

Oh, the places they'll go

Female sperm storage and sperm precedence in *Drosophila melanogaster*

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Abbreviations: Gld, glucose dehydrogenase; SFP, seminal fluid protein; SSO, female sperm storage organ

Among most animals with internal fertilization, females store sperm in specific regions of their reproductive tract for later use. Sperm storage enables prolonged fertility, physical and temporal separation of mating from fertilization and, when females mate with multiple males, opportunities for differential use of the various males' sperm. Thus, stored sperm move within the female reproductive tract as well as to several potential fates—fertilization, displacement by other sperm or ejection by the female. *Drosophila melanogaster* is a leading model system for elucidating both the mechanisms and evolutionary consequences of female sperm storage and differential male fertilization success. The prominence of *Drosophila* is due, in part, to the ability to examine processes influencing sperm movement and fate at several biological levels, from molecules to organ systems. In this review, we describe male and female factors, as well as their interactions, involved in female sperm storage and differential male fertilization success.

and sperm fate, in some cases at the expense of the female.⁴ When females mate with more than one male, male-male and male-female interactions within the female reproductive tract, particularly the SSOs, have profound consequences for sperm fate and, ultimately, male and female fitness. These consequences manifest most clearly as sperm precedence—the differential fertilization success of rival males. *Drosophila melanogaster* females mate frequently and store the sperm of multiple males in their SSOs. The wealth of well-developed genetic and genomic tools in *Drosophila* can therefore be applied to study the mechanisms and evolutionary consequences of female sperm storage. Here, we will review advances in our understanding of female sperm storage and sperm precedence in *Drosophila*. We will focus on molecular and cellular mechanisms underlying the functions of the SSOs, the effects of SFPs on sperm storage and use, and male and female factors affecting the precedence of one male's sperm over another's. For additional, comprehensive reviews of female remating, SFPs, and additional aspects of male-female interactions, see refs. 5-12.

Introduction

For many animal species, mating and egg laying occur at different times and locations. This temporal and physical separation is mediated by the female's ability to store sperm. Sperm storage includes the phases of sperm recruitment, maintenance, and release from particular regions of the female reproductive tract—frequently specialized sperm-storage organs (SSOs). Sperm storage allows the female to remain fertile for prolonged periods in the absence of potential mates, and to control the location and timing of oviposition.¹⁻³ Females can influence sperm fate by moving and supporting stored sperm. Male products, in particular seminal fluid proteins (SFPs), also influence storage processes

Female Sperm Storage

Sperm-storage organs. The *D. melanogaster* female possesses two types of SSOs located at the anterior of the uterus: a tubular seminal receptacle and the paired, mushroom-shaped spermathecae (Fig. 1). The seminal receptacle is a long, slender, closed-ended tube that narrows at the proximal end, whereas each of the spermathecae is composed of a duct that leads to the lumen of a cuticular capsule lined by secretory cells.^{7,13-16} Near the junctions between the spermathecal ducts and the uterus are two narrow ducts that lead to the female accessory glands (also known as parovaria), which have some known functions in immunity and fertilization in other insect species¹⁷⁻¹⁹ yet are poorly characterized in *Drosophila*. The spermathecae also function as glandular structures.^{14,19} Indeed, some *Drosophila* species do not store sperm in their spermathecae yet retain ducts and cells of presumably secretory function.²⁰ The sperm stored in the seminal

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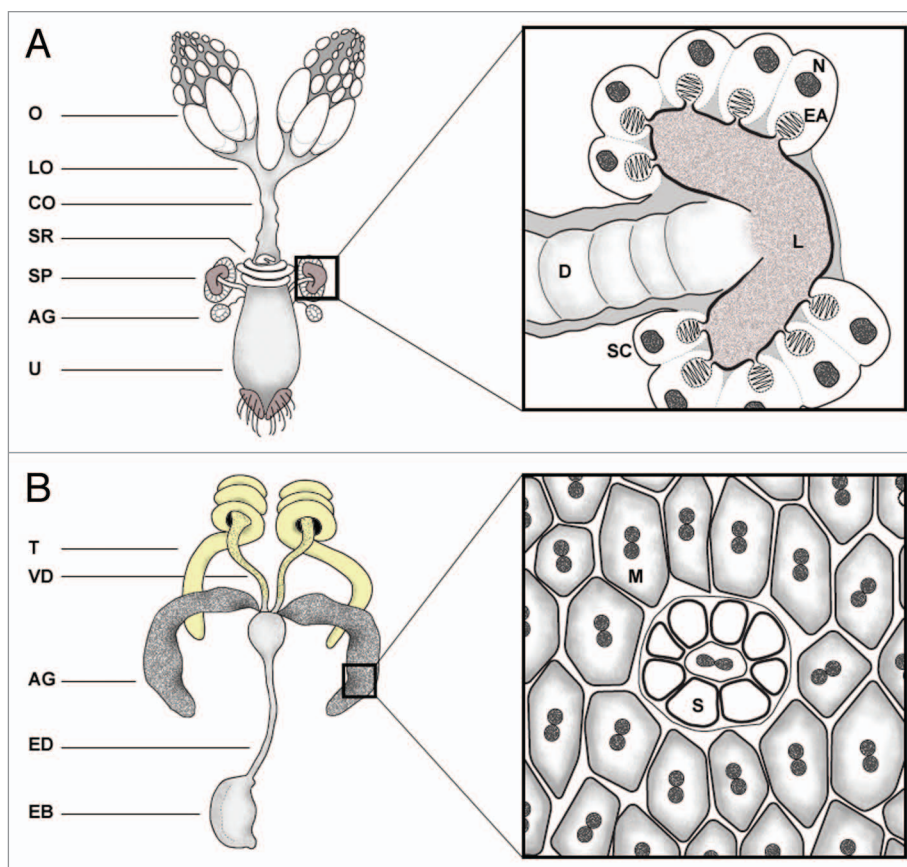


