaculate via early female sperm ejection
    - delayed entry of second male sperm into storage

(2) Increased sperm retention after an initial mating, which may manifest through
    - greater sperm transfer
    - more efficient sperm storage
    - delayed termination of sperm storage by females

METHODS

Fly stocks

To generate males with disrupted secondary cell secretion, we crossed esgF/O^{ts} flies (genotype: *w; esg-GAL4 tub-GAL80^{ts} UAS-FLP/CyO; UAS-GFP$_{nls}$ actin>FRT>CD2>FRT>GAL4/TM6)* to w^{1118} flies into which a *UAS-Dad* transgene had been backcrossed (hereafter, '*UAS-Dad'* males). For controls, we crossed esgF/O^{ts} flies to flies from a w^{1118} background (hereafter 'control' males). The *esg-GAL4* system incorporates a temperature-sensitive GAL80, which inhibits GAL4 and suppresses the activation of *UAS-Dad* expression at non-permissive temperatures. Consequently, by rearing flies at this temperature we can minimise disruption to the developing gland.

When the temperature exceeds 28.5°C, GAL4 is released to drive FLP-recombinase mediated recombination, which places GAL4 expression under the control of an actin promoter. This leads to constitutive expression of *UAS-Dad* in secondary, but not main, cells (Leiblich et al. 2012). To generate males additionally bearing fluorescent sperm, we separately crossed *UAS-Dad* and w^{1118} flies to a line bearing the *GFP-ProtB* construct, which marks the heads of sperm and allows for easy counting of sperm (Manier et al. 2010). We backcrossed the resulting *GFP-ProtB*; *UAS-Dad* and *GFP-ProtB* in w^{1118} into their non-GFP equivalents for 6 generations to minimise any genetic background effects. We verified that these GFP-transformed lines show the same phenotype (high post-mating receptivity, normal fecundity) as the untransformed lines (Supp. Fig. 1). GFP transformed flies are reared and used in exactly the same way as their non-GFP counterparts.

In sperm competition assays, we used competitor males from a Dahomey wild-type stock into which the spa^{pol} recessive allele had previously been backcrossed for four generations. Flies homozygous for spa^{pol} display a clear phenotypic marker of glassy, deep-red coloured eyes upon which paternity can be assigned. Where GFP-sperm transformed stocks are used, we used spa^{pol} males into which we had backcrossed the *RFP-ProtB* construct (Manier et al. 2010) as competitors. All females used in experimental matings were also from a spa^{pol} stock. Spermless males were generated by crossing spa^{pol} males to *tud1,bw1,sp1* 'Tudor' females. These 'son-of'Tudor' males lack a functioning germline, and therefore fail to produce sperm, but still transfer SFPs (Boswell and Mahowald 1985; Xue and Noll 2000).

Fly husbandry

All crosses to generate *UAS-Dad* or control males were conducted at 20°C on grape-agar plates, supplemented with a blob of yeast paste. We transferred approximately 200 eggs at a time from the plate into 250mL bottles containing 50mL of Lewis medium (according to methods outlined in Clancy and Kennington, 2001). These we left to develop at a non-permissive temperature of 20°C. Upon eclosion, we collected males under ice anaesthesia and separated them into groups of 8 to 12 in 36mL Lewis medium-containing plastic vials, supplemented with *ad libitum* yeast granules. To activate the expression of *UAS-Dad* (where present), we immediately moved these vials to 30°C where they remained for the full duration of experiments. To verify that phenotypes were specifically attributable to *UAS-Dad* expression, we repeated some experiments at a non-permissive temperature of 20°C. In these experiments, flies were moved to 20°C after eclosion where they remained for the full duration of experiments. The day before using *UAS-Dad* or control males in experiments, we gave each three matings to virgin females. We undertook this to deplete, as much as possible, the accessory gland lumen of any secondary cell products produced prior to the activation of the *UAS-Dad* transgene. We delivered a single female at a time, removing the female after mating. Following the end of the third mating, we moved the male to a fresh, yeast-supplemented vial.

The rearing, collection, and grouping of flies from all other lines was performed following the methods outlined above. However, in these cases rearing was conducted at 25°C with us moving flies to 30°C the evening before use in experiments. We reared all flies and performed all experiments in controlled-temperature rooms on 12:12 light:dark cycles. All flies were between 3 and 5 days old at the time of first experimental mating. We always matched the age distribution of the *UAS-Dad* and control males used in experiments.

Sperm competition outcome and post-mating response assays

We used a P1 double-mating assay to test for an association between defensive sperm competition outcome and secondary cell products. Defence refers to the ability of resident sperm to resist displacement by an incoming ejaculate (Boorman and Parker 1976). We aspirated single *UAS-Dad* or control males into yeasted vials containing an individual virgin spa^{pol} female. We monitored all matings, recording the time males were introduced, mating began, and when mating finished. From this data we calculated the duration of and latency to mating. Once matings ended, we disposed of the males and left the females in the mating vials to oviposit. Thus, we have a record of the offspring produced between matings, which we plot as 'day 1' in Figure 2a.

The following morning, we aspirated the mated females into a yeasted vial containing a pair of spa^{pol} males, grouped under ice anaesthesia the previous day. Again, we monitored all matings and recorded duration and latency. We introduced females in the order they had finished mating the previous day. Previous work has shown that *UAS-Dad* mated females remain highly receptive to remating (Leiblich et al. 2012), so we staggered the introduction of *UAS-Dad* mated females to minimise any systematic difference between treatments in inter-mating interval. Following the end of mating, we discarded the two males and moved the females to 25°C, transferring them into a fresh, yeasted vial every 24-hours. We allowed the resulting progeny to develop, freezing the vials after the adults eclosed. We then counted offspring and scored their eye phenotype in order to assign paternity. By adopting this same approach but reversing the mating order, such that *UAS-Dad* and control males mated secondarily to spa^{pol} mated females, we tested for an association with offensive sperm competition performance (P2) – that is, the ability to displace resident sperm. We performed three replicates of a repeat of the P1 experiment conducted entirely at a non-permissive temperature of 20°C.

We obtained P1 data across 6 experimental replicates. In each of these, we collected offspring for at least 24-hours after the female's second mating. In one replicate, we collected offspring for 6 days to test for the persistence of any detected differences. Within four of these replicates, we varied the identity of the second mating male. Here, prior to first mating to a *UAS-Dad* or control male, females were randomly assigned (a) no second mating, (b) a spa^{pol} second mating, or (c) a spermless, *son-of-Tudor* mating. In these variants, we collected offspring over four days after second mating to gain additional information relating to short- and longer-term patterns of fecundity stimulation.

Female ejection assays

In *D. melanogaster,* females eject the ejaculate from the uterus within 1 to 6 hours after mating (Lee et al. 2015). We followed the P1 experimental setup outlined in the preceding section, but moved females to 3D-printed, black plastic chambers immediately after a first or second mating. These chambers, of printing resolution 0.2mm, were cuboids of 34mm x 33mm x 9mm with a half-sphere concavity of dimensions 20mm x 20mm x 7mm. We used a glass coverslip to cover the concavity once a female had been introduced. We checked each chamber for the presence of an ejected sperm mass every ten minutes under a light microscope. We ran this experiment four times: twice for each of the females first (*UAS-Dad* or control) and second (spa^{pol}) mating. Data from two replicates were pooled within a mating order for analysis.

Post-insemination sperm dynamics assays

To test for a role of secondary cell products in driving patterns of female sperm use, we used the same P1 experimental design previously outlined but with *GFP-ProtB*

transformed *UAS-Dad* and control lines. We performed two separate experiments using these flies. In the first, we flash-froze in liquid nitrogen *UAS-Dad* or control mated females either 25-minutes or 5-hours after the start of their first mating to a *UAS-Dad* or control male. In the second experiment, we froze females 10-minutes or 24-hours after the start of a second mating to a *RFP-ProtB* transformed spa^{pol} male. We repeated experiment two twice and pooled the data for analysis. In each of these two experiments, we randomly assigned females a freezing time prior to mating.

We stored frozen females at -80°C until dissection, which we performed under light microscope in a drop of PBS. We retained the female reproductive tract from the vulva through to the common oviduct, sealed the slides using (Fixogum, Marabu), and stored slides at 5°C. We imaged the slides using a Zeiss 880 confocal microscope and processed the images by taking an average intensity Z-projection in the Fiji distribution of ImageJ (Schindelin et al. 2012) to condense Z-stacks into a single image for easier counting. We manually counted sperm using the multi-point tool in Fiji. We performed all dissections and sperm counts blind to treatment. We omitted any samples that showed no GFP sperm due to the possibility of heterozygosity for the *GFP-ProtB* chromosome in our stock populations.

Statistical analysis

All analyses were conducted in R statistical software (version 3.5.1)(Team 2013) in RStudio (version 1.1.456)(RStudio Team 2015). We used linear models wherever possible and assed the significance of variables by dropping individual terms from the full model using the 'drop1' function. Where the interaction term was non-significant we refitted the model without it. We determined model fit by visual inspection of diagnostic plots, as is generally recommended (Zuur et al. 2010). Where multiple measurements

were taken from the same female, as in analyses of day-by-day female fecundity, we used linear mixed effects models that accounted for female identity as a random effect. In our day-by-day analysis of female fecundity, our starting model contained a three-way interaction (male 1 x male 2 x day) along with two random effects (replicate and female ID). We used a stepwise algorithm ('step' function) to identify the best model by AIC. Associated *p*-values were generated using Satterthwaite's method. To analyse latency to mating and ejection, we ran Cox proportional hazard models using the *survival* package (Therneau and Grambsch 2000; Therneau 2015) and graphed the results using 'ggsurvplot' in the *survminer* package (Kassambara and Kosinski 2018). We analysed proportional data, relevant for paternity shares (P1 and P2) and some sperm count data, using generalised linear models. In all cases, we used a quasibinomial extension to account for overdispersion, which was detected by comparing the residual deviance to the degrees of freedom. In all analyses, we excluded the small number of individuals that failed to produce offspring between a first and second mating as these are assumed to be failed matings. In our analysis of first male sperm retention after a second mating, we winsorized one extreme outlier found to exert disproportionate leverage in our models.

RESULTS AND DISCUSSION

Disruption of secondary cell activity promotes defensive, but not offensive, sperm competition performance

We found that female receptivity to remating was substantially higher if females had previously mated to a *UAS-Dad* male (Coxph: *LRT*=69.935, $p<0.0001$; Fig. 1a), an effect that was lost at a non-permissive temperature (Coxph: *LRT*=0.001, $p=0.981$; Fig. 1b). Thus, we support previous findings that BMP-regulated secondary cell secretions are required for the normal post-mating reduction in receptivity (Leiblich et al. 2012; Corrigan et al. 2014).We further found that *UAS-Dad* males gained significantly higher P1 paternity shares in the clutch produced by females in the first 24-hours after remating (GLM: *Dev.*=8953.8, $p=0.034$; Fig. 1c). This effect was still present, and magnified, in clutches produced at 6-days post second mating (GLM: *Dev.*= 8296.7, $p=0.0003$; Fig. 1d). Elevated P1 isn't seen in *UAS-Dad* males reared at a non-permissive temperature, suggesting that the effect is directly linked to BMP-suppression in secondary cells (GLM: *Dev.* =7933.5, $p=0.630$; Fig. 1e). We found no effect of *UAS-Dad* on paternity share when the mating ordering was reversed (P2)(GLM: 24-hours, *Dev.*=1453.1, $p=0.567$; Fig. 1f; 4-days, *Dev.* =2776.6, $p=0.771$).

Female propensity to remating is linked to the density of sperm in storage (Manning 1962). As only a proportion of control-mated females remate, they may therefore represent a biased subset with fewer sperm in storage to begin with. If so, this effect might be driving the difference in P1. However, we found no significant correlation between female latency to remating and P1 either overall (GLM: *Dev.* = 9963.3, $p = 0.971$; Supp. Analysis 1) or as an interaction with male genotype (GLM: *Dev.* = 9963.2, $p = 0.909$; Supp. Analysis 1).

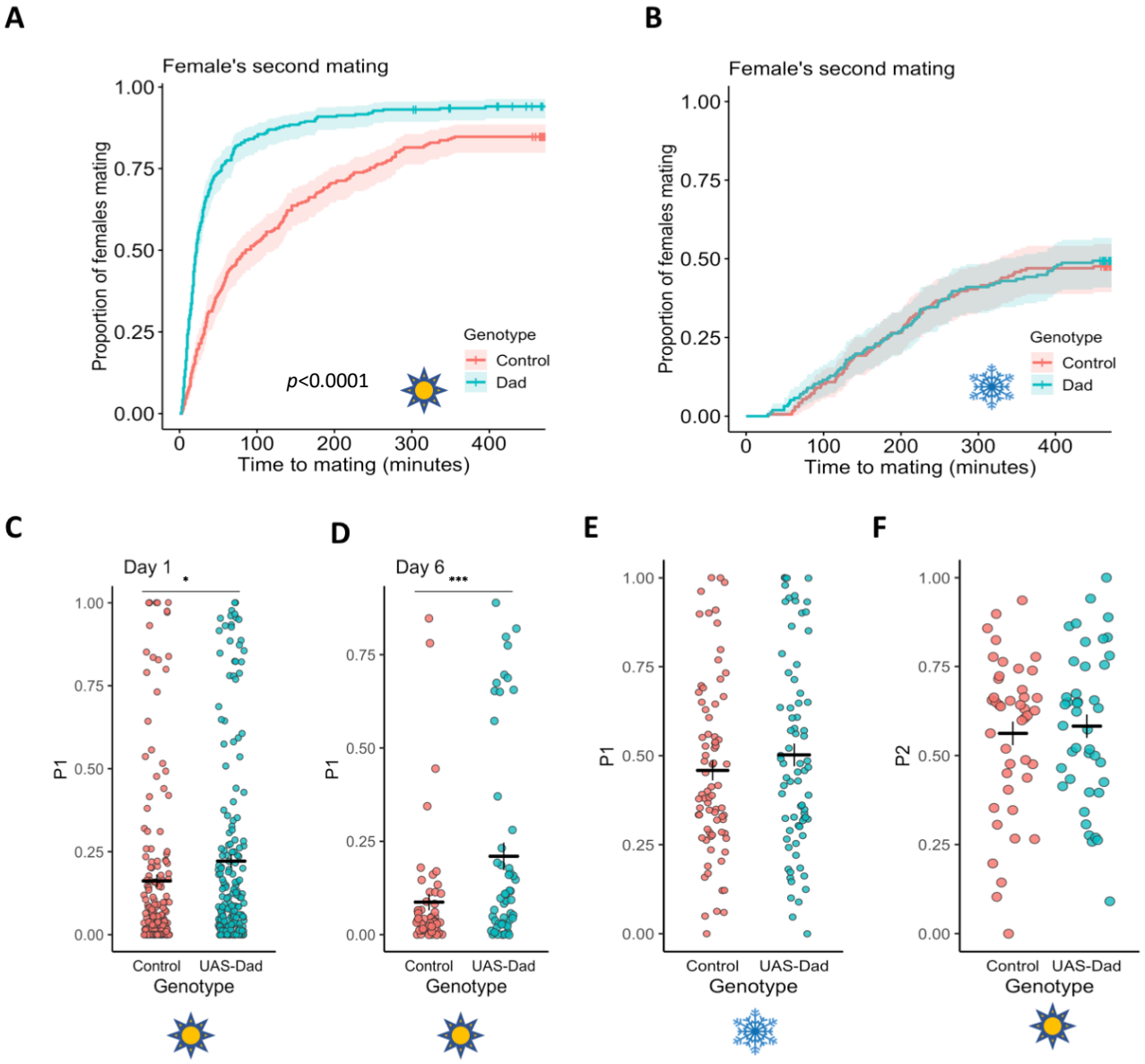


Figure 1. *UAS-Dad* males show improved performance in defensive sperm competition

(A) Latency to remating of females previously mated to a *UAS-Dad* or control male, censored according to whether a female remated. n_{Dad}=276, $n_{control}$=275. (B) As in (A) but at a non-permissive temperature of 20°C. n_{Dad}=156, $n_{control}$=166. (C) First male paternity share according to whether a female first mated to a *UAS-Dad* or control male. Offspring collected over the 24-hours following remating to a standardised competitor. (D) as (C) but offspring collected in a 24-hour period 6 days after the female remated. (E) As in (C) but at a non-permissive temperature of 20°C. (F) as in (B) but with the reverse mating order *i.e. UAS-Dad* or control male mates second. Suns indicate experiments conducted at 30°C; snowflakes, 20°C. * = p<0.05, ***=p<0.0005. In A-B, confidence intervals are at 95%. In C-F, bars give the mean ± 1 SE.

Displacement mechanism 1: *UAS-Dad* mated females show normal offspring production and *UAS-Dad* sperm are not more resistant to sperm-independent displacement

Displacement of resident sperm by an incoming ejaculate is known to occur in two episodes: a sperm-independent and a sperm-dependent episode (Manier et al. 2010; Lupold et al. 2013). To test for enhanced resistance of *UAS-Dad* sperm to sperm-independent displacement, we measured offspring production following a female secondarily receiving (a) no further mating, (b) a spa^{pol} male, or (c) a spermless *son-of-Tudor* male. If *UAS-Dad* male sperm are more resistant to sperm-independent episodes of displacement, then we would expect females mated to a *UAS-Dad* male followed by a spermless male to produce more offspring relative to controls followed by a spermless male. This would arise through *UAS-Dad* mated females retaining more first male sperm after receiving a spermless second ejaculate. Consequently, this effect would not show when a female receives no second mating. A difference in offspring production would also not be apparent when receiving a normal sperm and seminal fluid carrying second ejaculate (*e.g.* that from a spa^{pol} male), because second male sperm would make up for any first male sperm lost.

We detected no significant effect of *UAS-Dad* on day-by-day offspring production across second mating treatments (Supp. Table 1; Figure 2a). This suggests that *UAS-Dad* sperm are not more resistant to seminal fluid specific mechanisms of displacement. Moreover, the normal offspring production of *UAS-Dad* mated females is consistent with previous reports that secondary cell secretions do not affect female fecundity (Leiblich et al. 2012). Consequently, it seems likely that the improvement in defensive sperm competition performance in *UAS-Dad* mated females is driven by different mechanisms

than failures in sex peptide storage and release (as in Avila et al. 2010; Gligorov et al. 2013).

More generally, this analysis also reveals that females produce more offspring if they remate regardless of first male genotype, but that this effect is, for the most part, dependent upon second male sperm receipt (LME: $F_{8,2027}$=4.070, p<0.0001; Supp. Table 1; see also Lefevre and Jonsson 1962; Nguyen and Moehring 2018). Interestingly, females just receiving seminal fluid from a spermless second male produce more offspring than if they do not remate, but only during the 24-hours after remating (Day 2, None-Tudor, t_{1937}=-3.281. p=0.003). This short-term acting seminal fluid effect on offspring production is likely to be driven by fecundity-stimulating SFPs known to function independently of sperm, such as ovulin (Heifetz et al. 2005).

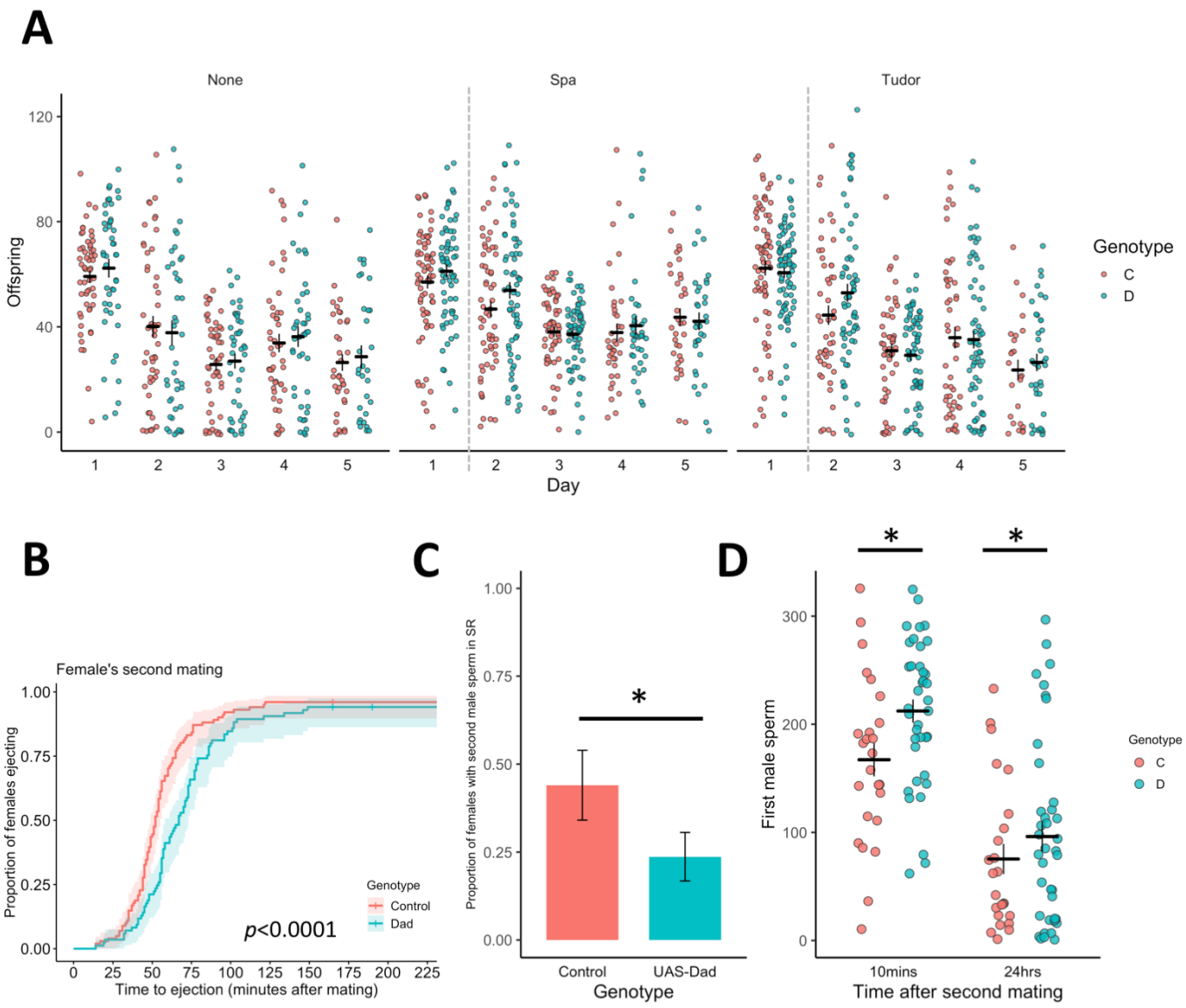


Figure 2. *UAS-Dad* mated females show normal fecundity, but altered use of a second male ejaculate

(A) Day by day offspring production according to whether a female first mated to a *UAS-Dad* (blue circles) or control (red circles) male, plotted separately for each of three second mating treatments: no second mating, a standardised *spa*^{pol} competitor, or a *son-of-Tudor* spermless mating. The dashed grey line gives the time point of second mating where applicable. (B) Time until ejection of a second male's ejaculate by females previously mated to a *UAS-Dad* (blue) or control (red) male. (C) The proportion of females where second male sperm has entered into storage 10-minutes after mating. (D) The number of first male sperm across the seminal receptacle and uterus 10-minutes and 24-hours after a female remates to a standardised competitor having previously mated to a *UAS-Dad* (blue) or control (red) male. * denotes $p<0.05$. In A,C and F, the bars give the mean ± 1 SE. In B, the confidence intervals are at 95%.

