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Insect Seminal Fluid Proteins: Identification and Function

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Abstract

Seminal fluid proteins (SFPs) produced in reproductive tract tissues of male insects and transferred to females during mating induce numerous physiological and behavioral post-mating changes in females. These changes include decreasing receptivity to re-mating, affecting sperm storage parameters, increasing egg production, modulating sperm competition, feeding behaviors, and mating plug formation. In addition, SFPs also have anti-microbial functions and induce expression of anti-microbial peptides in at least some insects. Here, we review recent identification of insect SFPs and discuss the multiple roles these proteins play in the post-mating processes of female insects.

Keywords

Egg production; mating receptivity; sperm storage; mating plug; sperm competition; feeding; reproduction; Acp

Introduction

In many insect species, mating initiates a behavioral and physiological ‘switch’ in females, triggering responses in several processes related to fertility. Receipt of seminal fluid—a mixture of proteins and other molecules—by the female is a major component of this switch. Insect seminal fluid proteins (SFPs) are the products of male reproductive tract (RT) secretory tissues—accessory glands (AGs), seminal vesicles, ejaculatory duct, ejaculatory bulb and testes. SFPs are transferred to females with sperm during mating. They are major effectors of a wide range of female post-mating responses, including changing female likelihood of re-mating, increasing ovulation and egg-laying rate, changing female flight and feeding behavior, inducing antimicrobial activities, and modulating sperm storage parameters. Absence of SFPs from the ejaculate adversely affects the reproductive success of both sexes. SFPs identified to date represent numerous protein classes, including proteases/protease inhibitors, lectins, prohormones, peptides and protective proteins such as anti-oxidants; these protein classes are present in the ejaculate of organisms from arthropods to mammals (1). While non-protein molecules are also present in seminal fluid (e.g. prostaglandins in crickets: 2; steroid hormones in mosquitoes: 3), research on the effects of seminal fluid receipt has largely focused on the action of SFPs. While the focus of this review is on insect SFPs, progress in the identification and function of SFPs in tick species is also included.

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The past few years have witnessed an explosion in the identification and functional analyses of SFPs in insects due to new proteomic and RNA interference technologies. Since earlier results in this field were reviewed by Gillott (4), Chen (5), and Leopold (6), we focus here primarily on recent developments, referring readers to those comprehensive reviews for details on earlier studies.

Dissection of the nature and function of insect SFPs has relevance beyond understanding insect reproductive molecules and their action. SFPs provide intriguing targets for the control of disease vectors and agricultural pests. As we discuss below, SFPs alter reproductive and/or feeding behaviors in a number of arthropods, including insects that cause economic damage or spread disease. In many of these species, there are no approved and effective methods to control the damage they cause. For example, vaccines for tick-borne pathogens have been developed for a limited number of tick antigens (7) and the principal vectors of malaria and dengue fever—the mosquitoes *Aedes aegypti* and *Anopheles gambiae*, respectively—have been notoriously difficult to control. Thus, the best current method of limiting these diseases is to control the spread of their insect vectors. In another area, increased resistance to pesticides has made population control by conventional means difficult for pests such as the bollworm/corn earworm, *Helicoverpa armigera* (8), the bed bug, *Cimex lectularius*, (9, 10), and ticks (11). As more is learned about the reproductive biology of specific arthropods, their SFPs may provide tools or targets for the control of disease vectors and agricultural pests.

The study of SFPs also provides insight into the evolutionary patterns of reproductive traits. Although the functional classes of SFPs are conserved, a significant fraction of individual SFPs show signs of unusual, often rapid, evolution at the primary sequence level. The forces driving this pattern are not understood, and the study of SFPs may allow for their identification and dissection. Comparative studies of SFPs, individually and in aggregate, are important because 1) lineage-specific SFPs may be involved in the reproductive isolation between species; 2) highly conserved SFPs or SFP classes may be essential for reproduction; and 3) SFP divergence between closely-related species may illuminate selective pressures underlying SFP evolution. Recent reviews have focused on the evolutionary dynamics of SFPs (12–15); therefore we will refer the reader to those and focus here on the nature and function of SFPs.

Identification of SFPs

The identification of proteins produced in secretory tissues of the male RT and demonstrated to be, or likely to be, transferred to females during mating is the primary step in SFP identification. Transcriptomic (EST, microarray; 16–27) and proteomic methods (28–39) have given a global view of proteins produced in arthtropod male RT glands and, in some cases, of proteins transferred to females during mating (Table 1). For the purposes of this review, proteins within the seminal fluid and transferred to females will be referred to as SFPs. Proteins synthesized in male AGs will be referred to as Acps.