Figure 1. Overview of *D. melanogaster* female and male reproductive structures and glandular tissues. (A) The female reproductive system is shown in ventral view, with anterior to the top. It contains a pair of ovaries (O), from which mature eggs pass to the lateral oviducts (LO), which join to form the common oviduct (CO). Eggs are activated¹⁵² in the common oviduct before passing to the uterus (U), where fertilization takes place. The entrance to the egg, or micropyle, is adjacent to the openings of the ducts to the spermathecae (SP) and seminal receptacle (SR). Aside from their role as SSOs, the spermathecae function as glandular structures. Each spermathecal duct (D), which is surrounded by a thin layer of muscle and epithelial tissue, leads from the anterior-dorsal uterus to the lumen (L) of a cuticular capsule where sperm are stored. Surrounding the capsule is a ring of polarized secretory cells (SC), with nuclei (N) distal to the capsule, that release the contents of the end apparatus (EA), a large membrane-rich secretory organelle, into the lumen.²⁸ Small accessory glands (AG) also connect through ducts to the anterior-dorsal uterus. (B) The male reproductive system is shown with anterior to the top. It contains a pair of testes (T), which connect through vasa deferentia (VD) to the anterior ejaculatory duct. A pair of lobed accessory glands (AG) also connect to the anterior ejaculatory duct. The male accessory glands are composed of a single layer of two distinct, binucleated, secretory cell types: the 'main' cells (M) and 'secondary' cells (S).¹⁵³ The spherical secondary cells are located primarily at the distal tip of each gland, interspersed among the predominant hexagonal main cells. Each lobe is surrounded by a sheath of muscle that presumably squeezes the secretions of the cells into the ejaculatory duct (ED) and bulb (EB) to mix with sperm and other SFPs.¹⁵³ Sperm are released from the vasa deferentia into the ejaculatory duct. Contractions in the ejaculatory duct propel the sperm and SFPs through the bulb and into the female at the time of ejaculation.¹⁶

receptacle, rather than the spermathecae, constitute the primary source of sperm used for fertilization.²¹ The morphological and physiological differences between the SSOs suggest the spermathecae and seminal receptacle function independently in sperm storage, yet there is also evidence of communication between the two organs (see “Female influences on sperm recruitment, viability and usage,” below).

Female reproductive physiology after a single mating. Sperm storage occurs in three major phases: recruitment to SSOs, maintenance of viability while in storage, and release from the SSOs. As early as 5 min from the start of mating, SFPs enter the posterior of the uterus followed by sperm.^{22–24} The secretions of the female reproductive tract are predicted to interact with the components of the ejaculate to form a sperm sac²⁵ and allow for the hyperactivation^{26,27} and possibly modification of sperm.²⁸

The activation of *Pkd2*, also known as *Amo*, a homolog of the TRPP2 calcium-permeable nonselective cation channel encoded by the Polycystic Kidney Disease 2 gene (*PKD2*) in humans, is required for sperm hyperactivation, reflected in higher tail-beat frequency of sperm, which swim predominantly tail first once inside the female reproductive tract.^{26,27} Shortly after sperm are transferred, and continuing after mating ends, a series of uterine conformational changes move the sperm from the posterior of the uterus to the anterior end and adjacent to the SSO entrances.^{29,30} According to different estimates, approximately 1,500 to 4,000 sperm are passed to females during a ~20 min mating, yet only 25–35% of sperm enter the SSOs.^{16,21,31} The majority of stored sperm, ~75%, are rapidly stored within the seminal receptacle, with the remainder accumulating at a slower rate in the two spermathecae.^{21,31} Although the majority of sperm is stored within

Table 1. Reproductive proteins with semonstrated roles in sperm storage and sperm precedence

Protein	Origins	Protein class	Effect	Reference
Sex peptide (Acp70A) ¹	AG	Prohormone	Decreases female receptivity, oogenesis, sperm release	70, 71, 148, 149
Glucose dehydrogenase (Gld)	SP, ED, EB	Enzyme	Sperm storage and release	41-44, 63
Acp36DE	AG	Prohormone	Sperm storage	30, 65, 66, 135
Esterase-6	ED	Enzyme	Sperm storage and release from storage	31, 75
Acp29AB	AG	Lectin	Sperm retention in storage	76
CG9997	AG	Serine protease	Sperm retention in the seminal receptacle	11, 63
CG1757	AG	Crisp	Sperm retention in the seminal receptacle	11, 63
CG1652	AG	C-type lectin	Sperm retention in the seminal receptacle	11, 63
CG1656	AG	C-type lectin	Sperm retention in the seminal receptacle	11, 63
Acp62F	AG	Protease inhibitor	Sperm precedence	68, 69, 136
CG11864	AG	Metalloprotease	Sperm storage and ovulation	62, 64
Seminase	AG	Trypsin-type serine protease	Sperm storage and ovulation	62
CG6168	AG	Protease	Female latency to remate and sperm precedence	59
Sdic	S	Sperm dynein intermediate chain	Sperm precedence, possibly motility	74
Wasted	S	Unknown	Sperm retention in SSOs, sperm chromatin decondensation, efficiency of egg entry	73

Abbreviations: AG, accessory glands; SP, spermathecae; ED, ejaculatory duct; EB, ejaculatory bulb; S, spermatozoa. ¹The female sex peptide receptor (SPR) has a hypothesized role in sperm storage and sperm precedence, although it has not been definitively demonstrated.