Displacement mechanism 2: *UAS-Dad* mated females do not terminate sperm displacement early

The second episode of resident sperm displacement by an incoming ejaculate is dependent upon second male sperm receipt. The degree of displacement is known to be linked to the length of time the second male's ejaculate is held by the female, which she ejects at a variable time point after mating (Lupold et al. 2013; Lee et al. 2015). Accordingly, we predicted that *UAS-Dad* mated females would eject quicker, thereby curtailing the degree of displacement, and boosting first male sperm retention. Contrary to expectation, we found that *UAS-Dad* mated females were significantly slower to eject second male sperm (Coxph: LRT=17.981, p<0.0001; Fig. 2b). Thus, *UAS-Dad* males succeed in P1 in spite of differences in female second male ejection, not because of them.

Displacement mechanism 3: Entry of second male sperm into storage is slower in *UAS-Dad* mated females

While the opportunity for displacement of resident sperm is bounded at one end by ejection, it will also be related to the entry of second male sperm into storage. We found that 10-minutes after mating, the proportion of females where second male sperm had entered into the seminal receptacle was significantly lower in *UAS-Dad* mated females (GLM: *Dev.* = 75.9, p=0.039; Fig. 2c), which could slow the initiation of displacement. At this stage, however, we found no significant difference between *UAS-Dad* and control mated females in the proportion of first male sperm that had been displaced from storage (GLM: *Dev.* = 499.77, p = 0.242; Supp. Fig. 2), but we predicted that any difference would be expected to accumulate throughout the course of the retention of the second male's ejaculate. To test this, we compared the number of first male sperm (*UAS-Dad* or control) in the female at both 10-minutes and 24-hours after second mating. We predicted

that the number of *UAS-Dad* sperm would show a shallower decline between these two-time periods as a result of lower displacement. Contrary to expectation, we found no evidence for an interaction between genotype and time-point after mating (LM: $F_{1,121}$=0.757, p=0.386; Fig. 2d). However, we detected that the total number of first male sperm was significantly higher in *UAS-Dad* mated females across both time points (LM: $F_{1,121}$=5.315, p=0.023; Fig. 2d). Therefore, while *UAS-Dad* sperm may show some enhanced resistance to displacement, sperm are already in greater quantities at the start of second mating.

As part of this analysis, we retained those control-mated females that failed to remate in order to test whether they produced greater offspring numbers than those that did. Greater offspring production may indicate that non-remating females hold more first male sperm and their absence from our sperm competition dataset would consequently bias our analyses towards females holding fewer. However, we found no significant difference in offspring production in the 24-hours following first mating between rematers and non-rematers (Supp. Analysis 2).

We next undertake a series of analyses to determine why *UAS-Dad* mated females enter into their second mating with greater numbers of sperm in storage.

Retention mechanism 1: *UAS-Dad* sperm are not transferred in greater numbers, and show slower entry into storage

The initial transfer of more sperm by *UAS-Dad* males could underlie an elevated P1. To test this hypothesis, we counted sperm in females frozen 25-minutes after the start of an initial mating with control or *UAS-Dad* males. We found no significant difference between the two male treatments (LM: $F_{1,55}$= 1.759, p=0.190; Fig. 3a). However, the

proportion of transferred sperm in storage at this point was significantly lower in *UAS-Dad* mated females (GLM: *Dev.* = 4079.4, $p = 0.007$; Fig. 3b).

Retention mechanism 2: *UAS-Dad* males show lower sperm storage

Despite transferring equivalent sperm numbers, *UAS-Dad* sperm may be stored in greater numbers. To test this, we counted sperm within the storage organs of females frozen 5-hours after mating to a *UAS-Dad* or control male. Contrary to expectation, we found that the total number of sperm that make it into storage is significantly lower in *UAS-Dad* mated females (control, 414±34; *UAS-Dad,* 301 ± 50; LM: $F_{1,53}$= 5.043, p=0.029; Fig. 3c) – a surprising result given their elevated P1. Mechanistically, this could be driven by defective activity of Acp36DE, which is known to promote sperm storage (Neubaum and Wolfner 1999; Bloch Qazi and Wolfner 2003; Adams and Wolfner 2007; Avila and Wolfner 2009, 2017). In keeping with this, recent proteomics work has suggested that *UAS-Dad* males transfer smaller quantities of Acp36DE to females (Chapter 4). Moreover, the seminal fluid of *UAS-Dad* shows broader compositional change (Chapter 4), raising the possibility that unknown SFP regulators of sperm storage that act either independently or in association with Acp36DE may also be reduced. Indeed, Acp36DE is known to depend on the presence of at least one other SFP for normal functioning (Avila and Wolfner 2017).

We also detect some sperm outside of storage in around a third of females frozen 5-hours after the start of mating. Of the 17 instances in which sperm were detected outside of storage, only 5 were from *UAS-Dad* mated females (Fig. 3d), which may suggest lower release of *UAS-Dad* sperm. However, in this small snapshot of samples, the difference between male genotypes was non-significant (GLM: *Dev.* = 68.021, p=0.112; Fig. 3d). Lower sperm loss between matings from *UAS-Dad* mated females could underlie the

switch from lower to higher numbers of sperm in storage relative to control-mated females that we detect occurs in the 24-hours following a first mating. This loss would have to be independent of the number of sperm used in fertilisations as *UAS-Dad* and control mated females produce equivalent offspring numbers. Instead, it could operate through *UAS-Dad* mated females using sperm more efficiently *i.e.* fewer sperm released per fertilisation. This would suggest an inherent leakiness in normal sperm storage that *UAS-Dad* sperm evade. Such a phenomenon may be related to the disconnect in the fecundity and receptivity post-mating phenotypes in *UAS-Dad* mated females: *UAS-Dad* mated females may fail to recognise that they are mated and consequently fail to release sperm normally despite the fecundity part of her being normally stimulated, unlike in *SP* knockdown (Chapman et al. 2003).

Retention mechanism 3: *UAS-Dad* mated females do not terminate sperm storage early

As the timing of ejection after a second mating is known to influence the degree of displacement, we predicted that it may similarly affect the quantity of sperm taken into storage in a first mating. If so, more rapid ejection by females after mating with a *UAS-Dad* male may prematurely terminate the process of sperm storage, thereby curtailing the number that make it in. Moreover, normal ejection requires the formation of the mating plug, for which Acp36DE is required (Lung and Wolfner 2001; Avila et al. 2015), providing a partial test of the hypothesis that the defective action of this protein underlies the sperm storage defect we detect. However, we found no significant difference between *UAS-Dad* and control-mated females in the timing of ejection (Coxph: LRT=0.892, p=0.345; Fig. 3e). Given that this phenotype is consistent with the normal formation of a mating plug, suggesting normal Acp36DE activity, an alternative mechanism underlying

the sperm storage defect could relate to the loss of secondary cell derived exosomes, the prostate-derived equivalent of which in mammals is known to fuse with sperm and stimulate motility (Aalberts et al. 2013). Given that *D. melanogaster* secondary cell derived exosomes are secreted under the control of BMP-signalling (Corrigan et al. 2014), it may be that their loss in *UAS-Dad* males similarly reduces sperm motility, negatively impacting their entry into storage. If true, then the lack of fecundity differences we detect would suggest that sperm motility is unimportant for fertilisation or that long-term motility is influenced by different, perhaps female-derived factors. However, we would expect the loss of motility-stimulating factors to equally compromise *UAS-Dad* entry into storage in mated females, which our P2 data does not support.

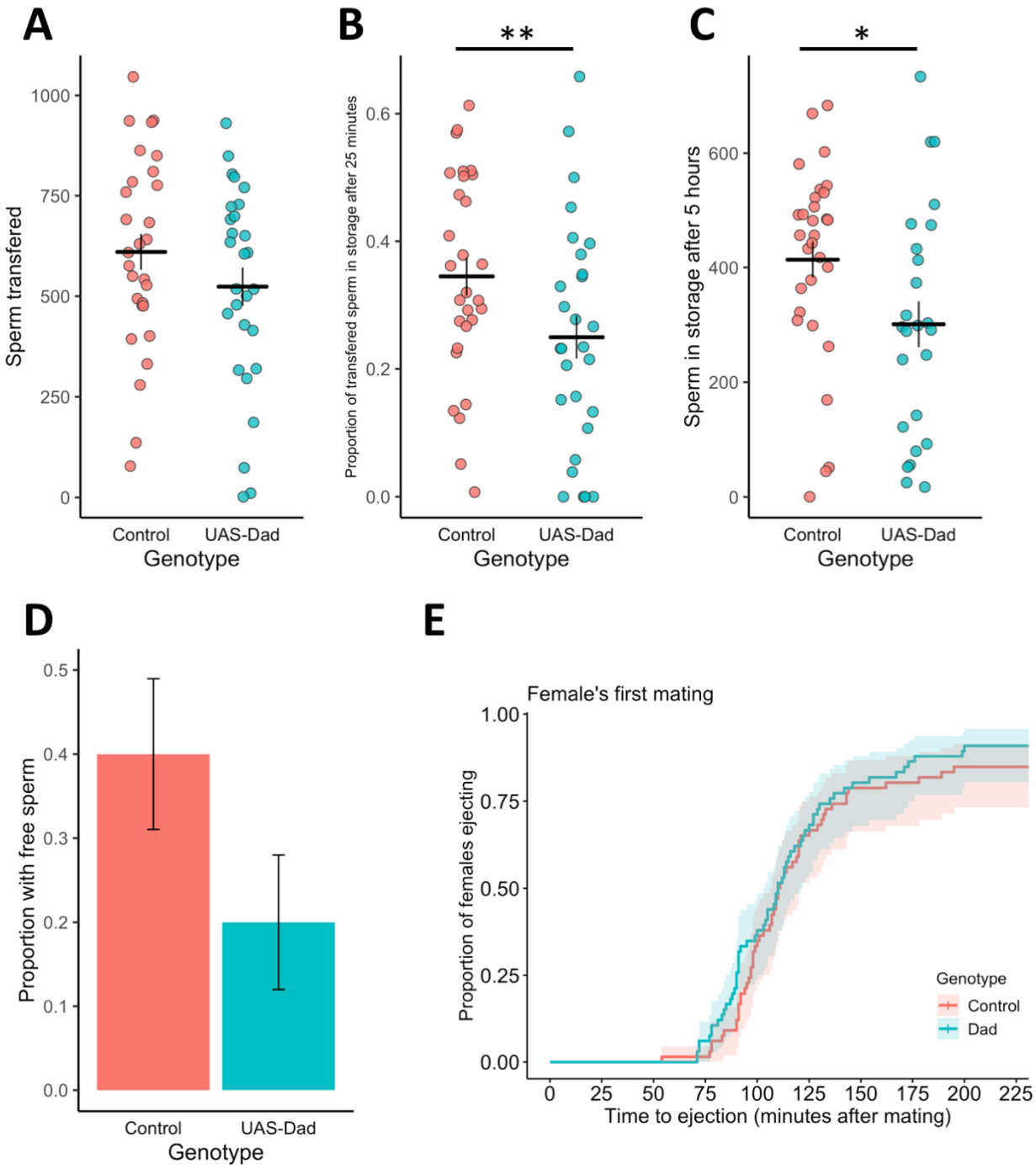


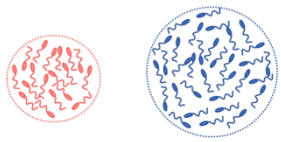
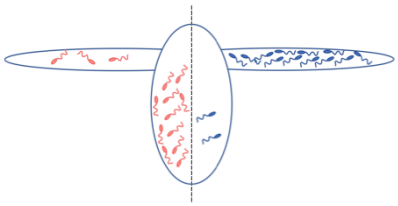
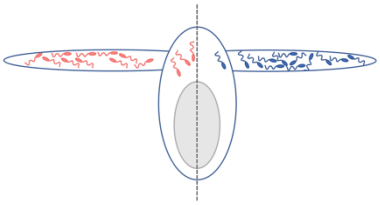
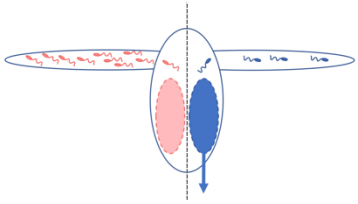
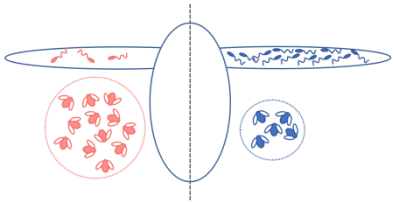
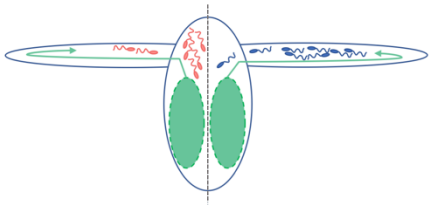
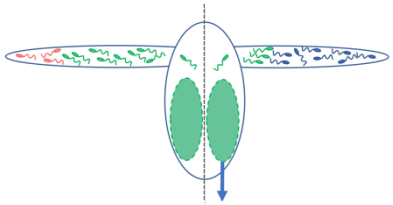
Figure 3. Use of a *UAS-Dad* ejaculate by females

(A) The number of sperm in the female reproductive tract 25-minutes after the start of mating to a *UAS-Dad* or control male. (B) The proportion of transferred sperm that has entered into the storage organs (seminal receptacle and spermathecae) at 25-minutes after the start of mating. (C) The number of sperm in storage at 5-hours after mating. (D) The proportion of females with sperm in the uterus at 5-hours after mating. (E) The timing to ejection of females (measured in minutes). In A-D, bars represent the means ± 1 SE. In E, confidence intervals are at 95%. * = $p<0.05$, **=$p<0.01$.

CONCLUSION

Our data reveal a complex of dysregulation that accompanies loss of BMP-controlled secondary cell activity (Fig. 4). This dysregulation divergently affects multiple male and female-driven determinants of sperm competition outcome. The sperm travelling within a *UAS-Dad* ejaculate show slower and reduced entry into storage, while the female shows altered treatment of a second male and his ejaculate: she is more willing to remate, second male sperm enters storage more slowly (perhaps minimising their displacing effect on resident sperm), and she delays ejection of the residual sperm mass. Our data also suggest she uses *UAS-Dad* sperm differently, perhaps more efficiently, leaking fewer between matings without compromising offspring production. This syndrome of phenotypic effects may be underlaid by distortion of ejaculate composition following the loss of secondary cell secretion, a feature that proteomics data support (Chapter 4). Consequently, multiple parts of the female post-mating response under SFP-control may be rendered simultaneously defective, while others remain unaffected. This syndrome points to a decoupling of aspects of the female post-mating response, perhaps arising through the partial or split stimulation of different post-mating neural circuitries (*e.g.* Haussmann et al. 2013), with the female behaving as though semi-mated.

That loss of activity from a glandular cell-type can impart divergent effects on female post-mating responses exposes division of labour between seminal fluid contributing cells. While some responses are built on an interplay between different cell-types, others may act independently, suggesting some modularity in ejaculate composition – new functions can be incorporated by addition or removal of cell-types. While this is accompanied by disruption to some functions, others remain unaffected. Thus, from an evolutionary perspective, simple loss of function mutations to cells could modify ejaculate-induced phenotypes without necessarily compromising fertility.

Model	Mechanism	Prediction	Result	Figure
	Number of transferred sperm	Greater in *UAS-Dad*	No difference	3a
	Number of sperm stored after initial mating	Greater in *UAS-Dad*	Lower in *UAS-Dad*	3b,c
	Number of sperm released per egg	Lower in *UAS-Dad*	Evidence of being lower in *UAS-Dad*	3d
	Timing of ejection of first male sperm	Earlier in *UAS-Dad*	No difference	3e
	Rate of sperm use through offspring producing	Lower in *UAS-Dad*	No difference (inferred from normal offspring production)	2a
	Resistance to seminal fluid mediated displacement	Increased in *UAS-Dad*	No difference	2a
	Timing of ejection of second male sperm	Earlier in *UAS-Dad*	Later in *UAS-Dad*	2b

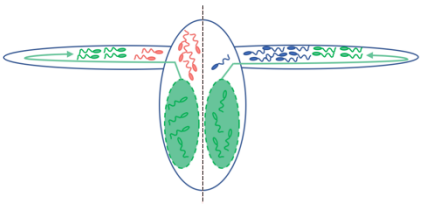
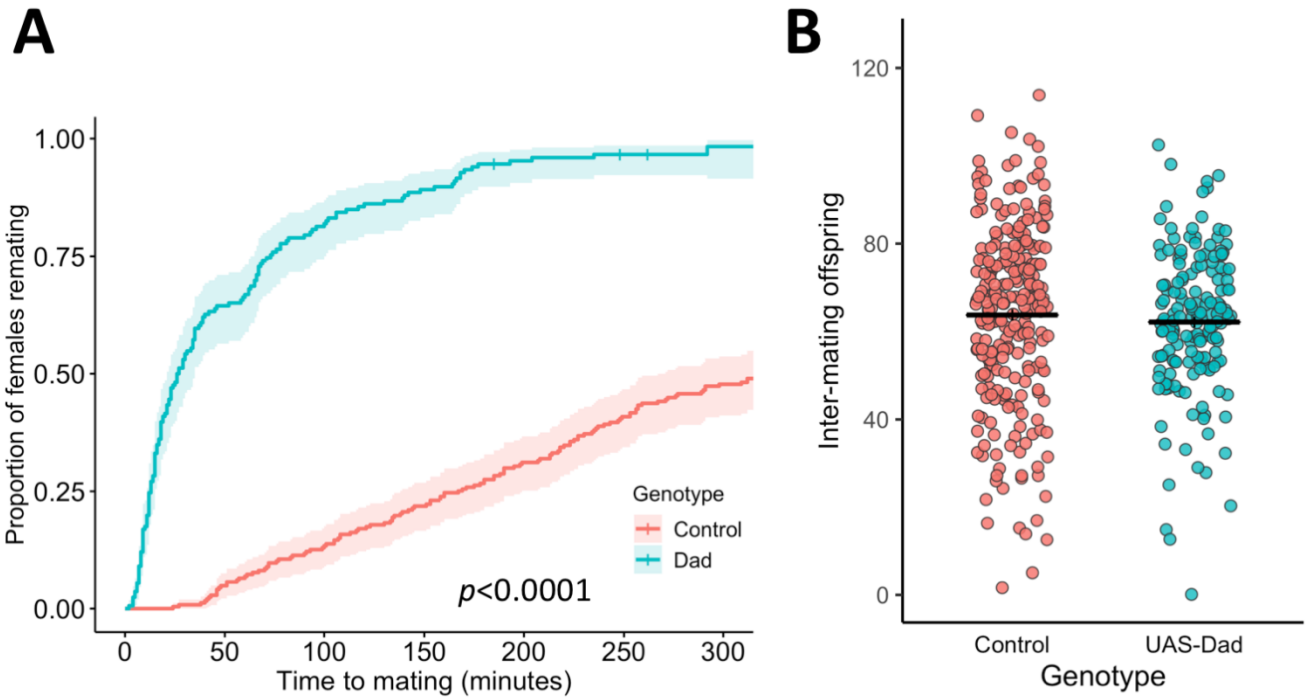
	Resistance to sperm-dependent displacement	Increased in *UAS-Dad*	Potentially increased in *UAS-Dad* given slower entry of second male sperm into storage	2c,d

Figure 4. Split responses of *UAS-Dad* sperm and *UAS-Dad* mated females to secondary cell disruption

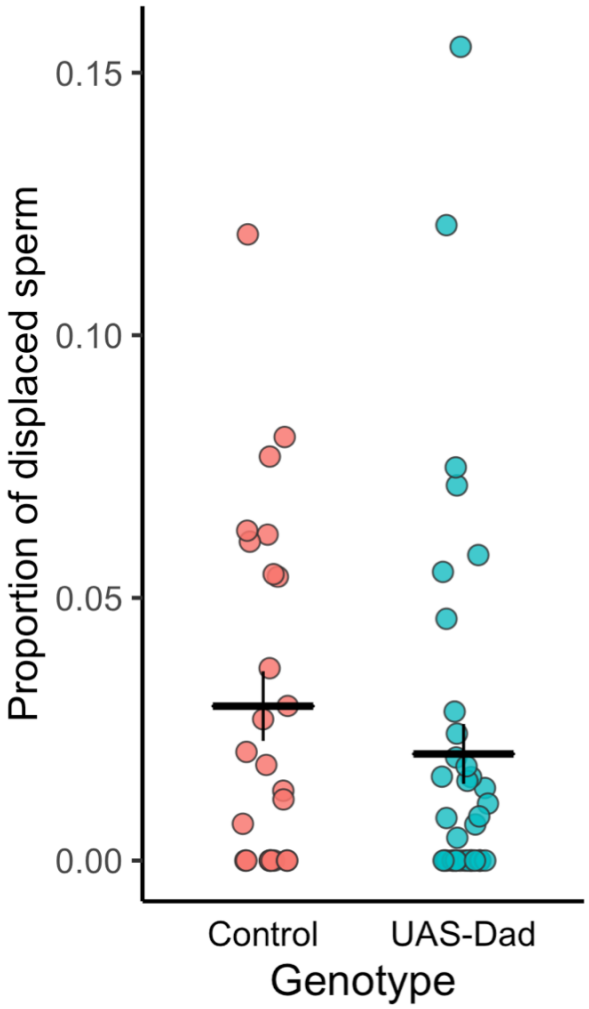
Each row represents an episode at which variance in sperm competition performance can manifest. This is depicted in a model alongside a verbal description of the mechanism. Blue writing describes mechanisms acting prior to a second mating, red gives mechanisms acting after. Predictions of how these episodes should be changed with *UAS-Dad* sperm, given the finding of elevated P1, are provided alongside what our data show and the relevant figure. All but the first row's model provides a mirror image (split by a dashed grey line) of a female uterus and primary sperm storage organ (seminal receptacle). On the left-hand side with red sperm is the control prediction, on the right-hand side in blue is the *UAS-Dad* prediction. Red ellipses give a control ejaculate, blue ellipses give a *UAS-Dad* ejaculate. Green gives a second male ejaculate. Arrows indicate the direction of movement of the relevant component. In row three, the grey ellipse represents an egg on passage through the uterus.