Most of the Acp/SFP identification studies in Table 1 examined RNA or proteins found in tissues of the male RT, but did not demonstrate SFP transfer during mating. A novel proteomic method directly identified 146 *D. melanogaster* SFPs (38 previously unannotated), 125 *D. simulans* SFPs, and 115 *D. yakuba* SFPs that are transferred to females during mating (30, 36). Findlay et al. (30) fed females a diet enriched in ^{15}N so that the females produced isotopically ‘‘heavy’’ proteins. After these females were mated to unlabeled males, only proteins transferred from males were detected when the female RTs were analyzed by mass spectrometry. This method was subsequently adapted to identify transferred SFPs in *Aedes aegypti* (Sirot et al. in prep.).

SFPs identified in these studies (Table 1) include peptides and prohormones and protein classes predicted to play roles in numerous functions including sperm binding (lectins and cysteine rich secretory proteins (CRISPs), proteolysis, lipases, and immunity-related functions. These proteins classes are seen in the ejaculates of several insects species, providing evidence that although the primary sequence of some SFPs evolve rapidly, the protein classes represented in seminal fluid are constrained (40). Further, examining the seminal fluid of the extensively studied *Drosophila* species reveals rapid gain/loss of Acp genes to be a common feature of *Drosophila* seminal fluid evolution (30, 40) suggesting that Acp genes evolve *de novo*, perhaps from non-coding DNA (41, 42). However, even as much of the knowledge of insect SFPs has been obtained via studies in *Drosophila* species, a recent proteomic study showed that *Apis mellifera* (honeybee) SFPs shared more sequence similarities with human SFPs than with *D. melanogaster* SFPs (28). Therefore, future studies of SFPs across representative taxonomic groups should shed light on the fascinating evolutionary history of these proteins.

Function of identified SFPs

Historically, insect SFP function has been analyzed by several approaches, including the injection of purified SFP(s) or protein fractions into virgin females, biochemical analysis, removal of putative SFPs by RNAi or mutation in *Drosophila*, or ectopic expression of SFPs in unmated *Drosophila* females. Moreover, the increasing availability of genomic and predicted protein annotations has made functional prediction of SFPs by sequence comparison much easier. For example, comparative structural modeling suggested the structure/function of 28 predicted Acps of *D. melanogaster* males (43). Flybase annotations to *D. melanogaster* genes were used to predict the functional classes of 240 candidate SFPs in *D. mojavensis* (34). Cross-species comparisons *to D. simulans and D. yakuba* led to the identification of 19 *D. melanogaster* proteins previously unreported as SFPs (30). The newly identified putative SFPs of these species fall into the same categories previously identified in *D. melanogaster* (43).

Aside from putative function based on sequence analysis, direct assessment of specific SFPs tissue targets within the mated female may hint at those SFPs function (e.g. localization to the sperm storage organs may suggest a role in sperm storage or maintenance, as seen for a network of *D. melanogaster* Acps; 44). Thirteen *D. melanogaster* Acps were shown to target to multiple tissues within the mated female RT, each having a unique targeting pattern (45). Additionally, a subset of Acps leave the female RT and enter the hemolympth (45–48), potentially reaching nervous and/or endocrine system targets.

More direct methods have also identified the roles of specific SFPs in processes such as the regulation of genes, behaviors and physiological processes such as sperm storage. These results are discussed below.

Transcriptome changes

Changes in female gene expression post-mating have been examined in *D. melanogaster* and, to a lesser extent, in *An. gambiae* and *Apis mellifera*. In *D. melanogaster*, the role of SFPs and sperm on transcriptional change in mated females has been dissected by microarray analyses. Levels of over 1700 transcripts are altered at 1–3 hrs post-mating in females (49, 50). The mating-dependent genes have predicted functions in a multitude of biological processes including metabolism, immune defense, and protein modification. However, only a handful of the mating-responsive changes in RNA level are greater than 2-fold, consistent with the hypothesis that sexually mature females are “poised” to respond to mating (50, 51). By 6 hrs post-mating, larger-magnitude changes in RNA levels are observed in a smaller number of genes (52). After a second mating, the expression of

immunity related genes is more pronounced (53), suggesting that previously mated females have sufficiently up-regulated metabolic and/or structural genes required for post-mating processes (e.g. ovulation and egg laying) to continue.

In the lower RT (the lower common oviduct, seminal receptacle, female accessory glands, spermathecae, and anterior uterus), the levels of over 500 transcripts are changed post-mating (54). A distinct shift—from gene silence to activation—is observed soon after the onset of mating (54). A dramatic peak in differential gene expression is seen at 6 hrs post-mating (54), consistent with the whole-body transcriptome results described above (52). In the oviduct, mating induces an up-regulation of immune-related transcripts and increases levels of RNA for cytoskeleton-related proteins (55). Some oviduct mating-responsive genes respond only to the first mating, while others to both the first and second mating (56). The female RT transcriptome suggests that the structural changes occurring after the first mating (presumably due to mating-responsive gene expression) are sufficient for continued post-mating processes.