~1 h after mating, total accumulation and movement among SSOs does not end until several hours later.^{30,31} The process of accumulation and movement corresponds temporally with changes in gene expression and tissue composition in the uterus, SSOs and oviduct.³²⁻³⁵ After this time, and before the first egg is ovulated, unstored uterine sperm are expelled.^{21,25,36} From a single mating, females can lay between 300 and 400 eggs and remain fertile for nearly two weeks.^{3,31,37-39}

Female Influences on Sperm Recruitment, Viability and Usage

Female input is required for sperm recruitment to the SSOs. The female influences all phases of sperm storage through interactions that occur at the tissue and molecular levels. As described above, conformational changes in the female reproductive tract correspond with sperm movement toward and into the SSOs. Furthermore, as the spermathecae and seminal receptacle have distinct morphologies, tissue compositions and evolutionary histories, it is not surprising that they appear to have distinct mechanisms of sperm storage. Storage dynamics (rates and quantities of sperm accumulating in the SSOs) differ between the seminal receptacle and spermathecae. Also, an intact female central nervous system is necessary for recruitment of sperm to the SSOs, but apparently more so for the spermathecae than the seminal receptacle. Disrupting the signaling between the female's central nervous system and the SSOs, either by masculinizing the central nervous system or by separating the female abdomen from

the thorax, resulted in significantly reduced sperm storage, particularly in the spermathecae when compared with the seminal receptacle.⁴⁰

Female-expressed proteins influence the recruitment of sperm to the SSOs (Table 1). Glucose dehydrogenase (Gld) is upregulated upon mating in the proximal and distal portions of the spermathecal ducts, yet absent in the seminal receptacle.⁴¹⁻⁴⁴ Although females mutant for Gld store a wildtype number of sperm in the seminal receptacle, they store fewer sperm in the spermathecae and often show an asymmetry in storage, with one spermathecal capsule containing many sperm and the other containing none.⁴¹

Although Table 1 only lists a single female-expressed gene with a demonstrated role in sperm storage (Gld), reflecting the paucity of molecular knowledge of female contributions to the process, there is evidence that there are more genes to be discovered. In particular, gene products made in the spermathecal secretory cells appear necessary for recruitment of sperm into storage in the spermathecae. The identification of regulatory regions of genes expressed exclusively in the spermathecal secretory cells has made it possible to develop genetic tools that allow the manipulation of these cells in a precise spatiotemporal manner. Genetic ablation of the spermathecal secretory cells using these regulatory sequences to drive an apoptosis-promoting protein in otherwise normal females results in decreased sperm recruitment to the spermathecae, but not the seminal receptacle.⁴⁵

Maintenance of sperm in the SSOs. Although sperm are recruited normally to the seminal receptacles of females lacking

the spermathecal secretory cells, sperm begin to lose motility within 1 d of mating.⁴⁵ This result implies that products of these cells act at a distance in the reproductive tract, and is consistent with prior results showing a loss of sperm viability in mutants impaired for the development of the spermathecae and female accessory glands.^{13,28} It is also consistent with the view that the glandular function of the spermathecae is as important as their function as storage receptacles.^{20,28} Notably, ablation of the spermathecal secretory cells after mating does not affect sperm maintenance in either the spermathecae or seminal receptacle.⁴⁵ The cells' secretions are therefore required before or around the time of mating and may potentially interact with SFPs or female targets within the reproductive tract.

The particular spermathecae-secreted gene products that are required for sperm storage are not known, although several notable functional categories are found in the lists of genes expressed highly in the spermathecae and, more broadly, in response to mating in the reproductive tract. These categories include serine proteases, which might participate in signaling cascades or process SFPs to active or inactive forms; proteins involved in carbohydrate and lipid metabolism, which might participate in sperm maintenance or maturation; and antimicrobial peptides, which might protect sperm or females from infectious agents transferred during copulation.^{28,32,34,35,42,46-51}

Female control over sperm release from the SSOs. Some of the same types of factors that influence recruitment of sperm also appear to influence sperm release. In particular, the nervous system plays an important role. Nerve terminals with putative octopaminergic-tyraminerigic function innervate the proximal portion of the seminal receptacle and extensively along the spermathecae. In both SSO types, nerve terminals are associated with muscle fibers.⁵² Female control of sperm release is coordinated by the neuromodulators tyramine and its derivative octopamine, which act via these SSO-associated octopaminergic-tyraminerigic neurons.⁵² The effects of removing tyramine or octopamine differ between the SSOs; in the absence of both tyramine and octopamine, sperm release from the seminal receptacle and spermathecae is reduced, whereas in the absence of octopamine alone, only sperm release from the seminal receptacle is reduced.⁵² Octopamine and tyramine also influence female fertility in that they are necessary for egg laying.⁵³⁻⁵⁵ The nature of the coordination between ovulation and sperm release from storage sites remains unknown. Although sperm release from storage corresponds with ovulation, it does not directly depend on it.⁵⁶ Gld also plays a role in sperm release. Gld-mutant females use sperm at a slower rate and retain fertility longer than wildtype controls.⁴¹