SUPPLEMENTARY MATERIAL



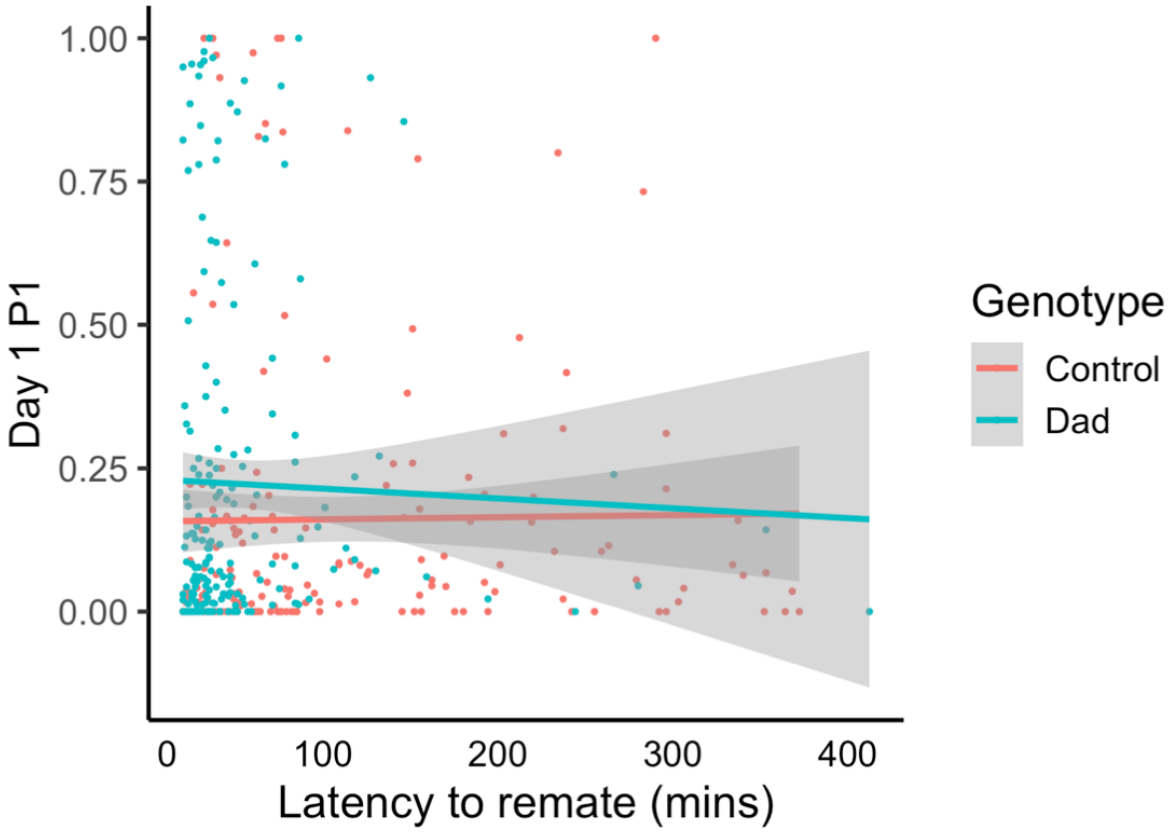
Supplementary Figure 1. GFP-transformed lines show expected *UAS-Dad* phenotypes

(A) Female latency to remating having first mated with a *GFP-ProtB; UAS-Dad* male or a *GFP-ProtB* control. n_{Dad}=166, $n_{control}$=247. Confidence intervals are at 95%. (B) The number of offspring produced by females in the 24-hours following mating to a *GFP-ProtB; UAS-Dad* male or a *GFP-ProtB* control. No significant difference between treatments ($F_{2,410}$=2.909, p=0.089).



Supplementary Figure 2. No difference in displacement of first male sperm at 10-minutes after the start of second mating

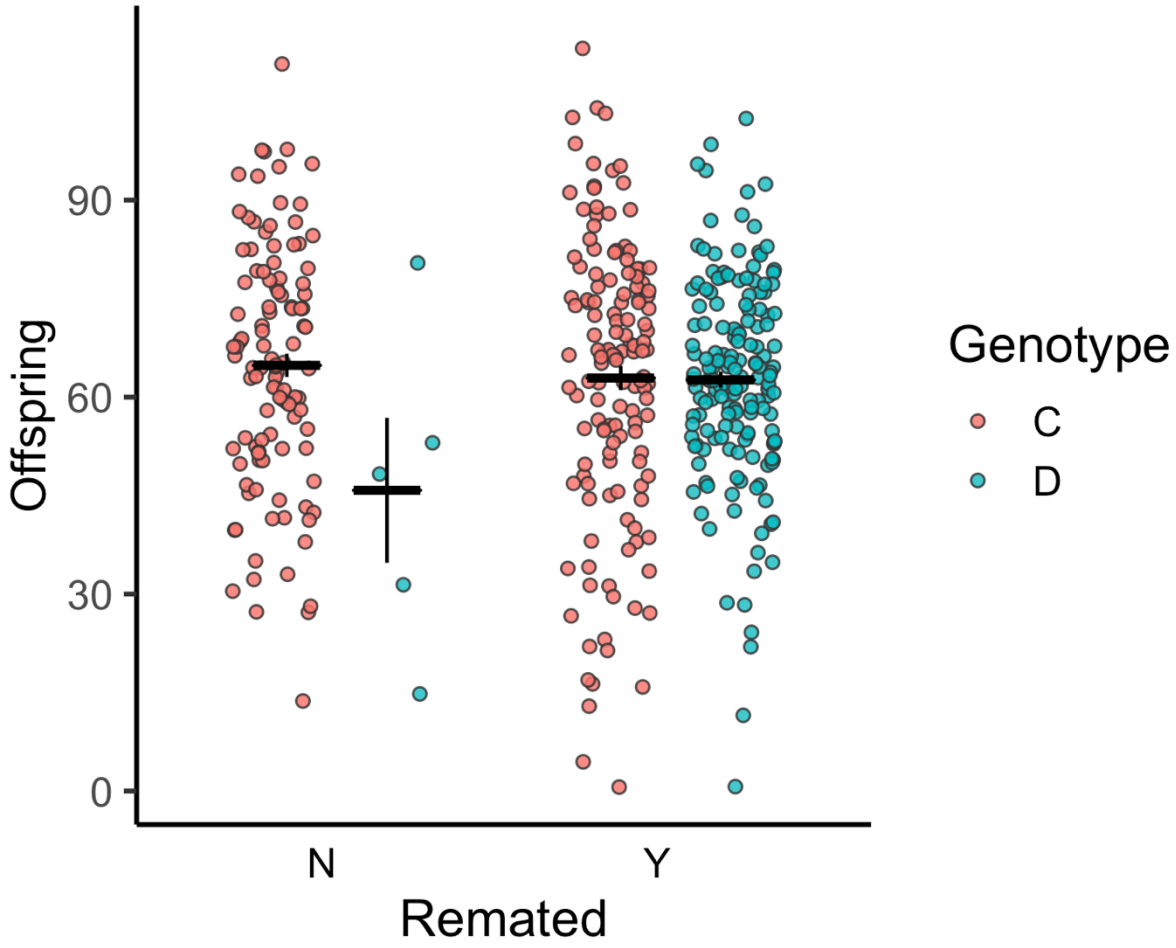
The proportion of first male sperm found outside of the storage organs 10-minutes after a female starts mating with a standardised competitor. Lines give mean ±1 standard error of a proportion.



Effect	*Df*	*Dev.*	*Scaled dev.*	*P(>Chi)*
*Genotype*Latency*	1	9963.2	0.0130	0.9091
Genotype	1	10121.4	5.3450	0.0208
Latency	1	9963.3	0.0013	0.9714

Supplementary Analysis 1. No relationship between latency to remate and P1

(A) A regression of P1 in the 24-hours after a second mating against the latency to that mating. Blue gives *UAS-Dad*-mated females and red gives control-mated females. (B) Significance of factors associated with a GLM testing for associations of male genotype, female latency to remating, and the interaction between them with P1 in the 24-hours after second mating. GLM uses a quasibinomial distribution with overdispersion parameter set as 29.597.



Effect	*Df*	*Sum sq.*	*RSS*	*AIC*	*F*	*P*
*Genotype*Remate*	1	965.2	122821	2360.1	3.2316	0.0730
Genotype	1	145.6	122966	2358.5	0.4849	0.4866
Remate	1	16	122837	2358.1	0.0533	0.8175
Run	1	23487.6	146308	2430.3	78.213	<0.0001

Supplementary Analysis 2. Non-rematers do not produce more offspring

The number of offspring produced in the 24-hours following mating to a *GFP-ProtB; UAS-Dad* (blue) or *GFP-ProtB* control (red), plotted according to whether they did (Y) or did not (N) remate. Two replicates pooled for analysis. The table gives the significance of effects from a linear model. Red denotes significance at the $p<0.05$ level.

	Eliminated	*Sum sq*	*Mean sq*	*NumDF*	*DenDF*	*F*	*P*
Male 1*Day*Male 2	1	2361	295.13	8	2015	0.9946	0.4281
Male 1*Male 2	2	23.5	11.74	2	592	0.0396	0.9612
Male 1*Day	3	1896.4	316.07	6	2052	1.0652	0.3812
Male 1	4	231.7	231.68	1	587	0.7810	0.3772
Day*Male 2	0	9659.5	1207.44	8	2027	4.0696	<0.0001

	Estimate	*Std. Error*	*df*	*t value*	*Pr(>|t|)*
(Intercept)	60.5527	2.0977	1765	28.866	<0.0001
Day2	-21.3990	2.4838	2000	-8.616	<0.0001
Day3	-34.2353	2.4925	2004	-13.735	<0.0001
Day4	-25.8096	2.5094	2008	-10.285	<0.0001
Day5	-32.0505	2.7599	2045	-11.613	<0.0001
Day6	-28.6347	1.9143	2164	-14.958	<0.0001
Day7	-36.2434	1.9143	2164	-18.933	<0.0001
Spa	11.0344	2.3502	1768	4.695	<0.0001
Tudor	0.8395	2.7112	1771	0.310	0.75688
*Day2*Spa*	7.7867	2.7778	1997	2.803	0.0051
*Day3*Spa*	1.9923	2.8862	2038	0.690	0.49011
*Day4*Spa*	-4.9767	2.9686	2053	-1.676	0.09380
*Day5*Spa*	6.1027	3.1927	2076	1.911	0.05608
*Day2*Tudor*	8.7870	3.3021	2053	2.661	0.0079
*Day3*Tudor*	2.2702	3.3047	2054	0.687	0.49219
*Day4*Tudor*	-0.4364	3.3216	2057	-0.131	0.89548
*Day5*Tudor*	-1.8680	3.8526	2100	-0.485	0.62782

Supplementary Table 1. Output from an analysis of daily fecundity patterns in relation to the genotype of a female's first and second partner

(Text overleaf)

Day	contrast	estimate	SE	df	t.ratio	p
2	None-Spa	-18.821	2.450	1859.54	-7.685	<0.0001
2	None-Tudor	-9.627	2.934	1936.53	-3.281	0.0030
2	Spa-Tudor	9.195	2.236	1984.58	4.112	<0.0001
3	None-Spa	-13.027	2.571	1993.91	-5.066	<0.0001
3	None-Tudor	-3.110	2.937	1938.31	-1.059	0.5399
3	Spa-Tudor	9.917	2.355	2111.99	4.211	<0.0001
4	None-Spa	-6.058	2.663	2097.87	-2.274	0.0597
4	None-Tudor	-0.403	2.956	1959.02	-0.136	0.9898
4	Spa-Tudor	5.655	2.443	2208.35	2.314	0.0540
5	None-Spa	-17.137	2.912	2329.57	-5.866	<0.0001
5	None-Tudor	1.029	3.543	2423.03	0.290	0.9546
5	Spa-Tudor	18.166	2.919	2529.18	6.223	<0.0001

Supplementary Table 1. Output from an analysis of daily fecundity patterns in relation to the genotype of a female's first and second partner

Output of a linear mixed effects analysis of day-by-day offspring production in relation to a female's first mate (*UAS-Dad* or control) and second mate (none, spermless *son-of-Tudor*, spa^{pol}). The first table gives the output of a stepwise AIC-based model selection process. The second gives a summary of the model of best fit. The final table gives pairwise, between-second mating treatment comparisons of differences in offspring production for each day after a second mating (where applicable). Red denotes significance at the $p<0.05$ level.

Chapter 4

Quantitative proteomics reveals division of labour between reproductive gland cell-types in Drosophila melanogaster

TITLE

Quantitative proteomics reveals division of labour between reproductive gland cell-types in *Drosophila melanogaster*

ABSTRACT

To build an ejaculate, males draw on contributions from multiple sources. As well as using sperm from the testes, males make use of accessory reproductive tissues, such as the prostate in humans, to compose fertility-enhancing seminal fluid. Each of these glands is further composed of several, distinct cell-types. Why males incorporate both glandular and cellular diversity into their reproductive systems is poorly understood. One suggestion is that a division of labour between glands and their constituent cells provides males with adaptive control over ejaculate composition. Here, we test how dysregulation of an accessory gland cell-type influences the protein composition of seminal fluid in *Drosophila melanogaster*. We apply quantitative proteomics to pre- and post-mating accessory glands from males genetically transformed to exhibit secretory defects in a specific cell-type ('secondary cells'). Our results show that disruption to the secretory activities of secondary cells imparts divergent effects on different portions of the seminal fluid proteome. While some SFPs are transferred in greater quantities to females, others show clear defects in their transfer, appearing to get stuck within the gland. Moreover, dysregulation of secondary cells changes the production of some SFPs suspected to be synthesised in the gland's majority cell-type ('main cells') thus exposing a level of communication between the different cells of the gland. The differential responsiveness of SFPs to secondary cell secretion at the level of production and transfer leads to compositional change in the seminal fluid – a feature that males may use to their advantage or that may represent an underappreciated driver of male fertility problems.

INTRODUCTION

Compromised ejaculate quality is associated with poor fertility, reduced sperm competitiveness, and offspring disease risk (Avila et al. 2011; Bromfield et al. 2014; Chen et al. 2016; Robertson and Sharkey 2016; Sharma et al. 2016; Levine et al. 2018; Watkins et al. 2018). While some negative effects accrue through changes to the number, morphology or molecular profile of sperm, others are driven by changes to the seminal fluid. In both mammals and insects, seminal fluid proteins ('SFPs') are crucial modulators of reproductive outcome (Hopkins et al. 2017). Across taxa, functions of SFPs include coordinating the movement of sperm, reshaping the female reproductive tract (physiologically and structurally), and inducing changes in female behaviour (Gillott 2003; Avila et al. 2011; Hopkins et al. 2018). The seminal fluid proteome is rich and diverse both in relation to the number of transferred proteins (>2000 in humans) and the molecular classes represented (Poiani 2006; Avila et al. 2011; Laflamme and Wolfner 2013; Rolland et al. 2013). Consequently, a detailed picture of reproductive health and fertility requires understanding of how sperm and non-sperm components of the ejaculate interact.

Multiple, independent lines of evidence support the conclusion that the composition of the ejaculate is a key driver of reproductive success. Firstly, males have been shown to independently manipulate sperm and non-sperm components of the ejaculate in response to cues predicting sperm competition (Chapter 2). This sophisticated manipulation of ejaculate composition is highly sensitive and is associated with improved reproductive performance under competitive conditions (Chapter 2; Bretman et al. 2009, 2013). Secondly, the ratio of sperm to non-sperm portions of the ejaculate is taxonomically variable. While bulls transfer very small volumes to females, boar and stallion ejaculates comprise much larger volumes of seminal plasma relative to the

number of sperm (Mcgraw et al. 2015). Such variation exposes evolutionary lability in ejaculate composition. Thirdly, multi-dimensional, between-population divergence in the seminal fluid proteome has been shown to correlate with variation in sperm competition in some species (Goenaga et al. 2015). How compositional change is mechanistically achieved, both in real-time (Sirot et al. 2011b) and over evolutionary timescales (Cameron et al. 2007; Dhole and Servedio 2014), remains unresolved.

A possible feature that provides control over ejaculate composition is the division of labour between tissues that contribute to the ejaculate (Hopkins et al. 2018; Bayram et al. 2019). While sperm are always produced in testes, seminal fluid is often a product of contributions from several different sources. Human seminal fluid, for instance, is composed of secretions from the prostate, seminal vesicles, and bulbourethral glands (Mcgraw et al. 2015). The number and relative sizes of these accessory reproductive glands ('accessory glands') varies between species. Dogs, for example, have lost the seminal vesicles, but have a much larger prostate relative to body size than bulls (Mcgraw et al. 2015). In insects, distinct glands can produce distinct parts of the ejaculate (Meslin et al. 2017; Bayram et al. 2019). Species in the suborder *Ensifera,* for instance, use their 'rough glands' to produce the edible component of the spermatophore (the 'spermatophylax'), while the 'smooth glands' produce the sperm-bearing ampulla (Lewis and South 2012).

As well as acting between glands, division of labour in ejaculate production may act within them via the operation of distinct cell-types. Accessory glands can be composed of many different cell-types, as in the bean-shaped glands of *Tenebrio molitor,* which are composed of at least seven (reviewed in Hopkins et al. 2018). It has been suggested that the sequestration of SFPs in different cells within a gland may afford spatio-temporal control over their actions (Findlay et al. 2014). Such a mechanism may

be essential in seminal fluid given that many SFPs are known to interact with one another (Ram and Wolfner 2007b, 2009; LaFlamme et al. 2014; Avila and Wolfner 2017; Singh et al. 2018). If and how the contributions of specific accessory gland cell-types act to optimally coordinate seminal fluid, and whether their disruption impacts the ejaculate remains largely untested.

To this end, we use quantitative proteomics to examine SFP production and transfer in transgenic *Drosophila melanogaster* males, in which the action of a subset of seminal fluid producing cells is disrupted. Specifically, we suppress BMP-signalling within a minority cell-type ('secondary cells') in the adult male accessory glands, the organ tasked with producing most of the >200 SFPs transferred to females. Our analysis of glands before and after mating provides a deep and sensitive method of discriminating patterns of SFP production, post-mating retention, and transfer (Sepil et al. 2018). Each of the two lobes of the accessory glands is made up a little over 1000 cells: ~960 'main cells' and ~40 much-larger secondary cells, the latter of which cluster in the distal tip of the gland (Bairati 1968) and show prostate-like qualities (Leiblich et al. 2012; Wilson et al. 2017). How the production of SFPs is distributed across these cells is poorly-resolved, but secondary cells are at least known to secrete concentrated packages of secretory material during mating (dense core granules; 'DCGs') under the control of BMP-signalling (Redhai et al. 2016). This same signalling pathway also controls the secretion of extracellular vesicles (exosomes) by secondary cells (Corrigan et al. 2014). Collectively, loss of these secretions leads to ejaculates that induce partial post-mating responses in females (Chapter 3; Leiblich et al. 2012; Corrigan et al. 2014). More drastic manipulations, such as inducing developmental defects, have further implicated secondary cells in the post-translational modification of SFPs, the loss of which leads to reduced fertility (Minami et al. 2012; Gligorov et al. 2013; Sitnik et al. 2016).

In this study, our prediction was that *UAS-Dad* males would show defective transfer of a set of SFPs, which remain stuck in secondary cells because they fail to be secreted into the gland lumen. As the secondary cells represent a small proportion of the total cell number, only a small number of SFPs should be affected.

METHODS

Fly stocks

We crossed esgF/O^{ts} flies (genotype: *w; esg-GAL4 tub-GAL80^{ts} UAS-FLP/CyO; UAS-GFP$_{nls}$ actin>FRT>CD2>FRT>GAL4/TM6)* with a *UAS-Dad* carrying or w^{1118} line to generate disrupted secondary cell and control flies, respectively (Leiblich et al. 2012; Corrigan et al. 2014; Redhai et al. 2016). *Daughters-against-DPP (Dad)* is a negative regulator of BMP signalling. *esg*-GAL4 is highly-expressed in secondary cells and does not drive expression of *UAS-Dad* in main cells (Leiblich et al. 2012). At low temperatures, GAL4 is suppressed by a temperature sensitive GAL80, which is rendered inactive at temperatures exceeding 28.5°C, releasing expression of the *UAS* construct (McGuire et al. 2004; Jiang et al. 2009; Leiblich et al. 2012). Thus, any disruption to the developing accessory gland is minimised at low temperatures. The females used in experimental matings were from a wildtype Dahomey background into which the *sparkling^{poliert}* recessive mutation was previously backcrossed for four generations. These flies carry a distinct eye phenotype and were used to align our methods with those adopted in a separate study (Chapter 3).

Mating experiment

We conducted crosses on grape-agar plates with a small quantity of yeast paste before distributing approximately 200 eggs into 250mL bottles containing 50mL of Lewis medium to standardise larval densities (as in Clancy and Kennington, 2001). These bottles were held at a non-permissive temperature of 20°C to prevent premature activation of the *UAS-Dad* transgene. At eclosion, we separated males under ice anaesthesia and placed them in groups of 10-12 in 36mL Lewis medium-containing plastic vials, supplemented with *ad libitum* yeast granules. These vials were immediately moved to 30°C to activate the suppression of BMP. We aged males for 3 to 5 days before each was mated with three virgin females in succession to void the accessory gland lumen of most secondary cell products synthesised prior to the activation of the *UAS-Dad* transgene. Following the third mating, we moved males to a fresh, yeasted vial.

The next morning, males were randomly assigned a mating treatment ('pre-mating' or 'mated') and paired within a genotype. We aspirated the mated treatment male within each pair into a yeasted vial containing an individually isolated 4/5-day old virgin female. At this same point, the pre-mating male from the pair was introduced to an empty, yeasted vial. We flash-froze mated males in liquid nitrogen 25-minutes after the start of mating, freezing their pre-mating partner at the same time. This paired freezing approach ensures that the distribution of freezing times is equivalent between mated and pre-mating males. Moreover, this method ensured that both pre-mating and mated males have equivalent exposure to a fresh vial. Frozen males were stored at -80°C until dissection. We ran this experiment five times in order to produce five independent biological replicates.

Sample preparation

For each sample, we pooled 20 pairs of accessory glands, which we dissected under a light microscope on ice in a drop of ice-cold PBS. We took care to remove the seminal vesicles and testes, and severed the glands from the distal end of the ejaculatory duct. Dissected glands were then transferred to an Eppendorf tube containing 25µl of PBS, which we stored at -80°C. In total, we had 20 samples: five for each of the four treatment permutations (mated, *UAS-Dad*; pre-mating, *UAS-Dad*; mated, control; pre-mating, control).

We prepared our samples for proteomic analysis in line with the previously published GASP protocol ('Gel-Aided Sample Preparation', Fischer and Kessler, 2015; Sepil *et al.*, 2018). The glandular tissue was first macerated using a clean pestle for a timed interval of 1-minute. To lyse the cells, we added 25µl of Pierce RIPA (Radioimmunoprecipitation Assay) Buffer, which we dripped over the pestle to flush residual tissue back into the sample. Next, we incubated the lysate with 50mM of the reducing agent DTT (Dithiothreitol) for approximately 10 to 20 minutes. To this, we added at room temperature an equal volume of 40% acrylamide/Bis solution (37.5:1 National Diagnostics) to facilitate cysteine alkylation to propionamide. Next, we added 5µl of 10% APS (ammonium persulphate) and an equivalent quantity of TEMED (tetramethylethylenediamine) to trigger the polymerization of acrylamide and form a gel plug. This plug was subsequently shredded via centrifugation through a membrane-less Spin-X filter insert (CLS9301, Sigma/Corning). Gel-fragments from this process were fixed in 40% ethanol/5% acetic acid before washing with a solution of 50mM ammonium bicarbonate, 1.5M Urea, and 0.5M Thiourea, which was then removed with ACN (acetonitrile). 250µl of dilute trypsin (Promega) was next added and the solution left at 37°C overnight to promote digestion of the immobilised peptides. The resulting peptides

were extracted via two repeated ACN replacements, dried, desalted in Sola SPE columns (Thermo), and then suspended in 0.1% FA (formic acid), 2% ACN before LC-MS/MS (liquid chromatography-mass spectrometry/mass spectrometry) analysis.

LC-MS/MS

For peptide analysis, we used a LC-MS/MS platform composed of a Dionex Ultimate 3000 and a Q-Exactive mass spectrometer (Thermo). Peptide loading took place in a solution of 0.1% TFA (trifluoroacetic acid) in 2% ACN on a trap column (PepMAP C18, 300µm x 5m, 5µm particle, Thermo). For separation, we used an easy spray column (PepMAP C18, 75µm x 500m, 2µm particle, Thermo) with a gradient 2% ACN to 35% ACN in 0.1% FA in 5% DMSO (dimethyl sulphoxide). For MS spectra collection, we used a resolution of 70,000 in profile mode on the Q-Exactive (ion target = $3x10^6$). We selected the top 15 most intense features selected for subsequent MS/MS analysis (resolution of 17,500). The following parameters were set: dynamic exclusion = 27 seconds; AGC target = $1x10^5$; isolation width = 1.6 m/z; and maximum acquisition time = 100ms.