Since some of the mating-dependent gene expression change is due to SFP receipt (50), transcriptome change in mates of males lacking specific SFPs was investigated. The ovulation inducing SFP ovulin and the sperm storage protein Acp36DE do not contribute extensively to female transcriptome change at 1–3 hrs post-mating (52). However, two other SFPs, Acp29AB and Acp62F, substantially affect the female transcriptome (52). Surprisingly, Acp29AB and Acp62F contribute to the up-regulation of genes involved in egg production and muscle development, even though analyses of mates to Acp29AB or Acp62F null males do not detect ovulation or egg-laying defects (57, 58).

The sex peptide (SP), a 36 amino acid Acp with roles in egg production, receptivity, feeding, receptivity and sleep behaviors in mated females (59–61) see sections below), affects expression of 52 genes in the head and abdomen of mated females. The majority of these RNAs changed only 2–3 fold (62). In the head, SP regulated RNA levels of genes for proteins involved in metabolism, proteolysis, signal transduction and transcription. In abdomens, the SP up-regulated antimicrobial peptide genes via the Toll and IMD pathways (63); a C-terminal motif of SP is responsible for mediating this effect (62). Despite the induction of antimicrobial peptide genes by mating, hemolymph challenge did not detect an immune response in mated females (64, 65).

RNA levels for 141 genes in *An. gambiae* females experience changes at 2, 6, and 24 hrs post-mating (66), with the number of genes with at least 2-fold change in expression levels increasing with time. Interestingly, changes in transcript levels of many of these genes persist for at least 4 days after mating. Mating responsive expression changes were examined specifically in the head, the gut, the ovaries, the lower RT (tissues below the ovaries), and the two major organs of the lower RT (the atrium, where the ejaculate is received and the spermatheca). In both the gut and the lower RT, several RNAs expressed tissue-specifically change in levels post-mating. Many of these are predicted proteolysis regulators. In the spermatheca, a predicted vitellogenin was also highly up-regulated post-mating. As with *D. melanogaster*, Rogers et al. (66) conclude that the female atrium is poised to respond to mating. However, they propose that the spermatheca may rely on signals received during mating to regulate genes involved in sperm storage and maintenance.

Large-scale transcriptional changes occur in the ovaries and brains of *Apis mellifera* queens post-mating (67, 68). In the ovaries, 366 transcripts are differentially expressed post-mating, with the regulated genes largely involved in cell division, gametogenesis, reproduction and oogenesis (67). The RNA levels of 971 genes are differentially expressed in female *A. mellifera* brains post-mating, with an over-representation of genes involved in protein

folding, protein catabolism, and the stress response (67). The types of genes regulated by mating in *A.mellifera* overlap with those seen in the previously mentioned *D. melanogaster* studies (e.g. genes involved in the immune response), suggesting that the post-mating transcriptional response may be conserved across species (68). In addition, insemination quantity affects gene expression in the brain (69), suggesting that ejaculate volume and, possibly, quantity of specific SFPs received, may act as a cue for this processes in mated *A. mellifera* females.

Antimicrobial functions of SFPs

Aside from roles in mediating the up-regulation of antimicrobial genes in mated females (50, 63), some SFPs have intrinsic anti-microbial function. Three *D. melanogaster* SFPs (from the AG and ejaculatory duct) have antimicrobial activity on E. coli growth *in vitro* (70). An additional three *D. melanogaster* SFPs have antimicrobial activity *in vivo*—able, upon ectopic expression, to reduce bacterial loads in females with *S. marcescens* (71); the relationship of these three genes to the three identified biochemically is unknown. Although analogous antimicrobial activities have not been detected in the seminal fluid of the bed bug *C. lectularius*, its seminal fluid does contain bacteriolytic activity, specifically, a lysozyme-like immune activity capable of degrading bacteria (72). These findings suggest that SFPs might play a protective role within the RTs of mated females, possibly aiding females' ability to clear microbes introduced during mating.

Structural and conformational changes of the female RT

The receipt of seminal fluid induces physiological and structural changes of female RTs. In the oviduct of *D. melanogaster* females, tissue-wide post-mating changes include the differentiation of cellular junctions, remodeling of the extracellular matrix, increased myofibril formation, and increased innervation of this tissue (55). Post-mating increases in neural activity to the oviduct occurs in the form of vesicle release from RT nerve termini and is modulated distinctly by mating, Acps receipt, and sperm receipt. Mating and/or the receipt of Acps or sperm have differing effects on vesicle release in different regions of the RT at different times post-mating, inferred from the intensity of labeled vesicles (51). Immediate post-mating change in neural vesicle release occurs in the lower common oviduct, seminal receptacle, and uterus. By 3 hrs post-mating—by which time females are ovulating at high rates and egg production has reached maximal levels (73)—vesicle release is inhibited in the common oviduct and lateral oviducts, with Acps modulating changes in nerve termini innervating the seminal receptacle (51).