Sperm usage might depend on the post-mating maturation of the female reproductive tract. Mating induces substantial changes in the physiology and structure of the reproductive tract. For example, as the rate of ovulation increases dramatically during the ~6 h after mating, the common oviduct exhibits changes in gene expression, undergoes tissue remodeling, and becomes highly innervated.³⁴ During this period, fertilization efficiency is lower when compared with the overall fertile period of females.⁵⁷ It is yet to be determined whether or not this lower efficiency is due to the inability of the maturing tissues of the

female reproductive tract to support a high fertilization rate.³⁴ The mechanism by which sperm leave or are released from the SSOs remains unknown, let alone how this mechanism changes as the reproductive tract matures.

Male Influences on Sperm Recruitment, Viability and Usage

Seminal fluid proteins. SFPs produced in the male accessory glands and ejaculatory bulb mediate physiological and behavioral changes that affect sperm storage and usage. Several studies have identified key SFPs that influence sperm dynamics and significantly impact female reproductive physiology and behavior^{6,58-61} (also reviewed in refs. 6, 8 and 58). SFPs act to promote the male's reproductive interests, which theory predicts should in some cases match the female's reproductive interests and in other cases run counter to them. Either way, the female sperm storage tissues and their secretions likely interact with male proteins to influence patterns of sperm usage. One apparent case of male-female cooperation is a proteolytic cascade inside the female that tightly regulates the cleavage and activation of prohormones that secure sperm in the SSOs then increase ovulation.^{62,63} CG11864, a predicted metalloprotease, undergoes self-cleavage after activation by the trypsin-type serine protease *Seminase*.⁶² While being passed to the female during mating, CG11864 in turn cleaves the SFPs *Acp36DE* and *ovulin*, which promote sperm storage and ovulation, respectively (see below).⁶⁴

Several SFPs facilitate the recruitment of sperm to the SSOs. A male lacking accessory glands transfers a normal number of sperm to his mate, yet the recruitment of sperm into the SSOs is reduced by 90%.³⁹ The 122 kDa glycoprotein *Acp36DE* is necessary for maximizing the total number of sperm stored in these organs.^{65,66} In the female, the active, cleaved version of *Acp36DE* stimulates conformational changes in the uterus that position sperm adjacent to the SSO entrances.³⁰

Sperm viability. It is unclear how SFPs promote sperm viability while stored in the female SSOs, as the majority of these proteins are no longer detected within the female shortly after mating.^{30,67} However, indirect evidence of effects on sperm maintenance come from studies of multiply mated females. For example, *Acp62F*, which localizes to the SSOs within one hour post-mating⁶⁸ is required for sustained success of the first male's sperm (see "Male influences on sperm precedence: sperm competition" below).⁶⁹

Sperm release from the SSOs. The most extensively studied SFP, sex peptide, binds to sperm and is slowly cleaved off during storage.⁷⁰ Sex peptide is required to promote efficient sperm release from storage⁷¹ and to maintain the sexual refractory period during which females reject subsequent courting males.^{70,72} Several SFPs promote the binding of sex peptide to sperm in a precise spatiotemporal manner during transfer to the female.^{11,62,67,71} Physical properties of the sperm themselves may also impact sperm release as sperm of males mutant for genes encoding sperm components—such as *wasted* and the *Sdic* (Sperm dynein intermediate chain) gene family—exhibit abnormal release from storage in single or double matings, respectively.^{73,74}

As with female influences, there appears to be overlap between male factors that promote storage in the SSOs and those that promote release from the SSOs. The SFPs Esterase-6 and Gld (see also “Female influences on sperm recruitment, viability and usage” above), promote both sperm storage and release from the SSOs.^{41,75} However, other SFPs appear only to influence release. The predicted lectin Acp29AB localizes to the spermathecae (and possibly also the seminal receptacle) and promotes sperm retention in both SSOs.⁷⁶ The SFPs CG9997 (a serine protease), CG17575 (a cysteine-rich secretory protein), and CG1652/CG1656 (C-type lectins) promote sperm retention in the seminal receptacle, but not the spermathecae.¹¹

A mating plug influences remating but not sperm storage. SFPs contribute to a temporary mating plug that forms posterior to the sperm mass in the uterus. In at least one other *Drosophila* species, *D. hibisci*, it appears that this plug acts to secure sperm in the SSOs,⁷⁷ but in *D. melanogaster* there is no evidence that the plug affects sperm storage or release.⁷⁸ Instead, the plug appears to play a role in delaying remating. The refractory period to remating in females takes several hours to develop⁷⁹ and, at least in laboratory settings, females can remate until this effect takes place.⁷⁸ The mating plug reduces remating rates in the first 4 h after mating.⁷⁸ PEBII and PEB-me, two SFPs made in the ejaculatory bulb, are required for the formation of this short-term barrier to reinsemination.^{23,24,78,80} The mating plug persists in the female reproductive tract until it is ejected by the female.^{21,36}