MS data processing

The following MS data processing pipeline has previously been outlined in Sepil *et al.* (Sepil et al. 2018). We imported the RAW data into Progenesis QIP (version 4.1.6675.48614), exporting spectra as MGF files using the 200 most intense peaks without deconvolution for searching. Peptide identification used the *D. melanogaster* UniProt reference proteome as a search target, with database retrieval conducted on 27/09/2017 (23306 sequences) in Mascot 2.5.1. Search parameters were set as follows: Oxidation (M), Propionamide (K), and Deamidation (N,Q) as variable modifications;

Propionamide (C) as a fixed modification; one missed cleavage site; 0.05 Da fragment mass accuracy; 10ppm precursor accuracy. Prior to importing the search results into Progenesis for quantification via the Top3 method, we applied a peptide-level 1% FDR alongside a further Mascot ion cut-off of 20. The resulting protein abundance data was subsequently normalised using the internal Progenesis algorithm to set of housekeeping proteins.

Data analysis

We detected 1839 proteins by mass spectrometry. To restrict our search to proteins with high-confidence identification, we limited subsequent analysis to proteins detected on the basis of at least 2 unique peptides (as in Borziak et al. 2016; Sepil et al. 2018). Thus, we retained 1194 proteins of which 88 are SFPs known to be transferred to females. Our assessment of whether a protein was a SFP was based on a reference list kindly provided by Mariana Wolfner (Cornell University, NY) and Geoff Findlay (College of the Holy Cross, MA) and updated to include the high confidence SFPs from Sepil *et al.* (Sepil et al. 2018). Where expression data is referenced, it was obtained from the modENCODE dataset on FlyBase (release FB2018_05)(Gramates et al. 2017). All analysis was conducted with R statistical software (version 3.5.1)(Team 2013) in RStudio (version 1.1.456)(RStudio Team 2015). All analyses were performed on $\log_2$ transformed data to standardise the variance across the dynamic range of protein abundances.

Our analysis of the global response of SFPs to genotype involved fitting a linear mixed effects model to SFP abundances, including genotype (*UAS-Dad*/control) and mating status (pre-mating/mated) as fixed effects, and protein identity as a random effect. Model fit was assessed by visual inspection of diagnostic plots. An initial model also included replicate as a fixed effect, but the model's residuals departed from normality

based on inspection of a qqplot. To improve the model fit we took a mean per-protein abundance for each of the four treatment permutations and refit the model without replicate. For this analysis, we also removed two outlier SFPs, S-Lap7 and Sfp87B, both of which showed high between-sample variance. The former shows considerably higher expression in the testes than the accessory gland and therefore may represent contamination from sperm. We assessed the significance of each factor by dropping individual terms from the full model using the 'drop1' function.

To analyse whether mating exacerbates the effect of secondary cell disruption, we calculated the between-genotype $\log_2$ fold change separately for pre-mating and mated glands. Fold changes were calculated by subtracting the per-treatment mean (taken across the five replicates) for each SFP in *UAS-Dads* from the equivalent value in controls. Consequently, negative values indicate greater abundance in *UAS-Dad* glands. We used a histogram to plot the distribution of fold changes separately for pre-mating and mated glands, and used a Wilcoxon signed rank test to test for a difference in their distributions. To examine how patterns of pre and post-mating change translate into what is transferred to females, we calculated the between-genotype difference in each genotype's respective pre- and post-mating fold change. This value describes the difference between genotypes in the degree of SFP loss during mating. We subsequently ranked and plotted the resulting fold changes.

Our hierarchical clustering analysis was conducted on the mean per-SFP abundance taken across the five replicates for each treatment permutation. We plotted the results using the *pheatmap* package (Kolde 2018). Row annotations for sex peptide network proteins are taken from: Ram and Wolfner 2009; Findlay et al. 2014; and Singh et al. 2018. Those for cellular localisation are taken from: DiBenedetto et al. 1990; Monsma et al. 1990; Bertram et al. 1992; Styger 1992; Wolfner et al. 1997; Gligorov et

al. 2013; and Sitnik et al. 2016. We conducted a PCA on SFPs using the 'prncomp' function in *stats* and plotted results using *ggbiplot* (Vu 2011). Ellipses on these plots denote 80% normal probability, raised from the default 67%. Variables were scaled to have unit variance and shifted to be zero-centred. We ran linear models on the PC scores to test for associations between PCs and our variables. The decision to model replicate as a fixed effect rather than as a random effect was based on the low number of levels (<6)(Bolker et al. 2009).

To identify differentially expressed proteins, we iterated a linear model over all proteins with mating, genotype, and replicate as fixed effects. To correct for multiple testing, we used a tail-based false discovery rate correction from the *fdrtool* package, which calculates a cut-off based on the distribution of *p*-values (Strimmer 2008).

RESULTS AND DISCUSSION

***UAS-Dad* males show defects in SFP transfer and altered protein production**

We first tested for global effects of secondary cell disruption on the abundances of SFPs across pre-mating and mated glands. Applying a linear mixed effects model across SFPs, we found significant effects of both genotype ($F_{1,253}$=6.859, p=0.0009) and mating status ($F_{1,253}$=461.522, p<0.0001) on SFP abundance. Specifically, the average effect of *UAS-Dad* on SFP abundance equated to an increase of 0.13 ($\log_2$ scale), while mating led to a decrease of 1.04 ($\log_2$ scale). Although we found no significant interaction between genotype and mating status ($F_{1,252}$=2.428, p=0.1205), an indication of differential transfer, the number of SFPs showing a directional trend for greater abundance in *UAS-Dad* males increased after mating: 66% (57/87) of detected SFPs are at higher abundance in pre-mating *UAS-Dad* glands and 74% (64/87) in mated glands. The distribution of between-genotype fold changes is significantly more *UAS-Dad* biased in mated glands compared to pre-mating glands (W=4672, p=0.018; Fig. 1a). This suggests that mating increases the difference between the *UAS-Dad* and control gland SFP proteomes.

Abundances being even higher in *UAS-Dad* glands relative to controls after mating is consistent with greater SFP retention following reduced transfer to females. Plotting the between-genotype differences in what is transferred to females (the pre-mating-mated fold change) makes clear that many SFPs are transferred in lower quantities by *UAS-Dad* males (59/88, 67%; Fig. 1b). This plot further shows that the size of the effect of secondary cell disruption on transfer varies between SFPs, a small number even show substantially larger transfer in *UAS-Dad* males. Alongside altered production, defective transfer may partially explain why *UAS-Dad* glands exhibit elevated SFP abundances prior to mating, having retained more following the previous day's triple matings (see Methods).

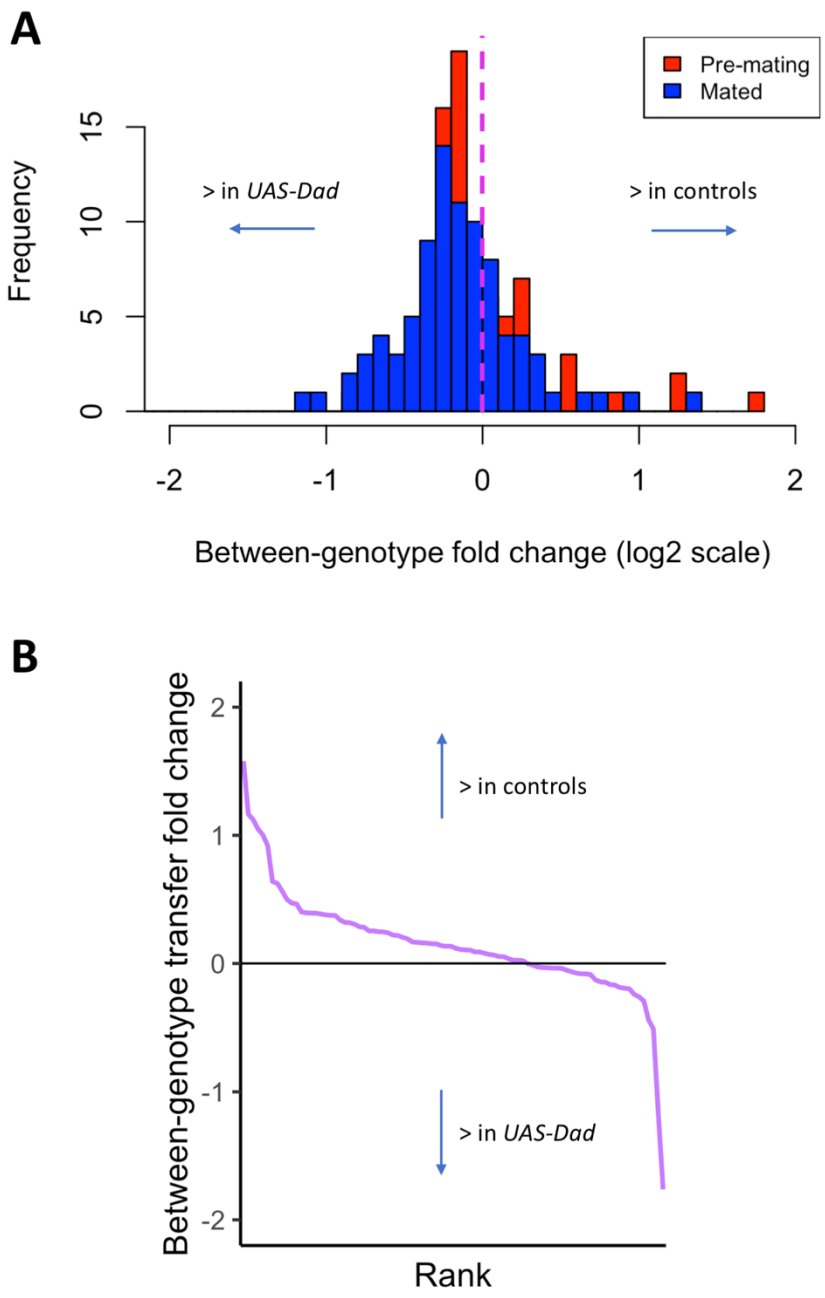


Figure 1. Signatures of defective SFP transfer following secondary cell disruption

(A) A histogram of the $\log_2$ fold changes between control and *UAS-Dad* males for each SFP detected in pre-mating glands (red) and those dissected 25-minutes after mating (blue). Values less than 0 indicate greater abundance in *UAS-Dad* males. Values greater than 0 indicate greater abundance in controls. The mated fold change histogram is overlaid on the pre-mating equivalent. (B) The difference between genotypes in each SFP's transfer during mating, ranked by size. The degree of transfer within a genotype is calculated by subtracting the mean mated abundance value from the mean pre-mating abundance value. Points lying above the line y=0 indicate greater transfer to females in controls. Points below the line indicate greater transfer in *UAS-Dad* males.

Secondary cell disruption divergently affects portions of the seminal fluid proteome

To test for patterns among SFPs in their response to secondary cell disruption, we undertook a hierarchical clustering analysis across genotypes and mating treatments (Fig. 2a). We detected a number of early-branching clusters that were small in protein number. Judging by the abundance patterns, these are composed of proteins either extremely responsive to genotype or inconsistently detected across samples, perhaps due to low abundance. To focus in on distinct and generalised patterns of abundance change, we further analysed four clusters (Fig. 2a,b). Cluster 3 was the only one showing a significant interaction between genotype and mating (LME: $F_{1,42}$=6.286, p=0.016), indicating a difference in the degree of SFP transfer to females during mating. Specifically, the degree of loss is smaller in *UAS-Dad* males.

Compromised transfer of SFPs following secondary cell disruption could occur at a number of non-mutually exclusive stages. Firstly, SFPs produced in or trafficked to secondary cells may get stuck inside them due to defective secretion. Indeed, DCG release from secondary cells is known to be significantly decreased following BMP-suppression (Redhai et al. 2016). Secondly, some secondary cell SFPs may be secreted normally, but exhibit aberrant post-translational modifications that lead them to get stuck within the gland lumen. This is consistent with a known role for secondary cells in modifying translated SFPs (Gligorov et al. 2013). Finally, some secreted secondary cell products may be required for the normal transfer of other SFPs to females. A number of SFPs are known to rely on the presence of others for effective transfer (Ram and Wolfner 2009), but how this occurs is unclear. Consistent with this hypothesis, Cluster 3 includes the highly-abundant, main-cell-specific protein Acp36DE (Wolfner et al. 1997). That the transfer of main cell products should be affected implicates the secondary cells in the normal transfer of the accessory gland's lumenal contents.

Cluster 1 and 2 showed significant associations with genotype (LME: Cluster 1: $F_{1,43}$=37.574, p<0.0001; Cluster 2: $F_{1,13}$=28.008, p<0.0001). In Cluster 1, SFPs were significantly more abundant in *UAS-Dad* glands across mating treatments, while Cluster 2 SFPs were significantly less abundant. The lack of a significant interaction term in either case (LME: Cluster 1: $F_{1,42}$=0.543, p=0.465; Cluster 2: $F_{1,12}$=3.265, p=0.096) suggests that where more of an SFP is produced, an equivalently elevated quantity is retained after mating, or vice versa. The differences in abundance between the genotypes captured in Cluster 1 and 2 may therefore arise through altered rates of protein production. This could be caused by the loss of secondary cell products from the accessory gland lumen mimicking the conditions of post-mating depletion, to which the main cells respond by stimulating protein production (see also Chapter 5). However, the low number of secondary cells relative to main cells means that their products are likely to represent a small portion of the total volume of the gland's secretion (Sitnik et al. 2016).

Cluster 4, which in covering 43% (38/88) of the detected SFPs is by far the largest cluster, showed no significant association with genotype (LME: genotype x mating, $F_{1,102}$=0.123, p=0.727; genotype, $F_{1,103}$=1.864, p=0.175) and therefore captures SFPs that are largely unaffected by disruption to secondary cells. This cluster includes key modulators of female post-mating behaviour, such as sex peptide and ovulin (Avila et al. 2011). That a large number of SFPs show no clear response to secondary cell disruption suggests that secondary cells exert specific effects on the transfer and production of a sub-portion of the seminal fluid proteome. This conclusion is further supported by the results of a PCA analysis. PC2, which captured an axis of variation significantly described by male genotype (Table 1), showed split loadings (42/88) indicative of variable, divergent responses among SFPs in the extent to which their abundance was affected by secondary cell disruption. PC1, which described the majority of variance, captured an axis of

variation associated with the interaction between mating and genotype (Table 1). There was high alignment in the responses of SFPs in PC1 (82/88 bore positive loadings), which accords with the general effect of the SFP proteome becoming more dissimilar between *UAS-Dad* and controls after mating. The two axes of variation intersect such that secondary cell disruption is followed by distinct compositional change in the SFP proteome (Fig. 2c).

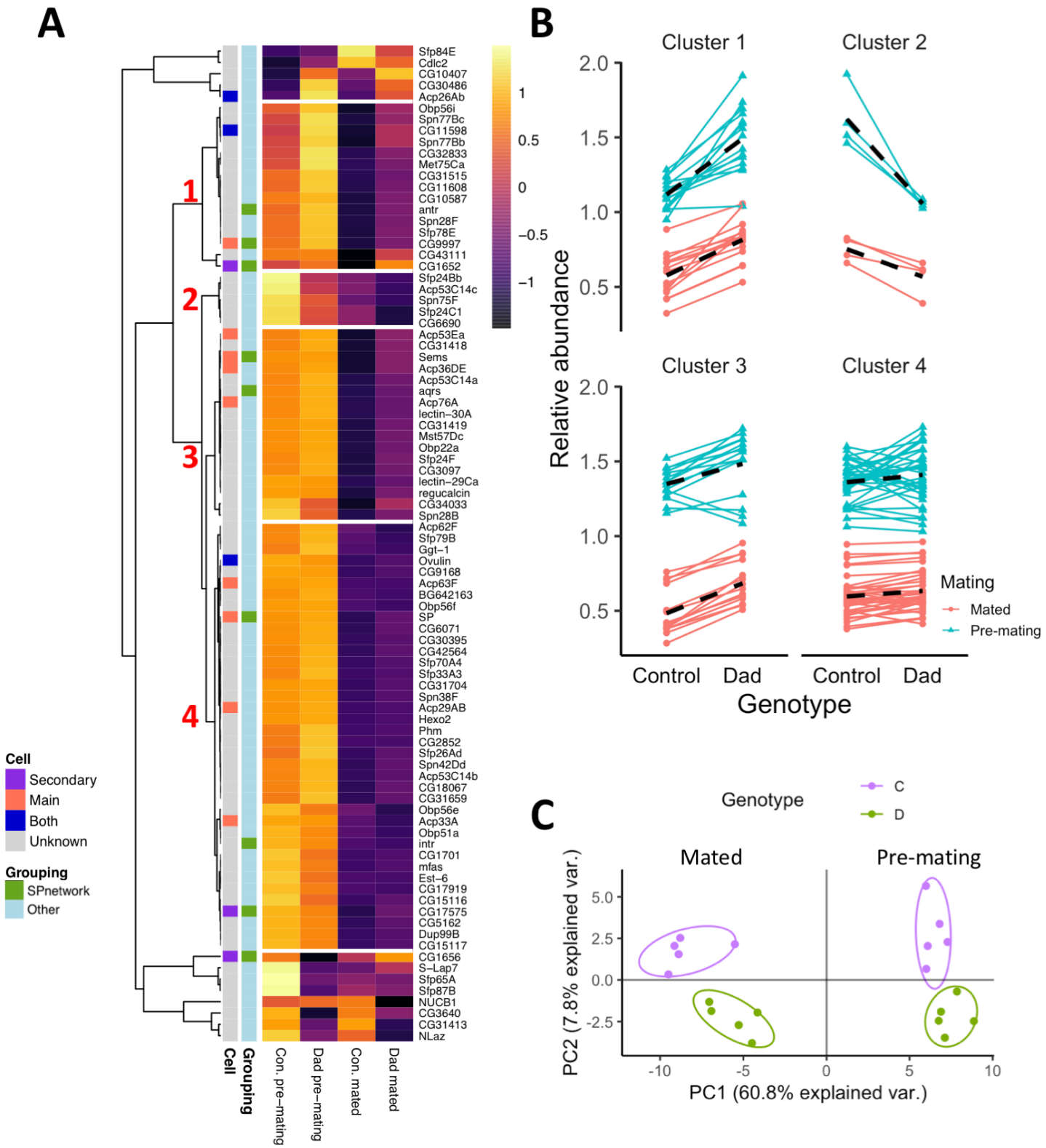


Figure 2. Divergent responses of SFPs to secondary cell disruption

(Text overleaf)

Figure 2. Divergent responses of SFPs to secondary cell disruption

(A) A heatmap showing the abundance patterns of all 88 seminal fluid proteins (SFPs) detected in a quantitative proteomics analysis of male accessory glands. All values are $\log_2$ transformed. Each cell gives the mean across five replicates for that protein in a particular treatment combination. On the left-hand side of the plot, the first two columns are from males dissected prior to mating, while the latter two are from males frozen 25-minutes after mating. Within each duo are plotted, from left to right, glands from controls and glands from males with disrupted secondary cell secretion (*UAS-Dad*), respectively. Row annotations highlight proteins known to act in the sex peptide network (a major functional grouping) and give a SFP's cellular localisation within the gland, if known (see Methods). Four clusters retained for analysis are numbered and bounded by white lines. (B) The average SFP response to competition in each of the four major clusters given in (A). Each of the detected SFPs is plotted separately for mated (red) and pre-mating (blue) glands for each genotype. The mean response is given by a dashed line. The abundance values are relativised by dividing the abundance of each SFP in a sample by the mean across all samples for that protein. A treatment mean across the five replicates is then taken for each SFP and plotted. (C) Output of a PCA analysis conducted on the normalised, $\log_2$ abundances of the detected SFPs. PC1 is given on the *x* axis and PC2 on the *y*. Points represent each of the 20 samples coloured according to the genotype of males (purple = control, green= *UAS-Dad*). Mated glands fall on the left of the plot, pre-mating glands on the right. Ellipses denote 80% normal probability.

A

	PC1	*PC2*	*PC3*	*PC4*
Variance explained (%)	60.76	7.77	6.15	4.35
Eigenvalue	53.47	6.84	5.41	3.83
No. positive loadings	82	42	47	44
No. negative loadings	6	46	41	44

B

PC1 *Effect*	*Df*	*Sum of sq*	*RSS*	*F*	*P*
*Genotype*Mating*	1	3.6962	11.3870	5.7672	0.0334
Genotype	1	18.56	29.95	21.190	0.0004
Mating	1	971.50	982.88	1109.1	<0.0001
Replicate	4	14.52	25.90	4.1433	0.0222

PC2 *Effect*	*Df*	*Sum of sq*	*RSS*	*F*	*P*
*Genotype*Mating*	1	1.1368	9.4912	1.6329	0.2255
Genotype	1	102.42	111.91	140.28	<0.0001
Mating	1	1.8380	11.329	2.5176	0.1366
Replicate	4	16.200	25.691	5.5474	0.0079

PC3 *Effect*	*Df*	*Sum of sq*	*RSS*	*F*	*P*
*Genotype*Mating*	1	2.0705	71.065	0.3601	0.5596
Genotype	1	4.3395	75.404	0.7938	0.3891
Mating	1	0.0131	71.078	0.0024	0.9616
Replicate	4	27.418	98.482	1.2539	0.3370

Table 1. Summary statistics from a PCA conducted on detected SFPs

The first table gives the variance, eigenvalue, and loadings associated with the first four principal components (PCs). The next three tables give the output from linear models fitted to each of the first three PCs, using the measured variables of genotype (*UAS-Dad* or control), mating status (mated or pre-mating), and replicate (5 in total). Significant associations at the $p<0.05$ levels are given in red.

Identification of high-confidence, secondary cell dependent SFPs

To identify high-confidence SFPs affected by secondary cell disruption, we performed a differential expression analysis. Differential transfer of individual SFPs to females would be indicated by a significant interaction between mating and genotype; however, after FDR correction, no SFPs showed a significant interaction. We detected significant responses of 11 SFPs to genotype (Table 2). A DAVID gene ontology analysis (Huang et al. 2009a,b) revealed borderline significant enrichment of the term 'serpin' in the differentially-expressed SFPs against a background of the SFPs we detected (enrichment score: 0.71; count =3; Benjamini FDR p =0.06). That the abundance of some SFPs thought to be present in secondary cells (CG1652, CG1656, CG17575, and ovulin) (Monsma et al. 1990; Bertram et al. 1992; Gligorov et al. 2013) do not show up in this analysis suggests that suppression of BMP-signalling does not block their production

The list of differentially-expressed SFPs included 4 at significantly lower abundance in *UAS-Dad* glands. These were NLaz, Sfp24C1, CG6690, and CG314137. The 7 proteins at significantly higher abundance in both pre-mating and mated *UAS-Dad* gland were antr, CG9997, Spn77Bc, Spn28F, Spn77Bb, CG11598, and Acp26Ab. Two of these (antr and CG9997) form part of the 'sex peptide network', which regulates the activity of sex peptide, the keystone modulator of female post-mating responses, within the female reproductive tract (Ram and Wolfner 2009; Findlay et al. 2014).