SFPs receipt also affects the lower RT, inducing a series of conformational changes in the uteri of mated *D. melanogaster* females, initiating in the first moments of copulation and continuing after mating has ended (74). At least part of this process aids in the storage of sperm, allowing them to access the storage organs. Acps, and not sperm, are the ejaculatory components required to trigger these changes (74). The Acp(s) that initiate this process is unknown. However, Acp36DE is essential for their progression. Incomplete progression of these changes in the absence of Acp36DE leaves sperm lagging in the mid-uterus instead of forming a dense mass adjacent to the sperm storage organ entrances (75). This finding, coupled to the abnormally low numbers of sperm stored in Acp36DE null mates (76, 77), suggests that the post-mating uterine conformation changes aid sperm movement, en masse, toward storage.

An. gambiae female RTs also undergo structural changes upon mating (66). In virgin females, the apical cytoplasm of atrium cells have extensive smooth endoplasmic reticulum surrounded by high numbers of mitochondria. The basal poles of the cells have a high density of rough endoplasmic reticulum. In mated females, both the smooth and the rough

endoplasmic reticula mostly disappear, and the mitochondria become distributed throughout the cells. Rogers et al. (66) propose that these structural changes may result in a barrier to re-mating in this species.

Sperm maintenance in, and release from, storage

In addition to roles of SFPs in sperm storage, SFPs are involved in the maintenance of sperm viability in, and their release from, storage (e.g. 78). Seminal fluid secretions from male AGs of both the honeybee *A. mellifera* and the leafcutter ant *Atta colombica* promote sperm viability (79, 80). However, AG secretions from one male do not positively affect the viability of another male's sperm (81). In ants and bees, the effects of AG secretions on sperm survival differ between monandrous and polyandrous species (81): AG secretions from monandrous species promote sperm survival, even when the seminal fluid and sperm are from different males. AG secretions from polyandrous species, however, are detrimental to sperm survival—even to sperm of related males—suggesting a sensitive recognition system exists during sperm competition (81). The negative effects of AG fluid on sperm survival are mitigated by spermathecal secretions in the *Atta* leafcutter ant, suggesting that females of this species are able to control ejaculate competition once sperm are stored (81). Similarly, *D. melanogaster* seminal fluid has a protective function, improving the survival of even rival sperm (82).

In. *D. melanogaster*, Acps are necessary for the efficient utilization of stored sperm, with the few sperm stored in the absence of Acps not used to fertilize eggs (78). Utilization of sperm involves their retention in, and release from, storage (83). The removal of 5 Acps, individually, from the male ejaculate lead to sperm retention in both storage organs after mating (83, Avila et al. in prep). Four of these proteins (CG9997, CG1652, CG1656, CG17575)—a serine protease, 2 C-type lectins, and a CRISP, respectively—are required for the localization of the fifth, SP, to sperm (44), acting in a functional pathway that targets SP to the storage organs in mated females (44). SP, responsible for eliciting numerous post-mating responses is unique in exerting its effects in mated females for several days. SP's effects persist long-term due to its physically binding sperm, maintaining its presence in the female RT as long as sperm remain in storage (84). Sperm binding is a function of the N-terminus of the peptide; SP's C-terminus—which contains the receptivity modulating activity—is gradually cleaved from sperm tails (84). These findings suggest that the phenotypes (in sperm storage but also in receptivity and egg laying—see below) elicited by the absence of CG9997, CG1652, CG1656, and CG17575 from the ejaculate may be attributable to the inability of SP to localize to sperm.

The effects of SFPs are not only in terms of sperm release. Acp29AB, a predicted lectin, is needed for sperm to be retained in the sperm storage organs. Sperm from males homozygous for a Acp29AB loss-of-function mutation enter into but are not well maintained within storage, consequently faring poorly in a sperm competitive environment (57). The latter result is likely due to the reduced numbers of stored sperm—a phenotype analogous to that seen in mates of Acp36DE null males (85).

Receptivity to re-mating

Decreased sexual receptivity of mated females occurs in a wide range of insects, and it has been suggested that inducing this change in females is of benefit to males by decreasing the likelihood of sperm competition. In *D. melanogaster*, the receipt of SFPs change female behavior—mated females actively reject courting males. The SP plays a central role in inducing this change in female receptivity (44, 61, 86–89). The four Acps required for SP's sperm localization also influence receptivity (83). How SP accomplishes its regulation of

female receptivity is not known, but its action requires a G-coupled-protein-receptor (87) and specific neurons (88, 89) in females.

C. capitata females are less receptive to male courtship and are less likely to mate for several days after a single mating than are virgin females (90, 91). Sperm storage may play a role in female receptivity in this species as females who store less sperm post-mating are more likely to re-mate sooner (90, 92). Additionally, *C. capitata* females switch from a male-pheromone odor preference to a host plant odor preference post-mating (91), a switch possibly mediated by factors in the male seminal fluid.