Female Multiple Mating

Remating prevalence and sperm precedence. Despite the continued fertility afforded by storing sperm, as well as the refractory effects of SFPs and the mating plug, females in the laboratory mate with another male before their sperm stores are entirely depleted, typically within ~5d after mating.⁸¹ Mature females appear to have control over whether or not to accept a mate,⁸² although newly eclosed females may not.⁸³ Female remating tendency shows natural variation, and polymorphisms in two genes, *Obp56a*, encoding an odorant binding protein, and *CG9897*, encoding a serine protease, have been associated with this variation.⁸⁴ Female receptivity to subsequent courting males is also influenced by male size,⁸⁵ quantities of stored sperm,^{86–88} and the previous receipt of SFPs^{8,9,89,90} whose effects are prolonged by the presence of sperm⁹¹ (reviewed in ref. 5). Female multiple mating in their natural environment is also well-documented.^{92,93} A large proportion (46%–100%) of captured females retain sperm from more than one male^{92–94} and as many as six males in their storage organs.^{94,95} Although remating frequencies may be slightly elevated in established laboratory populations relative to field-caught females,⁹⁶ female multiple mating is an integral part of the *Drosophila melanogaster* life history.

Because the sperm from more than one male co-occur within the female’s reproductive tract, an opportunity exists for non-random, differential fertilization success among the different males’ sperm. In *D. melanogaster*, the last mating male fertilizes, on average, ~80% of the subsequent offspring.^{38,97–99} This *last male sperm precedence* is an important determinant of male

reproductive fitness (measured as offspring production).¹⁰⁰ In principle, sperm precedence can be partitioned into distinct male and female components: competition between/among males, called *sperm competition*,^{101–103} and female choice between/among male ejaculates, called *female sperm preference* or *cryptic female choice* (cryptic because it is internal and not easily observable).^{2,104} However, because the outcome of sperm precedence depends on both male and female genotypes (reviewed below), it is difficult to disentangle sperm competition and cryptic female choice both experimentally and conceptually. Evidence from a range of animal systems indicates that sperm competition and cryptic female choice are powerful evolutionary processes shaping male and female reproductive behavior, morphology, physiology and biochemistry,^{2,4,102,103} as well as maintaining polymorphisms within populations.^{105,106} Males also allocate their ejaculate among mates in response to social environment or female condition, known as *cryptic male choice*.^{107–110}

Sperm precedence assays offer distinct benefits over single-pair mating assays when exploring causes and consequences of sperm fate. As described above, observing reproductive outcomes of sperm competitive (male) and preference (female) scenarios more accurately reflects the reproductive biology of this species. Also, the examination of sperm fate in competitive scenarios is often a more sensitive assay for sperm performance than is the examination of reproductive outcomes from single-pair matings, as shown for *Sdic* mutants.⁷⁴ Despite the challenge of identifying and characterizing specific mechanisms by which cryptic processes occur, the last ten years have been particularly fruitful in the following areas: (a) describing sperm localization and displacement dynamics within the female reproductive tract, (b) identifying male genes involved in differential fertilization success, (c) dissecting cryptic male responses to the social environment and female condition, and (d) elucidating genetic interactions underlying sperm competition and cryptic female choice.

Storage of multiple ejaculates. Female remating initiates a series of events ultimately resulting in the displacement of a portion of the previous male’s sperm and storage of the most recent mating male’s sperm.^{21,111–113} This phenomenon is typically examined as two components: the continued use of the previous mating male’s sperm (resistance to displacement), and the use of the most recent mating male’s sperm (ability to displace previously stored sperm). The development of transgenic males with either GFP- or RFP-labeled sperm has been valuable for elucidating interactions between competing ejaculates within the female reproductive tract in real time.²¹ Within minutes of a female’s remating, and before sperm transfer has begun, a small portion of sperm from the previous male leaves her SSOs and returns to the uterus.²¹ Sperm transfer occurs shortly thereafter (within 4–10 min of mating^{21,29,114}) and previously stored sperm continue to leave the SSOs during storage of the mating male’s sperm. Although the accumulation of sperm in storage appears to be maximized by one hour after the start of mating (reviewed in ref. 6), storage dynamics—including displacement of stored sperm by uterine sperm and mixing of the two males’ sperm due to their movement—continues for several hours.^{21,112} Eventually, unstored sperm are ejected^{21,36} in a process that precedes, and may

be independent from, oviposition.²¹ Second-male sperm compose the majority of the dumped mass.²¹

While it is clear that sperm are physically displaced from female storage organs, observations of decreased first-male paternity without clear decreases in the storage of his sperm led to the hypothesis that sperm are also functionally displaced, by death or incapacitation.^{111,113} Currently, alternative explanations exist for the observed decline in fertility, such as female sperm dumping³⁶ and differential fertilization efficiency.¹¹⁵ Vital staining of stored sperm reflects a small proportion of death over time in storage,^{36,116} although it is not attributable to remating.^{36,117} Additionally, observation of interactions between living sperm from two transgenic males failed to detect any effect of the presence of second-male ejaculate on the motility of first-male sperm.²¹ Support for the incapacitation hypothesis is further diminished by evidence that a second-mating male ejaculate does not distinguish self from other,¹¹⁸ and even supports sperm use and viability (the latter *in vitro*) from a previously mating male.^{91,117}

Sperm remaining in the female reproductive tract 5 h after mating are called the fertilization set. A direct relationship between the relative proportion of each male's stored sperm and short-term (72 h) relative fertilization success exists for the seminal receptacle, but not for the spermathecae.^{21,112} Within the spermathecae, displacement is more variable, as measured by the proportion of second-male sperm stored (there are more cases of relatively fewer second-male sperm).²¹ This, along with the direct observation that sperm of the two males are physically mixed within the seminal receptacle^{21,111} indicates that short-term sperm utilization in the seminal receptacle is a 'fair raffle'—a function of proportion—and that sperm within this fertilization (sub)set are unlikely to be preferentially selected by the female.