Differentially Expressed SFPs

Protein	qval		Log fold change			Functional class	Predicted function	Mating-related process
	Genotype	Mating	Virgin	Mated	Transfer			
Acp26Ab	0.0001	0.0252	-3.9921	-2.2305	-1.7616	Peptide/Prohormone	Post-mating behavior	Transfer of other SFPs
antr	0.0099	0.0001	-0.4067	-0.4717	0.0650	CRISP	Defence response	SPN
CG11598	0.0001	0.0001	-0.8491	-0.8736	0.0245	Acid lipase	Lipase activity	
CG31413	0.0021	0.4695	0.5486	0.6916	-0.1430	Thioredoxin	Protein modification process	
CG6690	0.0204	0.0001	0.5501	0.4407	0.1094	Thioredoxin	Protein modification process	
CG9997	0.0063	0.0001	-0.4432	-0.5971	0.1539	Serine protease	Proteolysis	SPN
NLaz	0.0001	0.0163	1.3765	1.3397	0.0368	Lipocalin	Lipid metabolic process	
Sfp24C1	0.0001	0.0001	0.9107	0.7763	0.1344	Serine protease	Endopeptidase inhibitor	
Spn28F	0.0011	0.0001	-0.6338	-0.7392	0.1054	Serpin	Negative regulation of proteolysis	
Spn77Bb	0.0003	0.0001	-0.6584	-0.8108	0.1524	Serpin	Negative regulation of proteolysis	
Spn77Bc	0.0363	0.0002	-0.5505	-0.4633	-0.0871	Serpin	Negative regulation of proteolysis	

Table 2. SFPs detected as significantly differentially-expressed in response to secondary cell disruption

11 SFPs showing FDR-corrected *p*-values of <0.05 in their association with secondary cell disruption. q-values are calculated by applying a tail-based FDR correction on *p*-values obtained from a linear model iterated over each detected protein. q-values are given both for the effect of mating status (pre-mating or mated) and male genotype (*UAS-Dad* or control). Fold changes are given on a $\log_2$ scale and calculated for each between-genotype comparison within a mating status. Fold changes are calculated by subtracting the *UAS-Dad* value from the control value, while transfer fold changes are calculated by subtracting the pre-mating/mated difference in *UAS-Dad* glands from the equivalent value in controls. Thus, in both cases, negative values indicate greater abundance/transfer in *UAS-Dad* glands. Functional information associated with each protein's FlyBase entry is provided, along with whether a protein has a known reproductive role. SPN = sex peptide network protein.

Evidence of inter-cellular signalling within the accessory gland

Of the SFPs we detect as being at significantly higher abundance following secondary cell disruption, two have previously been found in secondary cells. One of these is the lipase CG11598, which, curiously, was found in a transcriptomic study to be substantially downregulated when secondary cells fail to develop normally – an effect partially attributed to changes in production by main cells (Sitnik et al. 2016). The discordance between CG11598's direction of change between the previous and current study may reflect the divergent effects imparted by disruption to secondary cell development and suppression of adult cell BMP-signalling. Alternatively, it may reflect the general lack of alignment often observed between transcript and protein levels, the latter likely being more important in determining phenotypic differences (Khan et al. 2013; Liu et al. 2016).

The second secondary cell protein we find to be differentially-expressed is Acp26Ab, which is known to be present in both cell-types within the gland (Monsma et al. 1990). Acp26Ab stands out in the strength of response it shows to secondary cell disruption: 16 times more abundant in pre-mating *UAS-Dad* glands and 8 times as abundant in mated glands. The low number of secondary cells means that an abundance change of this magnitude is likely to only be accounted for by changes in main cell production. Interestingly, while Acp26Ab is known to be present in both main and secondary cells within the first day of eclosion, after 5 days it is only present within the 'phase-dark' vesicles of secondary cells (Monsma et al. 1990), which match the description of DCGs. It may be that Acp26Ab is trafficked from main cells to secondary cells, but when secondary cell secretion is disrupted, a feedback system between the cells is disrupted and the main cells over produce the protein. Indeed, it has been suggested that some secondary cell products may be involved in inter-cellular signalling pathways that influence rates of protein production within the gland (Sitnik et al. 2016). The

existence of such cross-talk may underlie the phenotypic discordance between secondary cell developmental (Minami et al. 2012; Gligorov et al. 2013; Sitnik et al. 2016) and secretory defects (Leiblich et al. 2012; Corrigan et al. 2014; Chapter 3). The more pronounced defects observed in developmental mutants may arise through the compounding, dysregulatory effects of disrupting channels of main cell-secondary cell communication during development.

Acp26Ab is of further interest as previous work has provided tentative evidence that its loss is associated with generally reduced transfer of a number of suspected main cell produced proteins (Ram and Wolfner 2007b). If Acp26Ab acts as a general regulator of SFP transfer then its over-abundance in a pre-mating male's accessory gland may interfere with the normal transfer of the lumenal contents. A similar logic could apply to the main-cell produced CG9997 (Gligorov et al. 2013), which we find at higher abundance in *UAS-Dad* glands, as it too has been implicated in the normal transfer of some SFPs (Ram and Wolfner 2009).

CONCLUSION

The phrase 'division of labour' reflects the assignment of different elements of a manufacturing process to separate parties with the aim of boosting efficiency. In this study, we identify that the prostate-like secondary cells of the *D. melanogaster* accessory glands make distinct contributions both to the normal transfer and production of seminal fluid. The biology of the secondary cells, wherein some material is packaged, stored, and released at mating, may allow for fine-scale, real-time manipulation of the composition of the seminal fluid that females receive, an ability we know males have but for which an underlying mechanism remains elusive (Sirot et al. 2011b). By controlling the release of regulator molecules, males may dictate what portion of the largely main cell produced

secretion within the gland lumen is transferred. Whether sensory information relating to mating context (*e.g.* Bretman *et al.*, 2011) can be translated into differential secretion by secondary cells is a key question for further work.

The same mechanisms of control over ejaculate transfer may act to a male's disadvantage: we find evidence that cell-type specific dysregulation can have proteome-wide implications for ejaculate composition, as well as altering the activities of the seminal fluid producing cells themselves. The secretory epithelium of the human prostate, as in the *D. melanogaster* accessory gland, is composed of two basic cell-types (Long et al. 2005). To what extent the inter-cellular integration we identify in flies might apply to humans is unclear, but there is emerging evidence of parallels in the activities of their respective accessory glands (reviewed in Wilson et al. 2017). Consequently, our work provides a warning against therapeutics or methods of male contraception directed at the prostate – disrupting one cell-type can affect other parts of the gland. Given that some reproductive cells, including both *Drosophila* secondary cells and human prostate cells, secrete sperm-binding exosomes, dysregulation may impact both sperm motility (Aalberts et al. 2013) and offspring phenotype (Chen et al. 2016; Sharma et al. 2016).

From an evolutionary perspective, the coordinated action we uncover between cells, suggests that barriers to integrating cellular diversity into reproductive glands are non-trivial. Whether the increased cellular diversity exhibited in the glands of species such as *T. molitor* provides further benefits, such as finer-scale control over ejaculate composition, faster responses to hormonal or neural stimulation, or enhanced spatio-temporal control over SFP interactions represents an exciting avenue for future work. As does unpicking the mechanisms by which species have overcome the challenges of integrating new cell-types into their reproductive systems while ameliorating the new opportunities for dysregulation that each additional cell brings with it.

Chapter 5

Loss of sex peptide stimulates seminal fluid protein production and distorts the seminal fluid proteome in Drosophila melanogaster

TITLE

Loss of sex peptide stimulates seminal fluid protein production and distorts the seminal fluid proteome in *Drosophila melanogaster*

ABSTRACT

Reproductive success crucially depends on the action of proteins in the seminal fluid. These seminal fluid proteins ('SFPs') can exert highly-specific functions in females, such as stimulating ovulation. Alongside this specificity, there is increasing evidence that many SFPs act interdependently as part of larger networks, which regulate the transfer or action of others. As a result, defects in one protein may have wider knock-on consequences for other SFPs. Here, we adopt a quantitative proteomics approach in the fruit fly *Drosophila melanogaster* to test whether loss of a keystone SFP, the sex peptide, feeds back to affect the broader composition of seminal fluid. Using a widely-used genetic knock-out, we show that loss of *Sex Peptide* leads to an increase in abundance of other SFPs in the accessory glands of virgin males. This increase correlates with a general upregulation of proteins involved in protein synthesis and leads to altered transfer of a cluster of different SFPs, some of which sex peptide is known to interact with inside females. Consequently, our data implicates sex peptide in a system controlling the normal transfer of a portion of the seminal fluid proteome. Collectively, our data reveal a high level of integration between SFPs, uncover cryptic roles played by SFPs within the male, and highlight a novel mechanism of dysregulation in a prostate-like organ.

INTRODUCTION

The ejaculate is a composite of sperm and seminal fluid. While the importance of the former for reproductive success has been appreciated for many centuries (Birkhead and Montgomerie 2009), the pivotal role played by the latter has only recently become apparent. So crucial is seminal fluid that failure to transfer it can render males of some species functionally infertile (Xue and Noll 2000), while disruption to its composition can lead to deleterious offspring phenotypes, such as metabolic disorders (Bromfield et al. 2014). Accordingly, concern has been expressed by some authors at the use of 'washed' sperm, which is free from seminal fluid, in assisted reproductive technologies (Mcgraw et al. 2015). Others still have drawn attention to the dependency of pregnancy outcome, fertility, and long-term female reproductive health on seminal fluid/female reproductive tract interactions (Robertson and Sharkey 2016). As human fertility rates decline (Levine et al. 2018), unpicking the mechanisms by which seminal fluid influences fertility must be a research priority.

Proteins carried in the seminal fluid ('SFPs') are key mediators of reproductive outcome across species (Avila et al. 2011; Mcgraw et al. 2015; Robertson and Sharkey 2016; Hopkins et al. 2017, 2018). Despite their cross-taxa importance, much of our understanding of the actions of SFPs comes from work in the fruit fly *Drosophila melanogaster.* Studies in this system have revealed that individual SFPs can directly stimulate key reproductive processes. For example, sex peptide is a master regulator of female post-mating change (Gioti et al. 2012), variously influencing aggression levels (Bath et al. 2017), dietary preferences (Carvalho et al. 2006; Walker et al. 2015), receptivity to remating, and fecundity stimulation (Chapman et al. 2003; Liu and Kubli 2003). However, it is also clear that many, perhaps even the majority, of SFPs function in supporting the activities of others. The best illustration of this comes from the

unravelling of the 'sex peptide network', a group of at least 8 SFPs that collectively act to promote the localisation of SP within the female sperm storage organs (Peng et al. 2005a; Ram and Wolfner 2007b, 2009; LaFlamme et al. 2012; Singh et al. 2018). While some sex peptide network proteins are late-acting, such as those facilitating the binding of sex peptide to sperm (Singh et al. 2018), others act further upstream and are necessary for the efficient transfer of their fellow network members (Ram and Wolfner 2009). Across the full spectrum of ejaculate proteins, there is evidence of varying degrees of specificity in the effect that an SFP can have on the transfer of others (high specificity: CG9997 on CG1652 and CG1656, Ram and Wolfner, 2009; low specificity: Acp26Ab on many, Ram and Wolfner, 2007).

The potential dependency of SFPs on the presence of others for effective transfer raises three important points. Firstly, it implies a level of intra-ejaculate organisation in the regulation and stabilisation of seminal fluid. If this is taxonomically widespread, then a breakdown of seminal fluid organisation may represent an underappreciated driver of male fertility problems. Secondly, from an ejaculate design perspective, it could help explain why seminal fluid proteomes are so richly diverse. Rather than hundreds or thousands of SFPs driving individual phenotypic effects in females, many may function as part of networks promoting a common phenotypic endpoint. Thirdly, it raises problems for reductive approaches to the functional annotation of SFPs. Phenotypes associated with a SFP knockdown/knockout may not be linked purely to the lost protein, but instead be a consequence of proteome-wide dysregulation that results from the high-connectedness of SFPs. Consequently, seminal fluid may best be considered as a dynamical system, rather than a collection of static, individual parts.

In this paper, we use quantitative proteomics to test whether ejaculate composition is changed following loss of the keystone SFP underlying most female post-mating

changes, sex peptide ('SP'). To do so, we compare the accessory gland proteome of virgin and recently-mated males from a *SP* knockout and rescue line widely used in evolutionary and molecular explorations of SP function (*e.g.* Liu and Kubli, 2003; Carvalho *et al.*, 2006; Fricke *et al.*, 2009; Gioti *et al.*, 2012; Walker, Corrales-Carvajal and Ribeiro, 2015; Bath *et al.*, 2017). By analysing both virgin and recently-mated accessory glands form each genotype, we can identify (a) differences in protein production, (b) differences in protein retention, and (c) by comparing the two, differences in transfer to females. Given that SP's functions are carried out downstream of other known SFPs, both in terms of the multi-day persistence of its effects and its dependency on early-acting network proteins, we make the following null predictions: (1) loss of SP does not lead to differences in SFP production; (2) loss of SP does not lead to differences in what is transferred to females.

METHODS

Fly stocks and husbandry

To produce males that synthesise no SP, we used *SP* gene knockouts previously developed through homologous recombination (Liu and Kubli 2003). Wild-type SP is synthesised via a 55-amino acid precursor. This precursor displays a 19-amino acid signal peptide, which is cleaved to produce the mature 36-amino acid form of SP (Chen et al. 1988). The SP^0 mutant was originally generated by inserting a TAG (UAG) stop codon in a region encoding the 19-amino acid signal peptide sequence (Liu and Kubli 2003). A rescue line was also generated in the same study, which includes a tandem arrangement of the non-functioning SP^0 alongside a wild-type SP^+ copy. To produce *SP* null males, we crossed SP^0*/TM3,Sb,ry* males to Δ*130/TM3,Sb,ry* females. Δ*130* flies carry a 3L chromosomal deletion that extends through the *SP* sequence (Nolo et al. 2001). For our controls, we crossed Δ*130/TM3,Sb,ry* females to males carrying the rescue SP^0,SP^+*/TM3,Sb,ry*. Each of these three lines was previously backcrossed for four generations into a wildtype Dahomey background. Females from these crosses were allowed to oviposit on a grape-agar plate, supplemented with a small quantity of yeast paste. We washed eggs from the surface of the plate with PBS into a flask, removed the supernatant, and, using a pipette, deposited 12.5µl of the egg-containing liquid into 250mL bottles housing 50mL of Lewis medium. This ensured we distributed an approximately equal number of eggs (~200) per bottle to standardise larval rearing densities (Clancy and Kennington 2001). We used this same approach to rear eggs laid by Dahomey females.

We collected *SP* null and control males as virgins (*i.e.* within 7-hours of eclosion) under ice anaesthesia. They were subsequently held in groups of 10-12 in 36mL Lewis medium containing vials supplemented with *ad libitum* yeast granules. We followed the

same procedure for Dahomey virgin females and males. All flies were then aged for 4 to 5 days before use in experiments. All rearing and experimental matings of these flies took place in controlled-temperature facilities held at 25°C on a 12:12 light:dark cycle.

Phenotypic verification of the *SP null*

To verify the loss of *SP* in the null, we tested for differences in the receptivity of females mated to *SP* null*,* rescue, and, as a further control, Dahomey males. Females that fail to receive SP are highly receptive to remating (Chapman et al. 2003; Liu and Kubli 2003), so this assay provides a clear indication of *SP* loss. We individually isolated males from the three genotypes in *ad libitum* yeasted vials before introducing a virgin female. Once mating had ended, we removed the male. 24-hours later, we individually aspirated mated females into yeasted vials containing a pair of Dahomey males and recorded the time until they started mating. We found that 13/15 *SP* null-mated females remated within 2-hours, while 3/17 Dahomey-mated remated and none of the 15 rescue-mated females remated (*SP* null versus control: χ^2_2=28.277, p<0.0001; Supp. Fig. 1), thus confirming the expected phenotype of the *SP* null males.

Mating experiment

We alternatingly aspirated males from each of the two genotypes (*SP* null and control) into yeasted vials that were either empty or contained a single virgin female isolated under ice anaesthesia the evening before. By comparing the proteome of virgin and mated males we gain access to what males transfer and overcome some problems that accompany analysing mated female reproductive tissue, such as dilution of low-abundance, male-derived proteins against the complex, female-derived proteome, and the rapid processing that some SFPs undergo once inside the female (Sepil et al. 2018).

In the case of males introduced into female-containing vials, we recorded the time a male entered the vial, the time the pair began to mate, and the time they finished. Through our alternation of introducing males into empty or female-containing vials, we could pair them such that when we came to flash-freeze a male in liquid nitrogen 25-minutes after the start of mating, we would simultaneously freeze the virgin male in the paired vial. By taking this approach we ensured that the distribution of freezing times was equivalent between mated and virgin treatments. After freezing, we held males at -80°C. We repeated this experiment three times so as to produce three independent biological replicates.

Sample preparation

A single sample consisted of 17 pairs of accessory glands, which we dissected on ice under a light microscope in a drop of ice-cold PBS. Grouping of accessory glands enhances our ability to detect otherwise low abundance proteins (Sepil et al. 2018). We severed the accessory glands from the distal end of the ejaculatory duct and removed the seminal vesicles and testes. Samples were held in 25µl of PBS and stored at -80°C prior to processing. In total, we had 12 samples: fully factorial permutations of mated status (virgin/mated) and genotype (*SP* null/control) across 3 replicates.

To prepare the samples for proteomic analysis we followed the GASP protocol (Fischer and Kessler 2015), which we have previously shown achieves highly-sensitive detection of SFPs (Sepil et al. 2018). We began by macerating the glandular tissue using a pestle for a timed period of one minute. We next added 25µl of Pierce RIPA (radioimmunoprecipitation assay) buffer to lyse the cells, dripping it slowly over the pestle to wash residual tissue back into the sample. The lysate was then incubated on ice with 50mM of DDT (dithiothreitol). We next added in succession an equal volume of

40% Bis/acrylamide solution (37.5:1 National Diagnostics), 5µl of 10% APS (ammonium persulphate), and 5 µl of TEMED (tetramethylethylenediamine). This led to the formation of a solid gel, which we loaded on to a spin shredder (CLS9301, Sigma/Corning) and fragmented. We fixed the resulting pieces of gel in a solution of 5% acetic acid/40% ethanol before undertaking two rounds of alternating thiourea/urea and ACN (acetonitrile) washes. This we followed with a further two rounds of ammonium bicarbonate and ACN washes. Then we added 250µl of dilute trypsin and left to solution to digest at 37°C overnight. We extracted the resulting peptides via two repeated acetonitrile replacements before drying and desalting them in Sola SPE columns (Thermo). Prior to LC-MS/MS (liquid chromatography mass spectrometry/mass spectrometry), the peptides were suspended in a 2% ACN/0.1% formic acid buffer.

LC-MS/MS

For peptide analysis, we used a LC-MS/MS platform composed of a Dionex Ultimate 3000 and a Q-Exactive HF mass spectrometer (Thermo). Peptide loading took place in a solution of 0.1% TFA in 2% ACN on a trap column (PepMAP C18, 300µm x 5m, 5µm particle, Thermo). We separated peptides using an easy spray column (PepMAP C18, 75µm x 500m, 2µm particle, Thermo) with a gradient 2% ACN to 35% ACN in 0.1% FA in 5% DMSO. For MS spectra collection, we used a resolution of 60,000 in profile mode on the Q-Exactive HF (ion target = $3x10^6$). We selected the top 12 most intense features selected for subsequent MS/MS analysis (resolution of 30,000). The following parameters were set: dynamic exclusion = 27 seconds; AGC target = $1x10^5$; isolation width = 1.2 m/z; and maximum acquisition time = 45ms.

MS data processing

The MS data processing pipeline we used has previously been described (Sepil et al. 2018). We imported the RAW data into Progenesis QIP (version 4.1.6675.48614) exporting spectra as MGF files using the 200 most intense peaks without deconvolution for searching. For peptide identification, we used the *Drosophila melanogaster* UniProt reference proteome as a search target, with database retrieval conducted on 30/03/2015 (21361 sequences) in Mascot 2.5.1. Our search parameters incorporated the following: Oxidation (M), Propionamide (K), and Propionamide (N-term) as variable modifications; Propionamide (C) as a fixed modification; one missed cleavage sites; 0.05 Da fragment mass accuracy; 10ppm precursor accuracy. Prior to importing the search results into Progenesis for quantification via the Top3 method, we applied a peptide-level 1% FDR alongside a further Mascot ion cutoff of 20. The resulting protein abundance data was subsequently normalised using the internal Progenesis algorithm to a set of housekeeping proteins.

Data analysis

We detected 2246 proteins in total, but restricted our analysis to the 1502 detected on the basis of at least 2 unique peptides (as in Borziak *et al.*, 2016; Sepil *et al.*, 2018). This subset contained 118 SFPs known from previous work to be transferred to females (Findlay et al. 2008, 2014; Sepil et al. 2018). We further included one SFP not previously demonstrated to be transferred to females (intrepid, *intr*) due to its known role within the sex peptide network pathway (Findlay et al. 2014). We conducted all analysis with R statistical software (version 3.5.1)(Team 2013) in RStudio (version 1.1.456)(RStudio Team 2015). In each analysis, we used $\log_2$ transformed values to standardise variance across the dynamic range of protein abundances. Where linear mixed models are used,

input values are generally treatment averages taken across replicates. This was undertaken so as to improve the model fit, which was inferred through visual inspection of diagnostic plots. When analysing combined datasets of many proteins, we include protein identity as a random effect. We assessed the statistical significance of factors by analysis of deviance using the 'drop1' function. Where the interaction term was insignificant, we re-fitted the model without it. Log_2 fold changes were calculated by subtracting the mean log_2 transformed values between treatments. The virgin-mated fold change within a genotype is taken to reflect the degree of transfer during mating (Sepil et al. 2018).

We performed PCAs using the 'prncomp' function in the *stats* package. We tested for associations between PCs and our measured variables (genotype and mating status) by extracting the PCA scores and fitting a linear model to them. Our PCA plots were produced using *ggbiplot* (Vu 2011), adding ellipses to denote 80% normal probability (increased from the less-conservative default of 67%). To assess the protein-by-protein contributions to each PC, we used the 'fviz_contrib' function in *factoextra* (Kassambara and Mundt 2017). Our hierarchical clustering analyses were performed and graphically displayed using the *pheatmap* package. For each protein, we took a mean abundance across replicates. We selected the first 5 earliest-branching clusters for further analysis. The protein S-Lap7 was removed from Cluster 3 due to it being an extreme outlier and showing high between-replicate variability in abundance. To visualise the general abundance profile across treatments that each cluster captured, we divided the non-log_2 transformed mean abundance for each protein by the mean calculated across all samples, before taking a treatment mean. This gives a measure of abundance change that is comparable across the substantial variance range of proteins.

RESULTS AND DISCUSSION

SP levels in the proteomic data

As expected, we detected significantly lower abundance of SP in the *SP* null treatment compared to controls (LM: $F_{1,12}$=30.726, p=0.0009). However, surprisingly SP levels in the null treatment were ~17% of control abundance, rather than the trace amounts we might expect if analysis software was generating artificial non-zero values from noise.

To check that the presence of SP in our null samples was not a result of carry-over from previous control samples run on the MS, we independently re-ran two *SP* null samples: again SP was detected, thus verifying our findings. We next examined the degree of confidence associated with the detection of SP. In the original Mascot search, we detected four peptide sequences across samples (DKWCR; DKWCR + propionamide (K); KPTKFPIPSPNPR + propionamide (K); and LNLGPAWGGRC). In our *SP* null samples, we detected only the latter peptide in each. However, using an alternative search engine (Peaks), we identified an additional SP peptide (WEWPWNR) in the *SP* null samples. This peptide went undetected in the original search as it contained no predicted cleavage site that would be recognised following an *in silico* digest. We also identified a cysteine modified DKWCR peptide in the *SP* null samples during this deeper analysis. Further examination of the normalised quantitation of the top three most abundant SP peptides showed that they shared the same abundance profile across samples (Supp. Fig. 2). Were we misidentifying rogue peptide fragments, we would expect the individual peptide profiles to diverge. Thus, these peptides were likely from mature SP in *SP* nulls.