Queensland fruit fly females, *Bactrocera tryoni*, when mated to irradiated sterile males (and thus subsequently storing little or no sperm) show no difference in sexual receptivity when compared to mates of non-irradiated males (93), suggesting that products of the seminal fluid, and not sperm, are responsible for the reduced post-mating receptivity observed. In support of this hypothesis, virgin *B. tryoni* females injected with male RT extracts experience diminished sexual receptivity and a shorter copulation duration when subsequently mated, similar to behaviors seen in previously mated females (94). That male AG size decreases after mating in *B. tryoni* suggests that this tissue is a major site of SFP synthesis (95).

In *An. gambiae*, male reproductive gland proteins also mediate female likelihood of re-mating (96, 97). This conclusion was initially suggested by studies involving females mated to hybrid males with reduced AGs (96, 97) and subsequently verified by injections of male AG homogenates into virgin females (98).

In several moth species, sexually receptive females produce sex pheromone to attract mates. Sex pheromone production declines substantially after mating: calling behavior cease and oviposition behaviors initiate (99). In several of these species, pheromone production is under neuroendocrine control, resulting from the release of pheromone biosynthesis activating neuropeptide (PBAN) into the female hemolymph (100). Reduction of female pheromone levels post-mating is a consequence of PBAN reduction in the female hemolymph (101). Synthetic *D. melanogaster* SP and the pheromone suppression peptide *Hez*PSP—from *H. zea* AGs (102, 103)—suppress pheromone production after injection into unmated *H. armigera* females. This effect occurs in a dose dependent fashion (104, 105). In addition, antibodies raised against the *D. melanogaster* SP detect signal from *H. armigera* male RTs (106).

There is growing evidence that SFPs have important effects on female post-mating behavior in lady bird beetles (107, 108), seed beetles (109–111), and ground beetles (112). Injection of testis extracts reduces the probability of mating at 3 hrs and 2 days after injection, whereas injections of AG extracts reduces the probability of mating only at 2 days after injection. Further, injection of a small molecular weight (<3kD) fraction of male RTs result in a short term decrease in the probability of mating (at 1 and 3 hrs after injection); whereas injection of a higher molecular weight (>14kD) fraction results in longer-term inhibition of mating (2 and 4 days after injection; 113). In the ground beetle *Leptocarabus procerulus*, injections of testes or AG homogenates into virgin females each independently decrease the probability of mating (112).

Egg Production

A frequent effect of seminal fluid receipt is an increase in egg production, ovulation and/or egg laying rates in female insects. Transferring SFPs that up-regulate these processes can benefits males, ensuring their sperm fertilize the maximum number of eggs before the female re-mates, and can also benefit females, allowing them to have increased egg

production only when sperm are present to fertilize those eggs. *D. melanogaster* SP stimulates egg laying in mated females(4, 27), and the long-term persistence of this activity requires the four Acps that localize SP to sperm (44, 83, 84).

The prohormone-like SFP ovulin (Acp26Aa) stimulates ovulation (114). Its mechanism is unknown but could involve two non-mutually exclusive mechanisms: direct ovulin interaction with neuromuscular targets along the lateral oviducts of the female RT, or indirectly by affecting the activity of the neuroendocrine system (51, 114). Ovulin is proteolytically cleaved in the female RT in a step-wise manner (reviewed in 12), a process dependent on at least one other Acp, CG11864 (115), a predicted astacin-family metalloprotease that is itself cleaved in the male RT during transfer to females (115). Ectopic expression of full-length ovulin, or either of ovulin's two C-terminal cleavage products, is sufficient to stimulate ovulation in unmated females (116), suggesting that ovulin cleavage may increase its activity by generating more bioactive components of the protein.

Little is known about SFP-mediated effects on ovulation in other insect species. In *Apis mellifera*, mating stimulates vitellogenesis and oocyte maturation in females (68, 117). In *Ae. aegypti*, male reproductive gland proteins modulate an increase in oviposition (reviewed in 4, 32, 118, 119). In *Anopheles* sp., there is indirect evidence that SFPs regulate female fecundity (120, 121): males have angiotensin converting enzyme (ACE) activity in their reproductive glands and females mated to males fed ACE inhibitors lay fewer eggs than females mated to control males. In *H. armigera*, crude extracts of the male AGs stimulate egg maturation and oviposition when injected into virgin females, similar to effects seen after mating (122). The receipt of the male ejaculate increases bed bug *C. lectularius* female reproductive rates, in terms of lifetime egg production, and females receiving more ejaculate enter reproductive senescence later than females who receive less ejaculate (123), suggesting that ejaculate components may compensate for the costs of elevated reproductive rates by delaying reproductive senescence in this species. Ejaculate volume affects seed beetle fecundity, as females receiving smaller ejaculates have lower fecundity than females receiving larger ejaculates, though this effect is not seen in all beetle species (109, 110). In the ladybird beetle, *Adalia bipunctata*, females ingest SFPs in the male spermatophore. Females prevented from consuming spermatophores have a longer latency to oviposition as well as a lower duration of resistance to re-mating than control females (108).