Female influences on sperm precedence, cryptic female choice. The female reproductive tract is the environment in which ejaculates from multiple males interact with each other and with female tissues and molecules.^{2,7} Therefore, females are predicted to have profound effects on the fates of stored sperm, although unequivocal demonstration of cryptic female choice has been challenging.¹¹⁹⁻¹²² These female effects appear to occur at the levels of behavior, morphology, physiology and biochemistry. Because a female's first mating induces additional reproductive tract maturation at both molecular and morphological levels,³²⁻³⁴ a male mating with a previously-mated female encounters an environment that differs from that of a virgin female. This may have implications for the fate of a male's sperm as the female continues to mate (and store sperm from) additional males. One experiment, deducing sperm displacement within 18 h post-mating based on offspring phenotypes, did not detect a difference between second-mated and third-mated females.⁹⁷ However, another experiment, also deducing short-term sperm fate by observing offspring phenotypes, did detect virgin effects on sperm-use patterns. Although this result is consistent with mating-induced reproductive tract maturation, it might also be explained by female age effects.¹²³ Together, these results highlight the need to further investigate how female mating history influences sperm fate over time.

Insight into the mechanisms of cryptic female choice—and the central role of sperm storage in the process—comes from multiple experimental approaches. At the behavioral level, females can dump stored sperm by a mechanism that is both sperm- and ejaculatory fluid-independent: second males transferring only seminal fluids (i.e., spermless) or transferring no ejaculate at all triggered the complete removal of sperm from the seminal receptacle in 32% of experimental females.³⁶ The extent to which the amount expelled can be modulated, what causes a female to dump sperm, and the ultimate fate of the sperm expelled into the uterus remain unknown. Also unknown are the contributions of factors demonstrated to affect aspects of sperm storage in single matings, such as the female nervous system, neuromodulators, and uterine conformational changes (reviewed above), to cryptic female choice. However, examination of SSO morphology, particularly with regard to interactions with sperm morphology, supports the existence of cryptic female choice and suggests possible mechanisms by which it occurs. Females with supernumerary spermathecae favor the use of the last mating male's sperm, but only within a few days after the second mating.¹²⁴ It is not clear whether this difference is related to an altered geometry of the reproductive tract, an altered secretory capacity, or another factor. However, the difference does not appear to be directly attributable to an increased storage capacity because these 'supernumerary' females produce fewer offspring than wild-type females.¹²⁴ Using lines independently selected for different seminal receptacle length or sperm length, Miller and Pitnick (2002, 2003) demonstrated that variation in seminal receptacle length influences sperm precedence though its interaction with variation in sperm tail length. Variation in seminal receptacle length alone was insufficient to affect sperm precedence patterns (although females with longer seminal receptacles recruited more sperm).^{125,126}

Male influences on sperm precedence, sperm competition. Because a male's fertilization success is a critical component of his reproductive fitness,¹⁰⁰ it is not surprising that males possess multiple mechanisms promoting their success in sperm competition. Disruption of sperm performance (e.g., viability, motility, ability to bind seminal fluid proteins, etc.) naturally or experimentally will likely alter sperm competitive ability, and therefore measures of sperm precedence. The ability to isolate components of the copulatory stimulus (copulation, male accessory gland products, and sperm) facilitates dissection of male effects on the ability to displace and resist displacement of stored sperm.^{91,127-129} Both direct examination of sperm presence and indirect inference from offspring phenotypes provide evidence that the second male's sperm enters the SSOs and displaces the previous male's sperm (^{21,112} and suggested by ref. 38). Moreover, direct examination of sperm localization in the female reproductive tract has revealed that a component of the mating stimulus that is neither sperm nor male accessory gland product is sufficient to cause physical displacement of sperm.^{21,36} Finally, SFPs influence levels of sperm competition through their effects on sperm recruitment, retention and distribution among SSOs (described below, and reviewed in refs. 8, 9 and 130).

In *D. melanogaster*, genes harboring polymorphisms affecting sperm competition occur across the X, Y, second and third chromosomes.¹³¹⁻¹³⁴ Relatively few of the X chromosome genes associated with sperm competitive ability code for SFPs. Characterization of these genes will provide novel insights into the mechanisms involved in sperm competition. For example, *Sdic* is necessary for sperm competitive ability; males lacking the *Sdic* family demonstrate a poor capacity to displace sperm relative to controls.⁷⁴ Although these sperm-tail proteins have a hypothesized role in sperm motility, mutant motility appears superficially normal in vitro and mutant males have normal fertility in single-mating situations.⁷⁴ This result highlights the sensitivity of sperm-precedence assays to detect subtle aspects of sperm performance on reproductive outcomes. At least seven SFPs have been identified (described in the following paragraph) that, when inhibited, influence sperm precedence through their effects on sperm storage dynamics or as yet-to-be identified mechanisms (Table 1; reviewed in refs. 8, 9 and 130). Their varied effects reinforce the idea that multiple processes affect the outcome of sperm competition.