One key possible explanation for the presence of SP in the null treatment is read-through of the stop codon, whereby protein translation can continue through a stop codon and progress until a second is encountered; a process that is thought to be common in *Drosophila* (Jungreis et al. 2011; Dunn et al. 2013). Indeed, of the three universal stop

codons, UAG, as used in the *SP* null*,* is generally recognised as the least efficient in terminating translation (Larkin and Place 2017). Why previous work on SP has failed to detect read-through could be due to the lower sensitivity of the detection methods used, such as Western blots. In phenotypic assays, females have been shown to only respond to SP when it is received in quantities that exceed a critical threshold (0.6pmol)(Schmidt et al. 1993). Therefore, limited transfer of read-through produced SP would likely go undetected in assays of post-mating behaviour, such as the one we employed. Although we were unable to completely exclude the possibility of contamination of the samples through the contamination of fly stocks with non-null individuals or contamination of the samples through vial mislabelling, the fact that SP levels were consistent across temporally-separated independent replicates within a *SP* null mating treatment (3 virgin, 3 mated) argues against contamination (Supp. Fig. 2). Thus, given our clear phenotypic verification of *SP* knockout, the low quantities of SP detected, and the consistency of the degree of detection across temporally-separated replicates, stop-codon read-through is most likely. We therefore continued with our study, omitting SP from further analysis to avoid the confound of attributing changes to the SFP proteome to changes in SP levels. References to 'all SFPs' from hereon in do not include SP.

Loss of SP stimulates SFP production

Our first prediction was that loss of SP does not change the degree of SFP production. To test this, we began by fitting a linear mixed effects model to all SFP abundances in virgin glands including protein identity as a random effect. Contrary to expectation, we detected significantly higher average SFP abundance in *SP* null virgin glands compared to rescue virgins ($F_{1,5882}$=9.555, p=0.002). This difference equated to an increase in average SFP abundance of approximately 6%. We next plotted the distribution of between-genotype

fold changes to examine whether the effect of elevated abundance in *SP* null virgin glands is driven by a large number of SFPs of small between-genotype differences or a small number of SFPs with large between-genotype differences (Fig. 1). The distribution appeared biased with elevated abundance of ~64% of SFPs (75/118) in *SP* null virgins. This suggests a distinct, but not universal, elevation of SFPs in *SP* null glands.

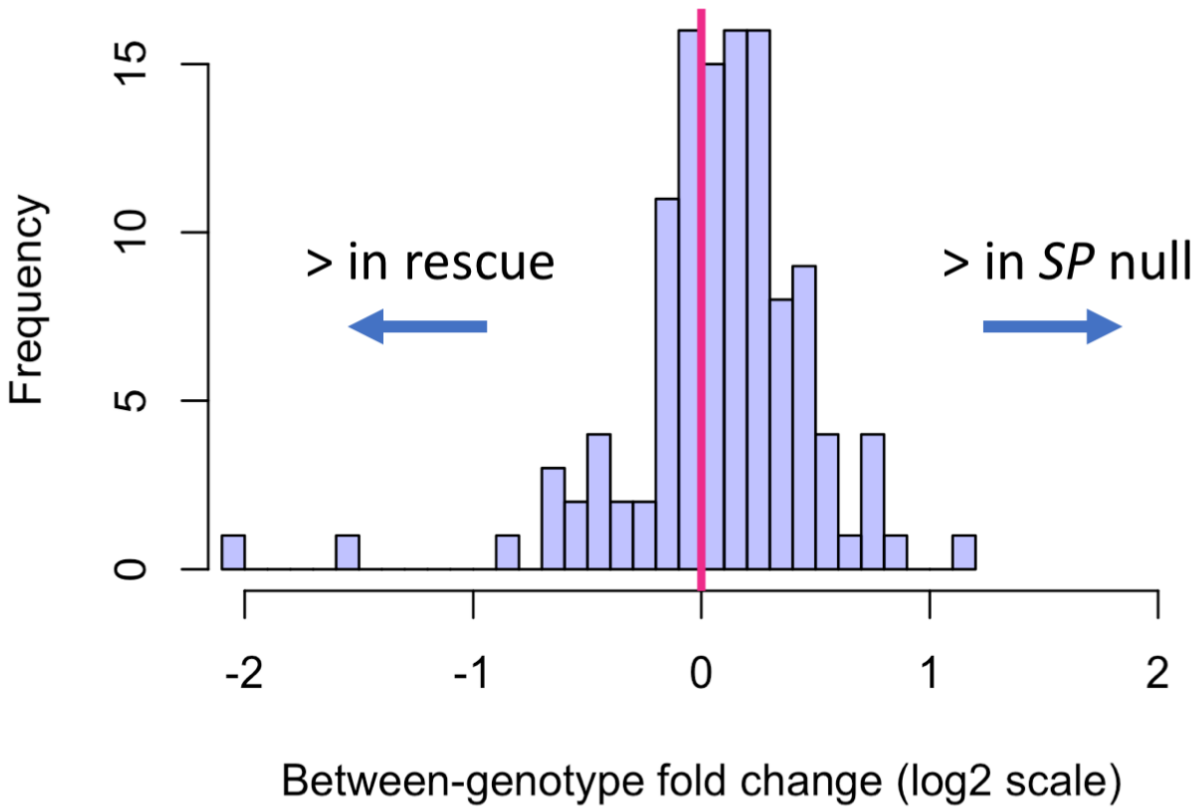


Figure 1. The distribution of between-genotype fold changes in virgin glands

Fold changes are on a $\log_2$ scale. Fold changes are calculated by taking the mean (across replicates) abundance for each SFP in virgin *SP* null and, separately, rescue virgin glands. The rescue value is then subtracted from the null value. Therefore, positive values indicate greater abundance in the *SP* null virgin gland. Negative values indicate greater abundance in rescue virgin glands.

Greater SFP abundance in *SP* nulls correlates with upregulated protein synthesis machinery

Elevated abundance of SFPs in *SP* null virgins may either be due to elevated rates of protein production or decreased rates of protein degradation. Accordingly, we would predict an associated increase in the abundance of proteins involved in one of these two processes in *SP* nulls. To test this, we identified major groups of proteins associated with protein production (translation initiation factors, ribosomal proteins), degradation (proteasome proteins), and transport (Rabs). Visual inspection of heatmaps, the structure of which was determined via hierarchical clustering, suggested no clear relationship between *SP* loss and the abundance of Rabs and proteasome components (Fig. 2). The latter did, however, show a clear signal of elevated abundance in response to mating in both *SP* nulls and rescues. Translation initiation factors and ribosomal proteins, showed a strong response to mating, but large numbers of proteins also appeared upregulated in virgin *SP* null males relative to virgin control males. This was reflected in a significant interaction between genotype and mating status (LME: $F_{1,321}$=4.183, p=0.042; effect size on $\log_2$ scale: -0.07), which suggests that the normal elevation of these proteins after mating is less pronounced in *SP* null glands because the levels of protein synthesis related machinery are already higher in the virgin state. Thus, the generally higher abundance of SFPs in *SP* null virgin glands is likely driven by elevated protein production, rather than decreased degradation.

Why should the loss of one SFP stimulate the production of others? How the rate of protein production within the *Drosophila* accessory gland is controlled is poorly resolved. However, evidence from two sources indicates a level of sensitivity in glandular activities to mating. It is known that mating stimulates the release of concentrated stores of secretory material ('dense core granules') from one of the accessory gland's constituent

cell-types (Redhai et al. 2016). Furthermore, mating is known to stimulate levels of protein synthesis within the gland (Schmidt et al. 1985). These processes could be driven by mechanical stimulation of the gland during mating, a neuropeptide circuit, or alternatively the secretory epithelium enveloping the gland may make use of a feedback system that links depletion to production. During mating, a third to a half of the total secretory material contained within the accessory gland lumen is lost (Kubli 1992; Sepil et al. 2018), which presumably leads to a change in the gland's internal pressure. Predictable changes in the outward pressure exerted by the lumenal contents would therefore offer a dependable cue as to when elevated rates of protein production are required. If the secretory epithelium does indeed tailor its rates of production to the gland's internal pressure, then loss of a highly-abundant SFP, such as SP, may mimic post-mating depletion and lead the gland to compensate by over-producing other SFPs. This hypothesis is consistent with the clear upregulation of protein synthesis machinery that we detect. Alternatively, SP may form part of an intra-glandular signalling network that mediates rates of protein turnover. While no such network has been discovered, previous work has hinted at their existence (Chapter 4; Sitnik et al. 2016).

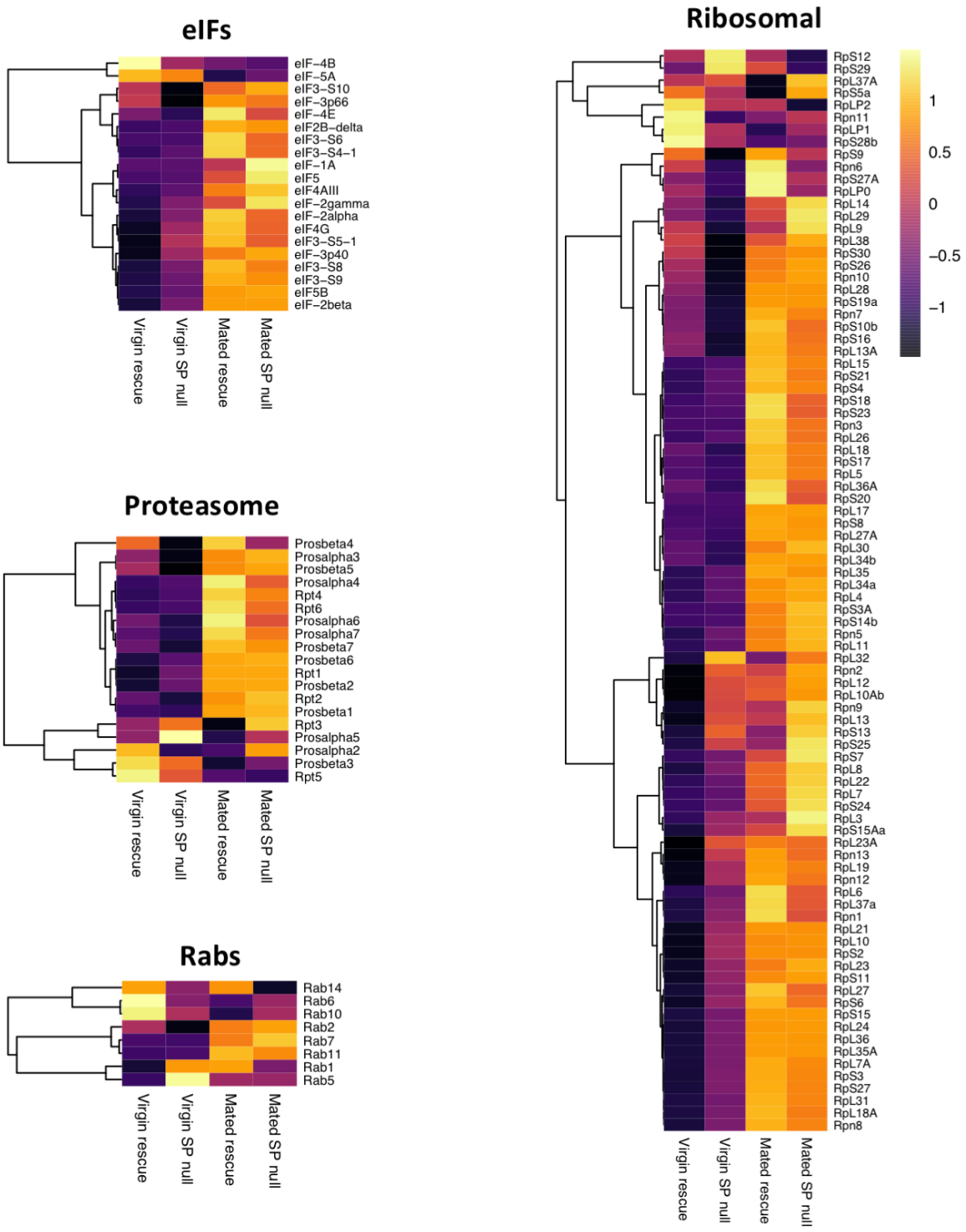


Figure 2. Signatures of upregulation in protein synthesis proteins in *SP* null virgins

Each heatmap captures a distinct protein functional grouping: eukaryotic transcription initiation factors (eIFs), Rabs, Ribosomal proteins, and proteasomal proteins. Each protein abundance is plotted for the rescue control virgin glands (far-left column), the *SP* null virgin glands (middle-left column), the rescue control mated glands (middle-right column), and *SP* null mated glands (far-right column). Protein location was determined by hierarchical clustering.

Loss of *SP* differentially affects the transfer and production of groups of SFPs

We identified that the SFP proteome in virgin *SP* null glands is split between a portion that is and is not upregulated. We may therefore expect a corresponding split between portions of the SFP proteome that are transferred in higher abundances and those transferred normally during mating. To test for multi-directional changes in the SFP proteome following *SP* loss, we used a hierarchical clustering analysis to identify distinct profiles of change across matings and genotypes. By applying this analysis across glands, we can test for generalised behaviours of groups of SFPs in terms of production, post-mating retention, and, as the difference between these, what is transferred to females. We included 5 detected structural proteins in this analysis to act as a protein outgroup. Their inclusion provides a comparison between the behaviour of the secreted and non-secreted portions of the glandular proteome in response to *SP* loss and mating.

Hierarchical clustering revealed the presence of 5 distinct, higher-order clusters. We ran linear mixed effects models on each cluster to test for associations with our measured variables (see Table 1; Fig. 3). Cluster 1 showed no significant association with any of the tested variables, and included 3 of the 5 reference structural proteins. Thus, we excluded it from further analysis.

Clusters 2,4, and 5 capture SFPs that are produced in significantly greater quantities in *SP* null virgin glands (Table 1; Fig. 3). This accords with our previous analyses demonstrating upregulation of SFPs in response to the loss of *SP*. Collectively, these clusters capture a substantial portion of the SFP proteome: 98 of the 118 SFPs we analyse. Cluster 3, however, describes a group of SFPs at lower abundance in *SP* nulls. A significant interaction between mating and genotype recorded for this cluster indicates that lowered production in *SP* null glands also translates into lower transfer to females (Table 1; Fig. 3). This cluster includes Dup99B, which shares considerable functional

similarity with SP, mimicking its receptivity-reducing and fecundity-stimulating qualities (Saudan et al. 2002). The reduced transfer of Dup99B and other members of this cluster following *SP* loss could therefore compound or confound the phenotypic effects detected in *SP* null-mated females.

SFPs in Cluster 5 are transferred to females in greater quantities, as inferred from a significant genotype by mating interaction (Table 1; Fig. 3). This elevated transfer is driven by two processes: greater production in virgins and lowered post-mating retention. Lowered retention of some proteins indicates that loss of *SP* dysregulates their transfer. Why loss of *SP* would lead to loss of control over SFP transfer during mating is unclear. One possibility is that SP aids in the organisation of the lumenal contents of the accessory gland. Consistent with this hypothesis, there is emerging evidence that SP maintains the structure of lipid-based 'microcarriers' within the accessory gland lumen (Wainwright *et al., in prep*). The function of these 'microcarriers' is unknown, but they may stabilise the transfer of SFPs, preventing their premature interaction, or regulate their trafficking to target regions of the female reproductive tract. Interestingly, despite accounting for just 26 of the 118 SFPs, Cluster 5 seems enriched for sex peptide network proteins, containing 5 of the 8 known: CG1652, CG1656, antr, intr, and aqrs (Ram and Wolfner 2009; Findlay et al. 2014; Singh et al. 2018). That we find evidence of known SP interactants responding similarly to *SP* loss indicates that these carriers could specifically promote the coordinated transfer of functionally-related SFPs.

Although Clusters 2 and 4 describe significant upregulation of SFPs in virgin *SP* null glands, in neither case does this translate into greater transfer to females during mating (inferred from the lack of a significant genotype by mating interaction, Table 1). Instead, these clusters separate from one another according to the extent to which the production of their constituent proteins is increased in *SP* null glands. While the

upregulation is modest in Cluster 4 (1.15x more abundant), the effect is much larger in Cluster 2 (1.47x more abundant). This differing sensitivity could be explained by divergent mechanisms regulating the production of these two protein clusters. No change in the transfer of SFPs to females within certain clusters indicates that loss of *SP* exerts SFP-specific effects on the transfer of accessory gland products. This further supports the previous suggestion that transfer mechanisms (potentially microcarriers) regulate the transfer of just a subset of SFPs rather than acting as more global influencers of SFP transfer. If so, the transfer of SFPs in Clusters 2 and 4 may be driven by independent, unknown mechanisms.

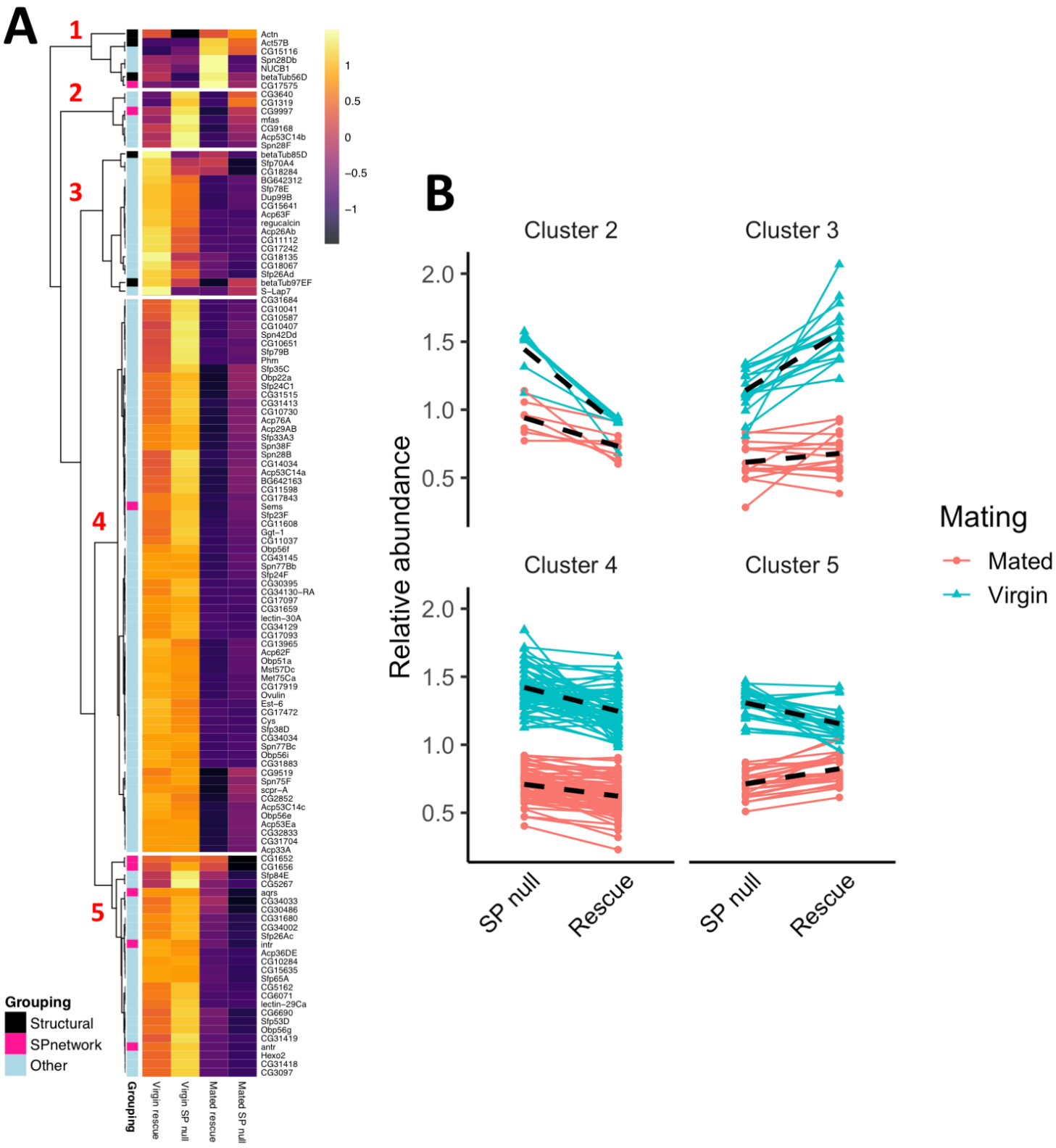


Figure 3. Seminal fluid proteins show diverse responses to *SP* loss

(Text overleaf)

Figure 3. Seminal fluid proteins show diverse responses to *SP* loss

(A) A heatmap that plots the mean abundance taken across three replicates for each seminal fluid protein (and 5 structural proteins as a reference). Each protein is plotted for the rescue control virgin glands (far-left column), the *SP* null virgin glands (middle-left column), the rescue control mated glands (middle-right column), and *SP* null mated glands (far-right column). Row annotations refer to whether a protein is structural or known to be involved in the sex peptide network. Five clusters, identified by a number in red, are taken forward for further analysis. (B) The generalised abundance pattern for Clusters 2 to 5 are given on the right-hand side of the panel. N= *SP* null; R= rescue control; red gives mated glands; blue gives virgin glands. The heatmap is generated using $\log_2$ normalised abundances, while the line graphs use mean-centred abundances to account for dynamic range in abundance across proteins

Cluster	*Variable*	*Df*	*Sum of sq*	*Mean sq*	*DenDF*	*F*	*P*	*Effect size*
1	Genotype*Mating	1	0.61843	0.61843	9	2.4364	0.1530	- 0.786
1	Genotype (R)	1	0.57977	0.57977	10	1.9972	0.1879	0.381
1	Mating (V)	1	0.69323	0.69323	10	2.3881	0.1533	- 0.416
2	Genotype*Mating	1	0.13749	0.13749	18	3.1034	0.0951	- 0.280
2	Genotype (R)	1	2.1672	2.1672	19	44.042	<0.0001	- 0.556
2	Mating (V)	1	1.8189	1.8189	19	36.963	<0.0001	0.510
3	Genotype*Mating	1	0.54912	0.54912	42	4.736	0.0352	0.383
3	Genotype (R)	1	1.3894	1.3894	43	14.596	0.0004	0.350
3	Mating (V)	1	15.993	15.993	43	126.959	<0.0001	1.033
4	Genotype*Mating	1	0.19178	0.19178	192	2.8732	0.0917	0.109
4	Genotype (R)	1	2.723	2.723	193	40.401	<0.0001	- 0.205
4	Mating (V)	1	70.612	70.612	193	1047.762	<0.0001	1.042
5	Genotype*Mating	1	0.7174	0.7174	69	21.51	<0.0001	-0.346
5	Genotype (R)	1	0.0023	0.0023	70	0.0542	0.8165	0.010
5	Mating (V)	1	11.7380	11.7380	70	272.1869	<0.0001	0.699

Table 1. Cluster-specific responses to *SP* loss and mating

Following the identification of 5 higher-order clusters through a hierarchical clustering analysis, we ran a linear mixed effects model on the proteins captured within each cluster to test for significant associations with our measured variables. Effect sizes are on a $\log_2$ scale and are relative to a baseline of a mated *SP* null. R = Rescue; V = Virgin. Red gives significance at $p<0.05$, orange at $p<0.1$.