Mating plug formation

In several insect species, a mating plug is formed within the female RT during and/or after mating. Mating plugs often contain SFPs, and their formation is dependent on receipt of SFPs. Mating plugs have a wide-range of functions, some involved in sperm competition (124–126), the formation of a physical barrier to re-mating, as in butterflies (127), or in switching off female receptivity entirely, as in the bumble bee *Bombus terrestris* (128).

In *D. melanogaster*, a mating plug is formed shortly after mating begins. This structure has two major regions: a posterior region comprised of ejaculatory bulb proteins (PEB-me, PEBII and PEBIII; (129, 130), and an anterior region comprised of Acps (129). Evidence for a role of the mating plug, and the SFPs within it, in reducing female receptivity has been shown in *D. melanogaster*: mates to PEBII knockdown males (who form smaller mating plugs) are more receptive to re-mating than controls in the short-term (4 hrs; 130). These results suggest that the mating plug mediates a short-term decline in receptivity before the long-term effects of other SFPs set in. A similar effect is seen in *Drosophila hibisci*, where the mating plug inhibits courtship by subsequent males and reduces female receptivity (131). In *D. hibisci*, the mating plug is also suggested to facilitate sperm storage by preventing the back flow of sperm away from the storage organs (132).

In *An. gambiae*, the mating plug is necessary for proper sperm storage, but does not prevent re-mating by the female (37). Further, a male AG-specific transglutaminase is necessary to form the mating plug (37). Interestingly, although transglutaminases are made in other mosquito species, male AG-specific transglutaminases are only found in mosquitoes that form mating plugs (37).

In *D. mojavensis* and related species females experience an “insemination reaction mass” (133). While not a mating plug *per se*, it fills the entire uterus and persists for hours, absorbing nutrients from the male ejaculate that are incorporated into female somatic tissue (134). Proteins with sequence similarity to larval clotting factors in *D. melanogaster* (34) found in the AGs of *D. mojavensis* along with proteins with fibrinogen domains found in *D. mayaguana* (135) and *D. mojavensis* (34) AGs, are good candidates for proteins involved in forming the clot-like insemination reaction mass.

Longevity

The longevity of mated females is decreased in some (e.g. *D. melanogaster*: 136) but not all (e.g. cricket: 137) insects. In *D. melanogaster*, Acps mediate at least part of this longevity reduction (138), for reasons that are as yet unknown, and recently one SFP, SP, has been shown to play a major role in Acp-mediated decrease in longevity (136). In addition, SP and three other Acps (the protease inhibitors Acp62F and CG8137, and the peptide CG10433) are toxic to *D. melanogaster* upon ectopic expression (71, 139), possibly reflecting of the negative effect of their action under normal mating conditions. However, the mechanism(s) by which these Acps decrease longevity is unknown and as ectopic expression produces protein levels higher than normally encountered during mating, the toxicity observed may not reflect the true effects of these Acps, suggesting that the longevity effects associated with mating may be an indirect effect of SFP receipt (58).

Feeding

SFPs affect the feeding behavior of some female arthropods. *D. melanogaster* SP increases female feeding post-mating (59). This behavioral change is substantially reduced in egg-less females and increased in virgin females with experimentally elevated rates of egg production, suggesting that increased feeding is tied to the post-mating increases in ovulation and/or oviposition (140). However, egg-less *D. melanogaster* females continue to show mating-dependent decreases in life span similar to that of fertile, wild-type females, suggesting that the decreased longevity observed in mated *D. melanogaster* females is not attributable to over-feeding or to the energetic costs of egg production (140).

In female ticks, the feeding cycle consists of a preparatory phase, a slow feeding phase, and a rapid feeding phase (141). After completion of this cycle, females will have increased in weight almost 100-fold—an engorgement process that lasts ∼6–10 days and completes before females lay an egg batch (141). The transition weight between the slow and rapid feeding phases is termed the ‘critical weight’. Most virgin females do not feed past the critical weight (142, 143). Initiation of the rapid feeding phase is dependent on the receipt of a testis/vas deferens derived engorgement factor called voraxin (144–146). Voraxin consists of two components (voraxin α and β) and was shown to be sufficient to stimulate engorgement of feeding when injected into virgin females (146). Additionally, female feeding to engorgement was reduced by 74% when reared on rabbits immunized with recombinant voraxin (146). Paradoxically, RNAi knockdown of voraxin had no effect on female engorgement after mating with knockdown males (147) and experiments in the American dog tick *Dermacentor variabilis* found that silencing engorgement factor α and β homologs via RNAi failed to reduce engorgement (148). Thus, the feeding role of these proteins has yet to be fully ascertained.