The ability to manipulate SFP levels has revealed their diverse roles in sperm fate. Some SFPs appear to affect sperm competitive ability by influencing the first male's representation in the fertilization set, as deduced from offspring phenotypes. For example, *Acp36DE*-deficient males experience lower fertilization success because fewer of their sperm accumulate in storage, resulting in a lower contribution to the subsequent fertilization set.^{65,135} Another SFP, *Acp29AB*, is required to retain sperm in female storage sites.⁷⁶ As a likely result of this, males who do not transfer *Acp29AB* to their mates have a lower capacity to resist sperm displacement relative to controls.⁷⁶ Still other SFPs support sperm utilization from storage sites. Sex peptide, *CG9997*, *CG17575*, and *CG1652/CG1656* interact with each other to support normal sperm release from the seminal receptacle.^{11,63} As a result of their effects enabling normal sperm depletion, decreased levels of each of these SFPs by RNA interference increase a male's resistance to sperm displacement.^{30,71} Finally, some SFPs' effects on sperm competition occur by as-yet unidentified mechanisms. *Acp62F*, a protease inhibitor that is toxic upon ectopic expression,^{68,136} is associated with increased displacement; *Acp62F* mutants exhibit elevated second-male sperm precedence.⁶⁹ While *Acp62F* mutants in single-mating scenarios do not alter their mates' egg-laying rates or sperm storage dynamics,⁶⁹ polymorphisms in this gene are associated with female fecundity and the ability to displace resident sperm.¹³² This last example also illustrates how examining the effects of natural genetic variation on sperm precedence provides an important complement to studies of mutants and is crucial to understanding how evolutionary selection shapes reproductive function (see 'The role of natural genetic variation', below).

Drosophila melanogaster males respond strategically to perceived level of sperm competition in ways that increase their fertility. One means of achieving this is by altering their copulatory behavior in response to the physical presence of other males. While the initiation of copulation may be under female control,⁸² its duration appears to be largely, although not completely

(described below), under male control.^{137,138} After sperm transfer occurs, additional time in copula increases the female's latency to remating¹¹⁴ and, possibly, fecundity,¹³⁸ (but see ref. 114) thereby increasing male resistance to sperm displacement. In the presence of rivals, males shorten both their latency to mating and copulation duration, resulting in decreased displacement of a previous male's sperm. However, when exposed to other males prior to mating, males had longer copulation durations resulting in higher short-term (24 h) fecundity, displacement of rival sperm, as well as resistance to sperm displacement by other males.⁸⁹ The latter was likely attributable to the induction of longer female remating latencies. Strategic responses extend to quantitative and qualitative shifts in SFP allocation. In the presence of a rival, males increase the quantity of sex peptide and ovulin transferred to the female¹⁰⁸ resulting in increased male fertility by stimulating female short-term fecundity and delaying both cryptic female choice and sperm competition by decreasing female remating rates. Consistent with predictions that males benefit by manipulating female latency to remating, but not fecundity (because fecundity does not increase with repeated matings^{138,139}) second-mating males transfer decreased levels of ovulin, but similar levels of sex peptide as males mating with virgin females.¹¹⁰

Male influences of sperm precedence, cryptic male choice. Males also respond strategically to female condition. The number of sperm a mating male transfers to the female corresponds with the extent of displacement from storage of the previous-mating male's sperm.²¹ Perhaps because of this, males copulate for longer durations with^{81,138} and transfer more sperm to¹⁰⁹ previously mated females than virgins. Males appear to be responding, in part, to female cuticular hydrocarbon composition. Male copulation durations with virgin females carrying the odor of a mated female did not differ from durations with mated females – both of which were significantly longer than copulation durations with virgin females.¹³⁸ In single-pair scenarios, males transfer more sperm to younger females than older females.¹⁰⁹ This might help explain why second-male sperm precedence decreases with older females (effect first detected 17 d post-eclosion),¹⁴⁰ which is unlikely to be due to decreasing sperm viability with female age.¹¹⁶ Instead, males may make a decreased investment in older females, resulting in insufficient storage of last-mating male sperm or compromised displacement of first-male sperm.

Female-male interactions influencing sperm precedence. Reproduction involves both cooperation and conflict. While successful reproduction requires males and females to coordinate the introduction of mature and activated gametes, characteristics increasing male reproductive success in a competitive environment do not necessarily improve female reproductive success and may even be detrimental to it.^{9,10,129,139,141,142} Several lines of evidence illuminate how male-female interactions determining sperm utilization patterns are mediated at the level of behavior and morphology. While males appear to have a large influence on copulation duration, it is also affected by the genotype of the female with whom they mate via yet unidentified traits.¹³⁷ The previously described effect of the interaction between variation in seminal receptacle length and variation in sperm length on

sperm precedence¹²⁶ highlights the complex interdependence of male and female traits that ultimately determines sperm fate.

The role of natural genetic variation. Observed variation in sperm precedence values and associated fertility components is due, in part, to genetic variation.¹⁰⁶ Examination of the genetic architecture of sperm precedence (the identification of genes involved in sperm precedence, their relative effects on sperm precedence and their interactions with each other) has provided valuable insights into the nature of male and female influences on sperm precedence, as well as the maintenance of genetic variation in the presence of strong selective pressures,¹⁰⁶ and has yielded novel genes with potential roles in sperm competition and cryptic female choice. These insights clarify the opportunities and limits for selection on male and female fertility phenotypes to result in evolutionary change.