Differential sensitivity to *SP* loss drives compositional change in the SFP proteome

Our hierarchical clustering analysis revealed distinct, generalised responses of different portions of the SFP proteome to *SP* loss across mating treatments. To better understand how these multi-directional patterns of change intersect to alter the composition of the SFP proteome, we used a PCA to distil our data into major axes of variation. We then tested those axes for associations with *SP* loss and mating status.

Across none of the first three principal components, which collectively describe 86.2% of the variance, did we detect an interaction between mating status and genotype (Table 2). Thus, the large majority of global between-sample variance in protein abundance is explained by factors other than an interaction. This is consistent with the conclusion from our previous analysis that the specific effects of *SP* loss on SFP transfer are restricted to a subset of SFPs.

Unsurprisingly, we found that mating status represented the dominant axis of variation in SFP abundance across samples and was significantly described by PC1 (Table 2). PC3, on the other hand, captured an axis of variation significantly associated with genotype (Table 2). A PC1 by PC3 plot showed a good degree of separation between genotypes and mating statuses, indicating distinct responses of the SFP proteome to both mating and *SP* loss (Fig. 4a). PC2 was not significantly associated with any tested variables and thus represents an unknown axis of variation in our dataset (Table 2).

Comparing the directionality of the loadings between PC1 and PC3 highlights the divergent effects that mating and *SP* loss have on the abundance of SFPs. In PC1, almost all SFPs (115/118) showed a positive correlation (*i.e.* bore positive loadings), which matches the expected SFP proteome-wide pattern of loss through mating. In PC3, however, SFPs were split in the directionality of their loadings (59/118 positive), suggesting a mixed-response of SFPs - both increases and decreases in SFP abundance -

to *SP* loss. This accords with our previous analyses that suggest the effects of *SP* loss on SFP transfer and production are not general. Moreover, this split response of SFPs points to compositional change in the SFP proteome. The strongest driver of change in PC3 (CG1319) bore a positive loading, indicating that its response to *SP* loss was marked upregulation (Fig. 4b). CG1319 is known to positively regulate ecdysteroid biosynthesis in flies (Palandri et al. 2015). Given that SFP production is suggested to be partially under the control of the ecdysone hormone (Sharma et al. 2017), upregulation of CG1319 could form part of the mechanism driving SFP over-production in *SP* null virgin glands.

All but one of the top 7 strongest contributors to PC3 were negative, indicating that most of the proteins particularly strongly affected by *SP* loss were reduced in abundance across glands (Fig. 4b). The list of strongly negative contributors included Sfp70A4, the protein directly adjacent to SP on chromosome 3, which our hierarchical clustering analysis also identified as showing a strong decrease in *SP* null glands. Interestingly, 3 of the top 6 negative contributors are known SP network proteins (CG1652, CG1656, and CG17575). Another sex peptide network protein, CG9997, falls in the top 5 strongest positive contributors. Taken alongside the results of our hierarchical clustering analysis, this further suggests that *SP* interactants may be disproportionately affected by the loss of *SP*. By association, other highly-sensitive SFPs presented in Fig. 4b, a list that includes three newly-discovered low-abundance SFPs (Sepil et al. 2018), may represent unknown contributors to the sex peptide network pathway.

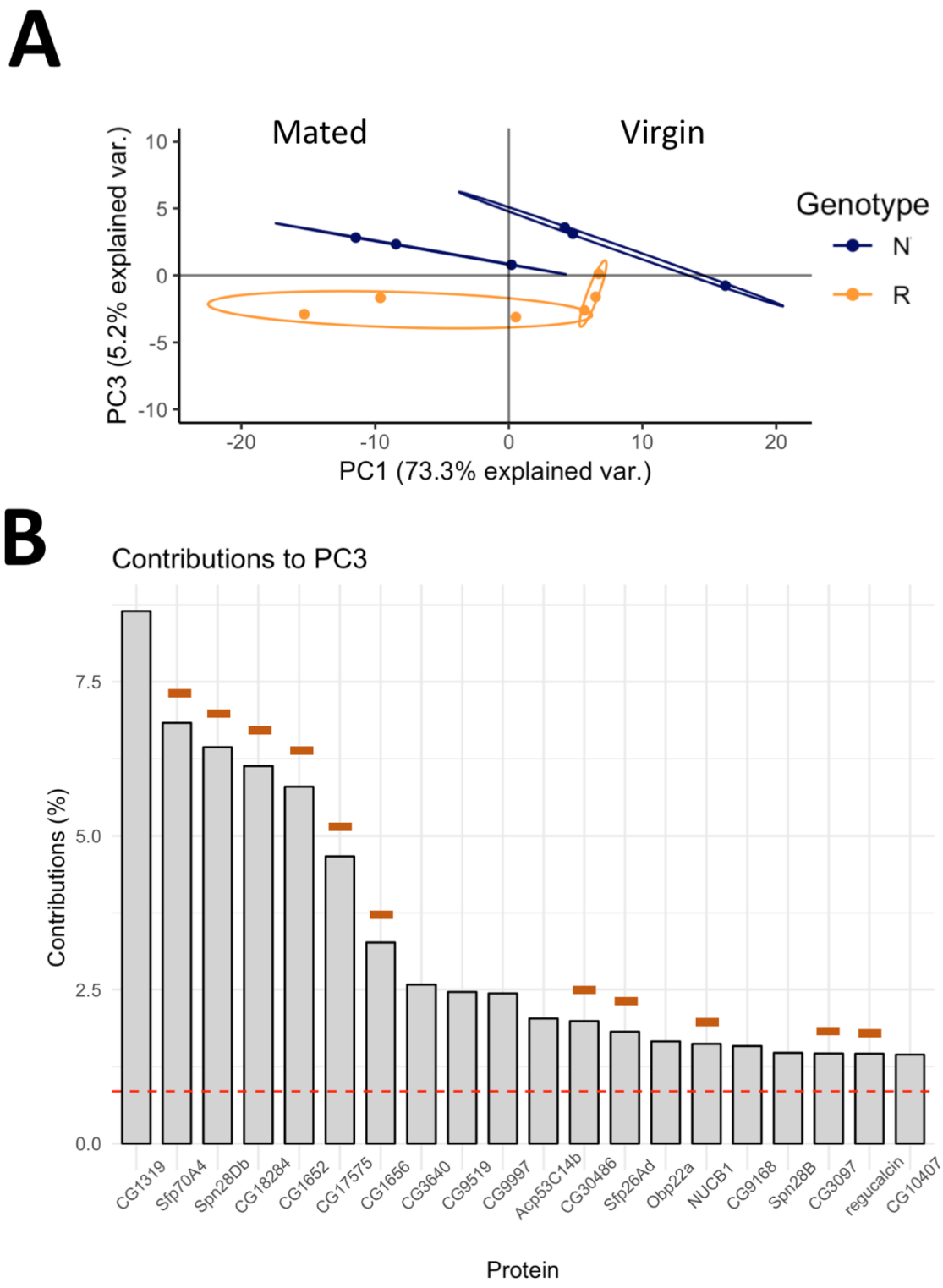


Figure 4. Variation in the sensitivity of seminal fluid proteins to *SP* drives compositional change

(A) A PC1 by PC3 plot. PC1 was shown to be significantly associated with mating status (virgin – ellipses on the right; mated – ellipses on the left). PC3 was shown to be significantly associated with genotype (*SP* null – blue; rescue – orange). Ellipses denote 80% probability. (B) The top 20 contributing SFPs to PC3, an axis of variation driven by genotype. Negative signs indicate whether a loading was negative *i.e.* if *SP* loss reduced abundance. Those without signs bore positive loadings. The red dotted line gives the expected contribution if all proteins were to contribute equally to PC3.

	PC1	*PC2*	*PC3*	*PC4*
Variance explained (%)	73.3	7.7	5.2	4.2
Eigenvalue	86.5	9.1	6.1	4.9
No. positive loadings	115	59	58	52
No. negative loadings	3	59	60	66

PC1	*Variable*	*Df*	*Sum of sq*	*RSS*	*F*	*P*
	*Genotype*Mating*	1	0.211	74.732	0.0170	0.9004
	Genotype	1	10.03	84.76	0.9395	0.3647
	Mating	1	648.04	722.77	60.7003	0.0001
	Replicate	2	219.08	293.82	10.2605	0.0083

PC2	*Variable*	*Df*	*Sum of sq*	*RSS*	*F*	*P*
	*Genotype*Mating*	1	11.7329	76.586	1.0855	0.3376
	Genotype	1	7.6292	84.216	0.6974	0.4312
	Mating	1	12.8743	89.433	1.1743	0.3144
	Replicate	2	2.8919	79.478	0.1322	0.8783

PC3	*Variable*	*Df*	*Sum of sq*	*RSS*	*F*	*P*
	*Genotype*Mating*	1	1.0790	10.2284	0.7076	0.4325
	Genotype	1	46.576	56.804	31.875	0.0008
	Mating	1	1.058	11.287	0.7243	0.4229
	Replicate	2	9.447	19.675	3.2326	0.1013

Table 2. Summary statistics from a PCA performed on 118 detected seminal fluid proteins

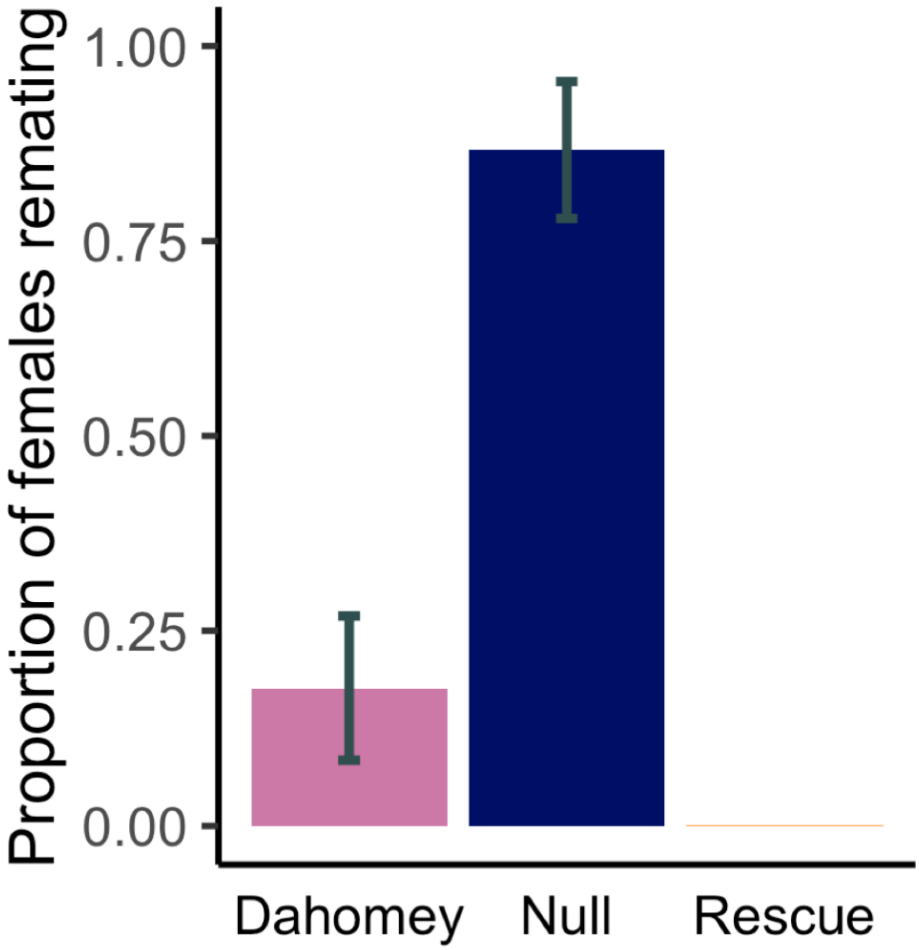
The top table provides statistics associated with each of the first four principal components. The following three tables provide the output from linear models fitted to the scores from each of the first three principle components (PCs). These test for associations between a PC and the measured variables of genotype, mating status, and experimental replicate. Red gives significance at $p<0.05$.

CONCLUSION

The changes to SFP production and transfer following *SP* loss revealed in this study have a number of important consequences. Firstly, researchers using *SP* knockouts should be wary of the extent to which the phenotypes they observe can be uniquely attributed to the removal of *SP* from the female-transferred ejaculate; broad compositional changes in the SFP proteome accompany the loss of *SP*. Secondly, our work points to levels of organisation within the accessory gland secretion that regulate the coordinated transfer of SFPs to females. This organisation may be mechanistically underpinned by recently described lipid-based 'microcarrier' structures within the accessory gland lumen (Wainwright *et al. in prep*). Given that loss of SP distorts the structure of microcarriers (Wainwright *et al. in prep*) and that SFP transfer is dysregulated following *SP* loss, it follows that these structures may function in the transfer of some SFPs. Associations of SFPs with higher-order ejaculate-borne structures, such as microcarriers, may facilitate their transport to target female reproductive tract tissues, prevent premature between-SFP interactions, or even guard sexually-antagonistic SFPs against female-mediated degradation. In light of the sensitivity of SFP transfer to the presence of other SFPs, males may be able to regulate the stability of organising structures within the accessory gland secretion by modulating the release of particular SFPs from the accessory gland cells. This could be used to exercise real-time control over the transfer of ready-made accessory gland secretion during mating (*e.g.* as in Sirot, Wolfner and Wigby, 2011). Finally, our work reveals a high degree of connectedness within ejaculates such that malfunctions in just a single component can have broad consequences both for the composition of the ejaculate and the activities of the glands driving its production. An awareness of the roles SFPs can play within males must be taken into consideration when designing novel fertility drugs and male contraceptives. In light of our data, an intervention that targeted

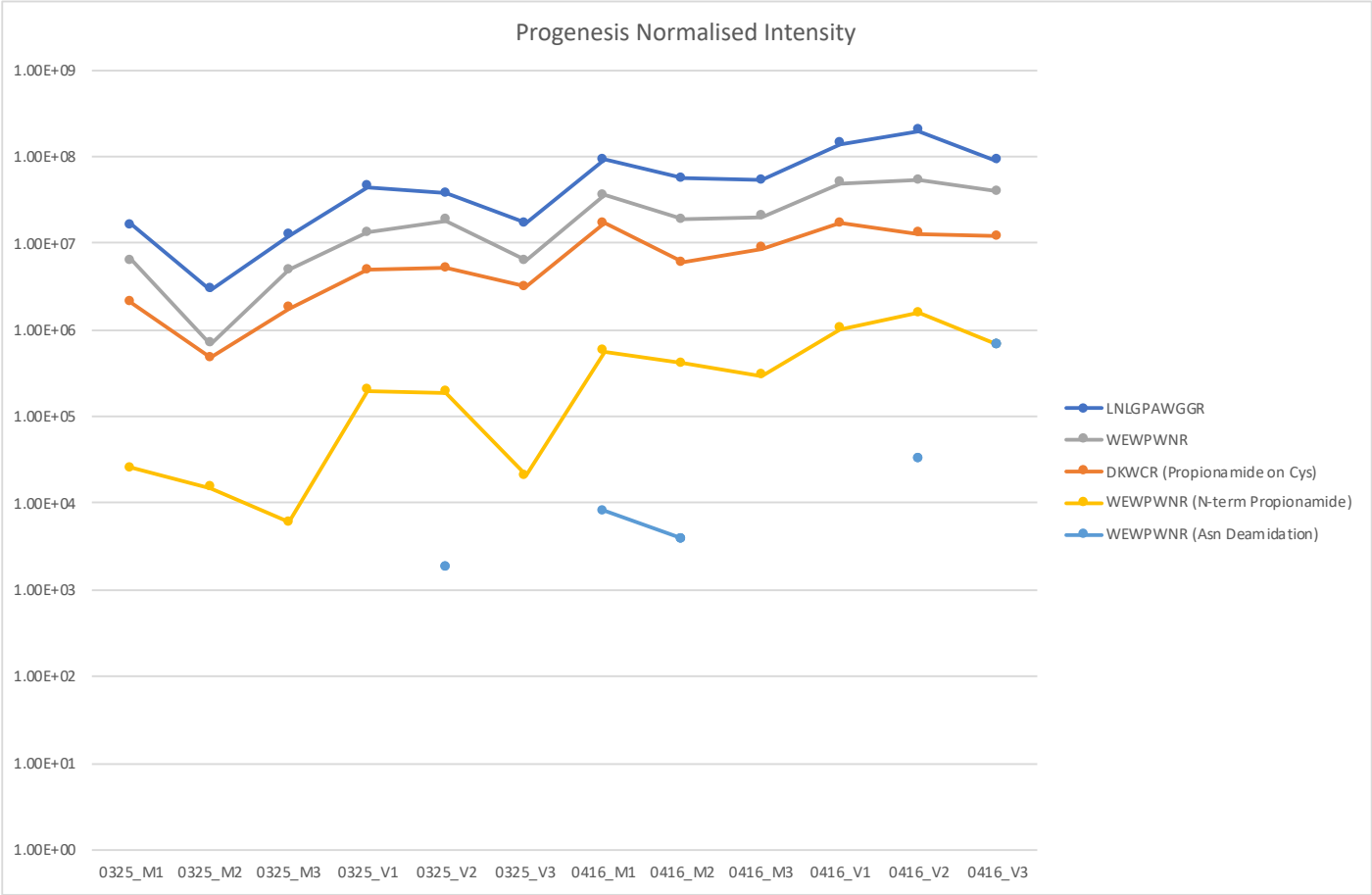
key human seminal fluid components could feedback to disrupt the normal biology of accessory reproductive glands, such as the prostate, and induce pathologies (see also Chapter 4). Moreover, it could change ejaculate composition in a way that aggravates the inflammation-inducing properties of seminal fluid within the female reproductive tract (*e.g.* Robertson and Sharkey, 2016). Ultimately, our work pushes us towards a new understanding of the ejaculate as a dynamic network of parts, a perspective that brings with it new challenges for biomedical research.

SUPPLEMENTARY MATERIAL



Supplementary Figure 1: Verifying the *SP* null phenotype

The proportion of females remating within a 2-hour period 24-hours after having previously mated to a wildtype Dahomey, *SP* null, or rescue control male. n_{Dah}=17, n_{null}=15, n_{rescue}=15. p<0.0001.



Supplementary Figure 2: Between-sample abundance patterns of each detected SP peptide Intensity profile for detected SP peptides. The principle (most abundant) form of each of the three sequences share the same intensity profile across samples. This indicates that mature SP is likely present in the null samples. 0325 = SP null; 0416=Rescue. M= Mating; V = Virgin. Numbers following the mating status refer to the experimental replicate.

Chapter 6

General Discussion

Summary

In Chapter 1, I made the case for treating the ejaculate holistically. I argued that we have limited theory in place to explain the evolution of seminal fluid and that evolutionary models of the ejaculate that neglect the action and composition of seminal fluid are incomplete. To address this deficiency, each of my data chapters represent an effort to test the causes and consequences of compositional change in the *Drosophila melanogaster* ejaculate.

In Chapter 2, I showed that males use independent allocation rules for sperm and SFPs. Males transfer low sperm numbers and low SFP abundances in the absence of competition; high sperm numbers and high SFP abundances at high competition; and high sperm numbers and low SFP abundances at low competition. I further showed that a subset of proteins are particularly responsive to mating, that different proteins are transferred in greater quantities according to one of two mechanisms (greater depletion and greater production), and that each of the three competition-specific ejaculate compositions is accompanied by distinct costs and benefits.

In Chapter 3, I showed that the loss of secretory activity from a particular cell-type in the accessory glands leads to improved defensive sperm competition performance. This improvement comes at the expense of the ability to reduce female receptivity to remating, but persists in the absence of any differences in offspring production. These split responses point to partial induction of the female post-mating responses, an idea supported by the syndrome of dysregulation in female-driven reproductive process that I uncover.

In Chapter 4, I showed that the loss of secretory activity in adult secondary cells disrupts the normal transfer of SFPs. While this may in part be due to some proteins becoming trapped within the secondary cells themselves, my data also suggest some level of disruption to the transfer of the predominantly main cell produced lumenal contents. I suggested that by controlling the secretion of master regulator products from the secondary cells, of which Acp26Ab may be a prime candidate, males could rapidly modify the transfer of a complement of accessory gland proteins within the gland lumen. I further show that loss of secondary cell activity alters the production of likely main cell proteins, exposing channels of inter-cellular communication within the gland.

In Chapter 5, I showed that loss of sex peptide feeds back to stimulate production of other SFPs within the accessory gland. This, I argued, may point to the presence of a mechanism linking a depleted glandular lumen, as would follow mating, to increased levels of protein synthesis. Alternatively, it may implicate sex peptide in pathways of cell-cell communication regulating protein production. I also showed that loss of sex peptide disrupts the transfer of other SFPs, particularly other sex peptide network proteins. This supports new work demonstrating a role for sex peptide in structuring the accessory gland secretion.

Future Directions

In this section, I group areas for future work into three themes relevant to the material presented in my data chapters. Firstly, I consider the mechanistic control of plastic ejaculate allocation. Secondly, I discuss what future work could focus on with respect to the evolutionary biology of plastic ejaculate allocation. Thirdly, I outline how my findings of ejaculate component inter-dependence inform our understanding of the evolution of ejaculate composition.

1. The mechanistic control of plastic ejaculate allocation

A substantial body of work now supports the ability of males to tailor the composition of their ejaculate in response to environmental stimuli (see Chapter 1 & 2 for supporting studies). However, the mechanistic basis of this plasticity is virtually unstudied. Here, I present four potential mechanisms, each of which builds on the findings presented in Chapter 2,4, and 5.

a). Controlling the production of ejaculate components

The experimental paradigms used in many studies of plastic ejaculate allocation, particularly in *D. melanogaster,* allow for compositional change to be driven by fairly simple mechanisms: produce more, transfer more. In Wigby *et al*. (2009), for example, males were held alongside a rival for 24-hours prior to mating and subsequently transferred greater quantities of two SFPs: ovulin and sex peptide. In Moatt *et al.* (2014), males were given a full week of exposure to rivals, at the end of which they had produced greater quantities of sperm relative to an isolated male (Moatt et al. 2014). With such lengthy exposure to a cue, males have ample time to upregulate the production of ejaculate components.

Similarly, in Chapter 2 I provided males with between 3 and 4 days of exposure to rivals – plenty of time to change the rate of production. That I found variation among SFPs in terms of the degree to which their abundance increased at high competition suggests a level of protein-specificity in the modulation of production – a feature that would enable fine-scale control over ejaculate composition if given sufficient time. How males exercise control of the expression of SFPs is unclear, but recent work has demonstrated that clusters of SFPs share micro-RNA binding regulatory sequences, which may facilitate fast-acting, sensitive co-regulation (Mohorianu et al. 2018). However, I failed to identify an association between the degree of SFP upregulation and putative micro-RNAs suggesting the operation of additional regulatory processes.

b). Controlled depletion and the stratified gland hypothesis

The methodology adopted in some experiments has revealed fine-tuning of ejaculate composition over time-scales too short for differences in production to be solely responsible. For example, in Pizzari *et al.'s* work on sperm allocation in roosters *(Gallus gallus)*, providing apparently sperm-depleted males with a novel female, without time to replenish lost reserves, led to increased sperm transfer (Pizzari et al. 2003). To achieve this, males must retain a reserve of ejaculate components, which they dig deeper into under certain conditions.