Activity levels

The increase in *D. melanogaster* female feeding observed post-mating coincides with a decrease in female 'siesta' sleep (a quiescent sleep-like state) post-mating (60). This effect is mediated by receipt of SP, which decreases siesta sleep by 70% (60), consequently increasing foraging and egg-laying activity of mated females. In conjunction with the negative impact of SP on female life-span (136), it has been suggested that SP's effect on female longevity may be the result of increases in stress due to sleep deprivation and to increased locomotor activity (60).

Flight behavior is altered post-mating in honeybee *A. mellifera* queens. At ∼1–2 weeks of age, queens mate multiply during "mating flights"—inseminated by an average of 12 males (149). Mating makes queens less likely to attempt flight again (68). Additionally, insemination by single vs. multiple drones affects several behaviors, including flight behavior (68), suggesting that queens might use ejaculate volume (and possibly contents) as a cue for flight attempts.

Female effectors of SFPs

Little is known about the female molecules that interact with SFPs and are subsequently responsible for inducing the myriad post-mating changes observed in insects and other arthropods. A notable exception is the receptor for the *D. melanogaster* SP, the sex peptide receptor (SPR). SPR, identified in an extensive RNAi screen, is a G-protein-coupled-receptor that acts through a cAMP-dependent pathway (87). It should be noted that the ejaculatory duct peptide DUP99B, having a C-terminus similar to that of SP, also interacts with SPR (87, 150). SPR's expression in neurons that express sex-specific *fruitless* transcript is necessary and sufficient to re-establish SPR's receptivity and egg-laying effects (87). Further, SPR expression is necessary in sensory neurons innervating the female RT that express the *pickpocket* marker, possibly reducing the output of these neurons to the central nervous system (88, 89). The ability of the *D. melanogaster* SP to interact *in vitro* with *Aedes aegypti* and *Bombyx mori* SPR orthologs (87) suggests that SFPs analogous to the SP are present in the seminal fluid of these, and potentially other, insects. This interpretation is consistent with the ability of *D. melanogaster* SP to induce post-mating responses when injected into in unmated *H. armigera* females (104, 106, 151).

Ultimately, SFPs must interact in the context of the female RT. Thus, progress in understanding the signaling mechanisms involved in insect reproductive processes may illuminate mechanisms of SFP modulation in female physiology and behavior. Recent reviews have addressed neuropeptide control of insect hormones and sexual receptivity (152) in the reproductive physiology of the locust *Locusta migratoria* (e.g. 153). SFPs may act up-stream of traditional neural signaling systems. For example, ovulation and subsequent egg laying are presumably mediated by contraction of the female RT. The biogenic amine octopamine (OA) is an important regulator of ovulation-related contractions of the female RT in the Orthopteran *Locusta* (154), the muscid fly *Stomoxys*, (155) and *Drosophila* (156–158). Further, RT extracts from male *Stomoxys* induce changes in muscle contraction in female RTs (155). The *Drosophila* receptor, OAMB, critical for the ovulation effect, is selectively expressed in oviductal epithelium (159) . OA mediates ovary muscle contraction and oviduct muscle relaxation. It has been proposed that these opposing effects may serve to expel the egg from the ovary while facilitating entry into the common oviduct (153, 157, 160). Perhaps signaling systems, such as OA, or their proximate downstream targets may serve as substrates that are modulated by SFPs.

Social behavior effects

The amount of SFPs transferred may depend, in part, on the mating status of males and females and their social environment before or during mating. Mating status of both sexes can affect the magnitude of female post-mating responses (161–163). In a number of insects, females mated to recently-mated males show less pronounced post-mating changes in receptivity and egg production than do females mated to virgin males (e.g., *D. melanogaster*: 162; *Anastrepha obliqua*: 163). In some insects, mates of nutritionally-stressed males have less pronounced post-mating changes in receptivity to re-mating than mates of control males (164). These studies suggest that males are limited in the amount of SFPs they can produce and/or store at a given time and that SFP production may be resource-limited.