Males from distinct genetic lines exhibit significant variability in their capacity both to displace sperm of other males and to resist displacement of their own sperm.^{38,99,111,112,131,134} These capacities are, to some extent, non-transitive; the outcome of sperm precedence depends on the particular combination of competing males. In some cases, these population-level differences in sperm displacement are not directly attributable to differences in quantities of sperm stored,¹¹² suggesting that more than one mechanism is involved in the ability to displace sperm and resist displacement. Female genetic variation is also associated with the extent of sperm displacement^{84,134,143} and differences in remating latency.^{84,134} Moreover, identification and examination of the relationships among genetic polymorphisms associated with different levels of sperm precedence and associated fertility components reveal significant male-female interactions.^{134,144} These male-male and male-female interactions likely reflect the multiple interacting traits involved in both male and female fertility and might serve to maintain genetic variation and decrease potential inbreeding^{144,145} (also see ref. 146). These interactions are also important, because they indicate that there is unlikely to be a single 'best male' genotype for sperm precedence,¹⁰⁵ and that the fertilization environment (competing males and even female¹⁴⁴) is critical for determining fertilization success.

Although it remains possible that an allele conferring fertilization success could go to fixation in a population, the high extant levels of relevant genetic variation suggest that such events are rare. Although selection on male and female traits involved in sperm precedence may be intense, it may not be consistent; non-transitivity of sperm precedence may maintain polymorphisms for genes with important effects on sperm precedence in both males¹⁰⁶ and females.^{134,144} Antagonistic pleiotropy is another phenomenon maintaining genetic variation, as evidenced by an allele of the gene encoding CG17331, a predicted endopeptidase, that both increases a male's ability to displace resident sperm and decreases a female's latency to remate.¹³¹ Pleiotropy is well-documented for male reproductive genes^{131,132,147} and may reflect common mechanisms among various components of fertilization success. For example, sex peptide acts in the female reproductive tract and central nervous system to affect female fecundity,¹⁴⁸ latency to remate,⁷⁹ cost of mating,¹⁴⁹ sleep and locomotion.¹⁵⁰ Epistatic effects are also well documented^{131,132} and possibly reflect

interactions among reproductive proteins, such as the interactions among sex peptide, CG9997, CG17575 and CG1652/CG1656 needed for appropriate retention of stored sperm.^{11,63}

Because SFPs have well-documented effects on sperm storage dynamics, female fecundity, female longevity, and sperm precedence,^{8,9,129,130} they are attractive candidates for augmenting male reproductive success. Some of the variation in both components of sperm competition (the ability to displace and resistance to displacement) and closely associated female responses such as fecundity, remating rate and sperm storage corresponds to variation in specific reproductive proteins.^{99,131,132,134,147} This relationship reflects ample available variation for natural and sexual selection to occur. Some of these relationships further characterize genes with already-identified functions, such as sex peptide and Esterase-6.¹³² However, associations between polymorphisms in uncharacterized male reproductive genes with components of sperm competition suggest functions for these genes that can be tested. For example, polymorphisms in CG6168, encoding a predicted protease,⁵⁹ are associated with female latency to remate, an ability to displace sperm as well as the ability to resist displacement.¹³² Based on these phenotypes, future experiments could examine whether CG6168 mutants appropriately retain stored sperm or processed SFPs. Female receptors for male SFPs likely mediate the magnitude of the female's response although additional genes are likely involved.¹⁴⁷ Quantitative trait locus mapping identified three regions of the female genome corresponding with each of three female traits affecting sperm precedence: ability to displace sperm, remating latency, and fertility.¹⁵¹ Although these accounted for significant levels of variation in female phenotypes assayed, large (> 85%) unexplained variation suggests additional factors influence expression of these characteristics.¹⁵¹ Narrowing the focus to examination of polymorphisms involved in a single male-female protein interaction, sex peptide and the corresponding female sex peptide receptor, revealed two significant allelic interactions affecting the male's ability to resist displacement.¹³⁴ These results suggest the value of additional assays examining the functional interaction(s) between sex peptide and sex peptide receptor as well as their effects on sperm precedence.

Future Directions for Female Sperm Storage and Utilization

The past 10 y have produced important insights into the timing and mechanisms of sperm movement within the female reproductive tract from a single male as well as overlapping ejaculates from multiple males. In particular, there is a better understanding of the SFPs involved in various aspects of sperm storage and how they interact with each other and the female to influence sperm movement and fate. In general, understanding female contributions to sperm storage and usage have lagged behind elucidation of male contributions. However, expression profiles of the SSOs, increased knowledge of the secretory tissues of the female reproductive tract, and documented interactions between SFPs and the female reproductive tract set the stage for continued exploration of female responses to the presence of sperm and SFPs. This holds promise for exploring male-female

reproductive interactions from the level of protein-protein interactions to coevolutionary dynamics. For example, understanding the functions of the numerous spermathecae-expressed proteases will likely shed light on cooperative and antagonistic aspects of reproduction.

The study of natural genetic variation also holds increasing importance. The revolution in next-generation DNA sequencing has been a boon to population-genetic studies in all fields. The study of sperm fate will benefit from the increased power and resolution that such studies can now achieve. This kind of study will be made even more meaningful by better knowledge of natural mating behavior and more efforts to study sperm precedence in experimental settings that more accurately reflect the environment in which they have evolved, especially settings involving more than two males or, even better, populations. After all, it is because of its rich and varied sexual life, as well as the extensive toolkit for its experimental manipulation, that *Drosophila*

melanogaster serves as such an excellent model for sperm storage and usage in animals.

Disclosure of Potential Conflicts of Interest

The authors declare no conflicts of interest and have no financial interests to disclose.

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