One way that males could manipulate the degree of depletion is by controlling the peristaltic action of reproductive tissues. Reproductive tissues are generally surrounded by highly-innervated muscular sheaths, which undergo waves of contractions to squeeze out their contents (Redhai et al. 2016). The receipt of social stimulation may be translated into neural impulses that direct either the strength with which the tissues contract to expel their contents or the timeframe over which contractions continue. Some authors have

suggested that by changing mating duration, which is known to vary in relation to social cues, *D. melanogaster* males can influence the degree of ejaculate transfer (Bretman et al. 2009, 2010a). A link between mating duration and the duration of glandular contractions could provide a mechanism for this. However, the data I presented in Chapter 2 demonstrate that the competition-sensitive elevation in mating duration only matches sperm allocation pattern – it does not reflect that for SFPs. Moreover, while a peristalsis-transfer link may work well for tissues with a homogenous secretion, such as the testes, it does not by itself allow for the rapid compositional changes in the accessory gland secretion that previous work has suggested occurs. For example, Sirot *et al.*'s (2011) finding that males transfer less ovulin but hold the transfer of sex peptide constant when mating with mated, rather than virgin, females.

The principle difficulty in adjusting seminal fluid composition in real-time is that much of it appears to sit, ready-formed within the accessory gland lumen. If, however, SFPs are stratified within the lumen, then variation in the extent to which the gland's contents deplete would translate into compositional change. Abundance gradients within the contents of the lumen could be facilitated by the secondary cells being confined to the distal portion of the gland. In light of Sirot *et al.*'s findings, we would expect the abundance of sex peptide to be evenly distributed across the lumen of the gland, but for the abundance of ovulin to exhibit gradated abundance along the proximal-distal axis. In keeping with this, sex peptide is known to be produced in the main cells, while ovulin is found in both cell-types (Monsma et al. 1990; Styger 1992). Whether males would be able to retain gradients of this nature in the face of glandular peristaltic action that appears to mix the contents of its secretion is unclear (*personal observation*).

Testing whether this 'stratified gland' hypothesis underlies protein-specific manipulation of ejaculate composition could make use of three approaches. Firstly, a link

needs to be established between the degree of peristalsis and the intensity of a social stimulus. This could be tested by dissecting and recording the strength of excitement in the neurons innervating the glands of males housed with rivals (according to methods in Tayler *et al.*, 2012). Secondly, the distribution of SFPs within the gland lumen needs to be examined. Many GFP-SFP constructs are available to test this (*e.g.* Ram and Wolfner, 2009; Singh *et al.*, 2018). Finally, to explore whether the secondary cells unique placement within the gland contributes to intraluminal gradients in SFP distribution, we need a better understanding of the unique contributions made by the two distinct cell-types in the gland. Applying single-cell transcriptomics to accessory gland cells should help to understand which products may be more likely to cluster in the gland's distal tips. The feasibility of applying single-cell methods to the accessory glands will require methods to maintain the structural integrity of the easily-damaged secondary cells during cell-sorting (*personal observation*).

c). The regulated release hypothesis

Redhai *et al*. (2016) has shown that some ejaculate components can be secreted into the gland lumen at the point of mating. This contrasts with much of the accessory gland secretion, which appears to sit ready-formed within the lumen. Given that males can link the timing of secretion to mating, they could conceivably house a base seminal fluid composition within the gland lumen, which they supplement with mating-context specific products immediately prior to or during expulsion of the gland's contents. A mechanism such as this would require the glandular cells to integrate information relating to the act of mating as well as the specific context under which that mating takes place (*e.g.* the sociosexual environment) and regulate the release of products accordingly. The example of secondary cell secretion of dense core granules represents a fertile opportunity to test

this. Redhai *et al*. have developed a pipeline for counting the number of dense core granules within secondary cells. Applying this approach to males housed in different group sizes, as per Chapter 2, would represent a straight-forward test of this hypothesis.

d). The carrier and master regulator hypothesis

The data presented in Chapters 4 and 5 contributes to a growing number of studies revealing that the transfer of some SFPs depends on the presence of others. In Chapter 4, I showed that loss of secondary cell secretions led to dysregulated transfer of the lumenal contents of the gland. I observed that a similar effect was achieved through sex peptide loss in Chapter 5. In this latter case, I suggested that this may arise through the malformation of recently-discovered 'microcarrier' structures within the gland lumen (Wainwright *et al. in prep*.). The function of these structures is currently unknown, but they my act to ensure that the SFPs they load reach target female tissues or prevent SFPs from interacting prematurely. Regulating the formation or structural integrity of these structures may allow for fine-scale control over the transfer of a ready-formed accessory gland secretion.

In Chapter 4, I drew on work exploring the cellular biology of secondary cells to suggest that products secreted at the point of mating may act as master regulators of SFP transfer. I identified Acp26Ab as a possible candidate given its known association with secondary cell vesicles (Monsma et al. 1990). These regulators may exert general or functional-cluster specific effects on the transfer of ready-made, lumen-based SFPs, and act by (a) directing post-translational modifications to SFPs that influence how they associate with one another or larger ejaculate-born structures, such as exosomes and microcarriers, (b) by disrupting the formation of these larger units of intra-ejaculate organisation, or (c) altering the overall consistency of the accessory gland secretion

through action on binding agents or changing the water content, both of which could dictate how readily the accessory gland contents is discharged from the gland. While option (a) may require longer time frames to mount a response, the latter two could conceivably act rapidly.

A first test of whether the carrier and master regulator hypothesis could allow for plastic control over seminal fluid composition might look for associations between microcarrier structure and social stimulation. Given the paucity of knowledge about these structures it is hard to make directional predictions about how they might change. However, once could imagine that having many, small carriers might allow for greater flexibility in the transport of accessory gland products to females, as could filtering their release from the glands based on size. A more detailed analysis could explore whether the association between social stimulation and microcarrier structure is disrupted in the absence of Acp26Ab or in BMP-suppressed secondary cell males (*UAS-Dad*). Such an experiment would provide a test of whether these products act as master regulators of compositional change through influencing carrier structure. If these products are secreted specifically at the point of mating, then the glands of mating males may need to be examined. To the best of my knowledge, changes in the gland at the time of mating have yet to be tested, but such an experiment could provide fascinating insight into the activation of an ejaculate.

e). Secondary cells as an architect of plastic seminal fluid allocation

The four plastic ejaculate allocation mechanisms I presented above are not mutually exclusive: some SFPs may be produced in greater quantities; others (such as secondary cell products) may conform to distributional gradients; and carrier structures themselves may exhibit distributional gradients or be modified through the late addition of

intracellular stores of master regulators. In each case, however, there is reason to believe that the secondary cells occupy an orchestrating role. Firstly, at least some of their secretory activity is sensitive to sources of stimulation (specifically mating) (Redhai et al. 2016). Secondly, there is reason to be believe that they can secrete signalling molecules to propagate a response to stimulation across cells: mating-specific release of the BMP-ligand decapentaplegic is known to promote biogenesis of some ejaculate components via allocrine mechanisms, but there is further evidence of paracrine effects on neighbouring cells (Redhai et al. 2016). Thus, it may be that secondary cells can relay social information across accessory gland cell-types to direct the construction of particular ejaculate compositions. Thirdly, their localisation in the distal tips of the glands increases the probability that their secretions conform to distributional gradients within the lumen of the gland. Fourthly, in Chapter 4 I provided evidence that their secretions can exert broad influence over the transfer of assumed main cell products.

A reasonable test for the role of secondary cells in driving plastic ejaculate allocation therefore might involve using a mutant line such as *UAS-Dad* to block their activities. However, the work presented in Chapter 3 reveals the difficulties that would be encountered in such an approach. By blocking the secretory activities of the cells, males don't just lose the ability to respond plastically, but they also lose the secretion of many elements generally needed to promote normal post-mating responses, such as the reduction in receptivity to remating.

An approach that may suffer less from off-target effects to normal reproductive processes is seeking to establish whether sensory information relating to mating context can be transmitted to the secondary cells. Redhai *et al.* (2016) have suggested that secondary cells may make connections with a neuropeptide circuit that has been shown to innervate the accessory gland and be critical for the transfer of both seminal fluid and

sperm (Tayler et al. 2012). A neuropeptide circuit such as this may be further modulated through the activity of socially-sensitive hormones, such as 20-hydroxyecdysone which is known to be elevated under stressful social conditions (Ishimoto et al. 2009). Ecdysone receptors have already been shown to be essential for normal accessory gland development, demonstrating a clear role within the biology of the gland (Sharma et al. 2017). Supplementing males with socially-sensitive hormones and exploring how both the secondary cells and seminal fluid proteome changes would allow for a simultaneous test of (a) hormonal underpinnings of plastic ejaculate allocation, and (b) the relative sensitivity of different glandular cell-types.

2. The evolutionary biology of plastic ejaculate allocation

(a). Differences in the production cost of ejaculate components and the operation of plastic ejaculate allocation

In Chapter 2, I provided evidence that the costs of seminal fluid production exceed those of sperm. This was evidenced by the divergent ways in which repeated mating affected low and high-competition males: high-competition males showed a steeper rate of reproductive decline over successive matings than did low-competition males. Given that the difference in ejaculate composition between these male groupings was the abundance of SFPs transferred, I suggested that this decline may represent the specific cost of SFP transfer. Moreover, that the rates of reproductive decline are more pronounced between low and high-competition males than between no and low-competition males, the latter differing only in sperm transfer, suggests that the costs of seminal fluid production may exceed that of sperm. In support of this, there is evidence from bedbugs (*Cimex lectularius*) that seminal fluid represents a costlier ejaculate component than sperm (Reinhardt et al. 2011). Similarly, work in red-sided garter snakes (*Thamnophis sirtalis*

parietalis) has shown the metabolic cost of seminal fluid production represents between 5% and 18% of daily energy expenditure (Friesen et al. 2015).

Accordingly, divergent production costs between seminal fluid components should influence the extent to which males gain by allocating them prudently. If this is true, we might predict that males evolve the ability to independently manipulate the transfer of different ejaculate elements, and, if seminal fluid is generally more expensive to produce across taxa, for the flexibility in SFP transfer to be more pronounced. An interesting test would be to set up experimental evolution lines in *D. melanogaster* where the costs of producing particular ejaculate components have been artificially changed. Corresponding evolutionary change in ejaculate allocation strategies could then be monitored. Mechanistically, this could use genetic manipulations that exacerbate the degree of oxidative stress associated with producing an ejaculate component in a particular tissue, such as the testes. In *D. melanogaster,* proteins involved in oxidative stress tolerance are under master regulation by the Jun N-terminal kinase (JNK) signalling pathway (Wang et al. 2003). Compromising the activity of this pathway could elevate the per sperm cell rate of tissue damage, elevating the rate of reproductive aging, and increasing the costs associated with sperm production relative to SFPs. We would therefore predict that sperm allocation may evolve to show greater sensitivity to social stimulation.

(b). Establishing the breadth of male responsiveness in *D. melanogaster* and beyond

A fairly clear research programme has started to emerge in relation to understanding what cues males are responding to. Bretman *et al.* have demonstrated a robust link between elevation in mating duration and the presence of rivals (Bretman et al. 2009, 2010a, 2011b, 2013a). By exploring what conditions lead to this elevation, it has been suggested

that we gain insight into what cues males are manipulating ejaculate composition in response to. However, a series of findings complicate the interpretation of elevated mating duration. Firstly, male sperm transfer can be increased independently of changes in mating duration (Lüpold et al. 2011; Garbaczewska et al. 2013). Secondly, while there exists one neuropeptide circuit that couples sperm and seminal fluid transfer to mating duration (Tayler et al. 2012), another controls ejaculate transfer independently of changes in mating duration (Crickmore and Vosshall 2013). Thirdly, in Chapter 2 I showed that while changes in sperm transfer recapitulate changes in mating duration, changes in SFP transfer do not. Fourthly, changes in mating duration, if they do relate to the degree of SFP transfer, clearly do so in a protein-specific manner (Wigby et al. 2009; Sirot et al. 2011b). Accordingly, we must recognise that behavioural analyses alone are insufficient for categorising the breadth of male responses to social stimuli and the compositional changes in the ejaculate that may accompany them.

The proteomics approach outlined in Chapters 2,4, and 5, provides a highly-sensitive method of inferring and categorising changes in ejaculate composition, while simultaneously providing insight into the mechanisms underlying their differential transfer. Further work might apply the pipeline developed in this thesis and in Sepil *et al.* (2018) to a variety of reproductive contexts. Firstly, given the remarkable sensitivity to the intensity of competition outlined in Chapter 2, it would be worth exploring changes in ejaculate compositions over a broader range of competition-intensities. Secondly, this pipeline could be applied to Sirot *et al.'s* (2011) experimental set-up to explore how instantaneous, rather than prolonged pre-copulatory, exposure to cues influences ejaculate composition. Such an approach would complement work on the mechanisms facilitating rapid change in ejaculate composition (see Section 1 of this chapter). In addition to rival males, this approach could further investigate compositional change in

relation to female mating status (as in Sirot *et al.* 2011), or even use related rival males to examine whether males differentially alter ejaculate composition in response to kin. Altered ejaculate compositions could underlie the reduced harm that females suffer when held with related males (Carazo et al. 2014).

Rather than looking at social drivers of ejaculate composition, future experiments could target variance in internal state. For example, the proteomics pipeline could be applied to males reared under different nutritional geometries, taken from different age classes, or of varying infection statuses. We might expect plastic ejaculate allocation to become more exaggerated under reduced nutrition, where conservation of resources becomes increasingly important (Perry et al. 2013). By looking across age classes we should gain insight into how age-related changes to the accessory gland, as demonstrated by Leiblich *et al* (2011) and which are well-documented in humans, affect the ability to direct compositional change. Infections may further interfere with the efficacy of the mechanisms directing plastic ejaculate allocation, change the costs/benefits ratios associated with particular compositions, or even be manipulated by pathogens themselves. For example, there is emerging evidence that Wolbachia infection can change the expression of SFPs in *D. melanogaster,* perhaps to match their distinct evolutionary interests (He et al. 2018). Finally, looking at interactions between these different conditions will allow us to test whether plasticity can integrate multiple extrinsic and intrinsic cues to direct the transfer of highly-specialised ejaculate compositions.

As it stands, it is unclear just how sophisticated and sensitive plastic allocation should be. Theory to explain if and to what extent males plastically alter the composition of their ejaculate is reasonably limited. The theory developed to explain the evolution of plasticity *per se* is, naturally, relevant. DeWitt *et al.*'s classification of the costs and limitations of plasticity provide a catalogue of factors that may limit the efficacy of

plasticity (DeWitt et al. 1998). These include the reliability of the cues to which individuals respond, the lag-time between a relevant stimulus being received and actualising a phenotypic response, and the costs of producing or maintaining the machinery which detects and directs plastic change. Experiments to test how these constraints act to limit the efficacy or economic feasibility of plasticity might include using systems where sensory information is sparse – such as cave-dwelling vertebrates and invertebrates – or where there the sociosexual environment represents a poor cue of sperm competition risk. An example of this latter situation could be colonially-nesting Procellariformes (albatross, petrels and shearwaters), where long foraging trips leave males with little information about the probability of their partner being visited by extra-pair copulation-seeking individuals who themselves may travel from geographically separate regions of the colony. Alternatively, experiments could utilise systems where the benefits of being able to accurately partition resources are particularly high, such as in prospermatogenic species where males are unable to replenish lost components of their ejaculate (Boivin et al. 2005; Boivin 2013).

Despite the promise and precision of a proteomics approach to exploring plastic ejaculate allocation, a complete picture must move beyond sperm and protein and consider other ejaculate elements. For example, future work may combine proteomics with metabolomics, to gain an insight into plastic change in the metabolite composition of seminal fluid, which has recently been shown to associate with fertility in bulls (Velho et al. 2018). The further deployment of transcriptomics would provide insight into compositional change in the regulatory RNA make-up of the ejaculate. Many of these RNAs are known to associate with vesicles. Therefore, isolating and analysing the contents of seminal exosomes, which are known to affect post-mating phenotypes in *D.*

melanogaster (see Chapter 3), represents a key next step in developing our understanding of both plastic ejaculate allocation and reproduction more generally.

(c) Context-specific benefits of plastic ejaculate allocation

The principle prediction of plastic ejaculate allocation theory is that it benefits the male to instigate fine-scale tuning over the composition of his ejaculate (Perry et al. 2013). In Chapter 2, I showed that a high-competition ejaculate leads to elevated rates of offspring production, an effect that, through comparison to the low-competition male, could be attributed to a change in the seminal fluid proteome. By increasing the rate at which females use sperm, a male can ensure that many of his sperm are used prior to the female remating and, therefore, prior to those sperm encountering competition. This mechanism is a clear adaptation to sperm competition. However, Bretman *et al.* further found that rival exposure led males to obtain increased paternity shares after the female's second mating (Bretman et al. 2009; but see Bretman et al. 2013), which I failed to find. Why the benefits of plastic ejaculate allocation should have manifested differently in my experiments may be attributable to a number of phenomena. Firstly, Bretman *et al.* reared males in groups of 1 or 4, rather than 1,2, or 8. Given the unprecedented level of sensitivity to the specific intensity of competition that I uncovered in Chapter 2, it may be that the composition of an ejaculate from a male reared in a group of four is different enough to one from a group size 2 or 8 male to induce these divergent post-mating effects. Secondly, although we used the same ancestral fly stock as Bretman *et al.,* there may have been some between-population divergence either in how plastic ejaculate allocation manifests in the flies or in the female response to those changes. Thirdly, differences in the food composition may influence either ejaculate composition or the female response to particular compositions. Each of these hypotheses serves to highlight how the fitness

benefits enjoyed by males, and the mechanisms through which they manifest, can be variable and context-specific – a phenomenon that is increasingly appreciated (Fricke et al. 2010).

(d) The broad(er) fitness effects of plastic ejaculate allocation

The fitness consequences of plastic ejaculate allocation should extend beyond the male. As discussed throughout this thesis, seminal fluid components are increasingly recognised as influencing offspring phenotype (*e.g.* Bromfield et al. 2014; Watkins et al. 2018). While much of this work has been conducted in mammals, there is a precedent for similar effects to operate in insects. The phenomenon of telegony describes how offspring sired by a male can inherit characteristics shared by his partner's previous mates. In both burying beetles (*Nicrophorus vespilloides*) and neriid flies (*Telostylinus angusticollis*), this effect has been shown to operate through inheritance of body size (Crean et al. 2014; Pascoal et al. 2018). In burying beetles, smaller males promote greater fecundity stimulation in females who then lay a greater number of eggs (Pascoal et al. 2018). This exacerbates larval competition, leading to the development of smaller males. The effect of ejaculate components on offspring phenotype thus means that plastic ejaculate allocation may influence the fitness of a male as well as both the fitness of his offspring and that of any other males that mate with the female. Indeed, this could represent a novel form of ejaculate exploitation, where males preferentially mate with females mated to particular males in order to reap the transgenerational benefits. Future work, both in *D. melanogaster* and beyond, should seek to identify how plastic ejaculate compositions influence offspring fitness and how this might feedback to influence the allocation strategies themselves.

Given that many seminal fluid components have long-term consequences for female fitness, it is important that we better understand the influence of plastic ejaculate allocation on their partners. These consequences could act in multiple directions. Firstly, theory predicts that females should pay attention to signals of partner quality in order to acquire fitness-enhancing seminal fluid components (Bonilla et al. 2016; Crean et al. 2016). However, where males exercise control over how they allocate those products it may be hard for females to select males accordingly. Secondly, we might expect females to use the same cues that males are responding to anticipate especially deleterious ejaculate compositions. If, say, a female was surrounded by a large number of males, she may anticipate a high-competition ejaculate, which may include greater abundances of toxic, life-shortening ejaculate components, such as Acp62F (Lung et al. 2002) and sex peptide (Wigby and Chapman 2005), as the data in Chapter 2 suggest. She may then use this information to mount a response against these products, such as through the expression of miRNAs (Fricke et al. 2014). Whether the female reproductive tract proteome shows a social responsiveness similar in nature to that observed in the accessory gland remains unstudied.

3. The interdependent ejaculate – prospects for evolution and medicine

An alternative title for this thesis might have been 'The Interdependent Ejaculate'. In each chapter, I have uncovered multi-level interactions and independencies between different ejaculate components: the fitness benefits of a plastically-manipulated ejaculate composition crucially depend on the relative levels of sperm and SFP (Chapter 2); loss of particular secretions from an ejaculate-contributing cell leads to improved performance in some reproductive process, but a reduction in others (Chapter 3), as well as changing the transfer of proteins produced by adjacent cells (Chapter 4); loss of one SFP can

feedback to influence the transfer and production of many more (Chapter 5). This degree of interconnectedness within the ejaculate has received limited attention in the literature.

Male seminal fluid proteomes are rich, diverse, and evolve rapidly (Hopkins et al. 2017). However, the incorporation of a newly-evolved SFP into a male's arsenal faces a number of barriers. Not only must it either fit into pre-existing SFP networks (*e.g.* 'the sex peptide network' or ovulin network) or act alone, but it must fit into pre-existing transfer mechanisms. To what extent this interdependency constrains or promotes the evolution of seminal fluid is unclear, but requires further investigation if we are to understand variation between taxa in the composition of their ejaculate. The study of ejaculate composition would no doubt benefit from comparative tests that track how seminal fluid proteome size and molecular complexity changes within and between lineages, particularly in relation to key features such as female reproductive tract size, the intensity of post-copulatory sexual selection, and the method by which the seminal fluid proteome is transferred to females (*e.g.* within a spermatophores or via insemination). Similarly, how these features correlate with the diversity of the reproductive glands driving seminal fluid production may provide insight into the causes and consequences of variance in their number.

The interdependent ejaculate perspective extends beyond the seminal fluid proteome evolving in relation to itself to how it co-evolves with other elements of the ejaculate. A number of ejaculate elements, including exosomes and certain SFPs, have been show to fuse or bind with sperm (Peng et al. 2005a; Corrigan et al. 2014; Singh et al. 2018). Thus, any changes to sperm morphology, which shows extraordinary inter-specific variation (Lüpold and Pitnick 2018), may feedback to influence the efficacy with which sperm-associating ejaculate components operate. Moreover, changes to the seminal fluid proteome may influence the physical properties, such as the viscosity, of the medium in

which sperm ultimately have to move. A comparative study of the relationship between sperm morphology and seminal fluid complexity would represent an exciting initial test of this.

It is not just evolutionary biologists who should concern themselves with the interdependence of the ejaculate. In Chapters 4 and 5 I made the point that every mechanism that allows for control over the action or transfer of ejaculate components represents a novel route to potential fertility problems. Male reproductive glands have evolved to cope with competing selective forces: they must be able to replenish lost material rapidly, but also allow for real-time manipulation over the content of their secretion. Accordingly, it is likely that they have evolved integrated mechanisms that influence the production and transfer of SFPs. Loss of one component, may therefore have knock-on consequences for other SFPs and for the action of the gland itself. The design of fertility drugs and novel male contraception must take this interconnectedness into consideration to avoid inducing glandular pathologies, elevating harm caused by the ejaculate to the female reproductive tract (*e.g.* induction of inflammatory responses; Robertson and Sharkey 2016), and to minimise deleterious off-target effects on seminal fluid mediated mechanisms of transgenerational inheritance.

Conclusion

The distinction between the terms ‘sperm’, ‘seminal fluid’, and ‘ejaculate’ isn’t just one for the pedant. In this thesis, I have highlighted that the ejaculate is a composite of parts that show varying degrees of connectivity. Males can individually alter the transfer of different ejaculate elements to their benefit, utilise regulated secretion to influence how the seminal fluid proteome is transferred during mating and how it ultimately affects the female, and show unprecedented organisation within their seminal fluid. To understand variation in fertility outcome, the problematic pathologies of male reproductive glands, and, most fundamentally, the evolution of ejaculate composition, we must start to treat the ejaculate as a flexible and dynamic system.

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