Given this potential limitation and the importance of SFPs in determining male reproductive success (e.g., via effects on sperm storage, egg production, and re-mating), selective pressures should exist for males to allocate the ejaculate in a manner that maximizes their reproductive success. One way this could be accomplished would be to allocate more SFPs to females mated under conditions of higher sperm competition risk (elevated either because the female has previously mated or because other males are in the vicinity of the mating pair). There is support for such “strategic allocation” of sperm in a number of insect species, including beetles, crickets, and medflies (reviewed in 165). Recent evidence has demonstrated strategic allocation of SFPs as well (reviewed 12). Briefly, male *D. melanogaster* transfer more sex peptide when they are exposed to another male before and during mating than when they are alone with the female before and during mating (166) . Other evidence is consistent with the hypothesis that strategic SFP allocation increases male reproductive success. For example, the mates of males exposed to other males before mating have longer latencies to re-mating and higher fecundity than mates of males not exposed to other males before mating (167). Thus, *D. melanogaster* males are able to adjust their ejaculate composition in response to risk of sperm competition, an adjustment that appears to increase male reproductive success. Future research in this area should test for strategic SFP allocation in other insect species.

Conclusions

SFPs have roles in modulating many female behavioral and physiological processes across a wide range of insect species. The recent rapid pace of technological advances in transcript and protein identification has resulted in greatly increased knowledge of suites of SFPs in a number of insect species, and roles of individual SFPs in female post-mating responses are being elucidated. However, several questions still need to be addressed.

First, how do male SFPs interact with each other and with female molecules to effect the changes observed in mated females? Downstream female effectors with, or through/which SFPs exert their functions remain unknown, with the exception of *D. melanogaster* SP and its receptor SPR. Proteins secreted from female RTs, including the sperm storage organs, offer an exciting list of candidates to test for roles in mediating SFP responses (168–170). That hundreds of SFPs are transferred to females suggest that, potentially, many molecular pathways are involved in female post-mating responses. Interactions have been shown to affect protein localization (e.g. SP to sperm; 44) and proteolytic cascades (115) in *D. melanogaster*, but much needs to be done to characterize these and other pathways.

Second, what extrinsic factors affect the production and transfer of SFPs and the magnitude of their effects on mated females? Few studies have investigated this question, but those that have suggest that effects of SFPs on female post-mating response are influenced by a

number of factors. For example, adult female nutrition alters the magnitude of the effects of SP on different phenotypic traits, which show that the responses to mating in general, and SFPs in particular, can vary under different environmental conditions (171, 172). Furthermore, males transfer different amounts of SFPs in different contexts, such as a competitive environment (166). These effects observed in the lab suggest that modulation of SFP action and allocation in the natural setting will be important to consider for fundamental reasons and also in insect pest control.

A third question relates to the unusual evolutionary characteristics of SFPs. Conservation of protein classes indicates fundamentally conserved roles for SFPs, yet individual SFPs tend to evolve rapidly. How do different proteins come to play such roles in different species, and what forces lead to the rapid sequence evolution? These are only a small sampling of the fascinating questions that await answers.

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Terms/Definitions

Accessory gland protein	proteins made by, and expected to be secreted from, the accessory gland of male insect reproductive tracts.
Seminal fluid proteins	proteins expressed from tissues of the male reproductive tract and likely transferred to females during mating

Acronyms list

AG	accessory gland
SFP	seminal fluid protein
RT	reproductive tract
Acp	accessory gland protein
SP	sex peptide
SPR	sex peptide receptor

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Summary Points

1. SFPs have roles in modulating female behavioral and physiological processes in numerous insect species. SFPs are being identified in an increasing number of insects.
2. Seminal fluid proteins and protein of Diptera, Lepidoptera, Hymenoptera, Coleoptera, Orthoptera, Hemiptera, and Ixodida species are described.
3. Mating and SFP mediate female post-mating responses in processes such as transcriptional and RT structural changes, up-regulation of anti-microbial peptide genes, altered receptivity to re-mating, sperm storage, mating plug formation, post-mating feeding and female activity levels.

Future Directions

1. Determining how male SFPs interact with each other and with female molecules to effect the changes observed in mated females.
2. Determining how extrinsic factors (e.g. nutrition, differing social conditions) affect the production and transfer of SFPs and the magnitude of their effects in mated females.
3. Determining how SFPs regulate similar reproductive processes across numerous species in the face of selective pressures and rapid evolution, and examining the forces that drive these changes.

Table 1

Recently identified SFPs and RT-expressed genes in insects

Order	Family	Genus sp.	Method	No. identified	
				In male RT	Transferred
Diptera	Drosophilidae	*Drosophila simulans*	EST screen[26], proteomic[3640]	57[26]*a*	122[40], 3[36]
		D. melanogaster	cumulative review[27], EST screen[57], peptide purification[129150], EST screen/bioinf. candidates*b*[23] proteomic[33353640] microarray[1927]	112[27]*a*, 46[19] [27], 440[33] 13[35]*a*	138[40], 8[36] 14[27], 3[57129] [150]
		D. yakuba	EST screen[23], proteomic[3036]	119[23]	107[30], 8[36]
		D. erecta	EST screen[23]	114[23]	—
		D. mojavensis	EST screen[21], proteomic[34]	57[21], 786[34]	—
	Tephritidae	*Ceratitis capitata*	EST screen[24]	13[24]	—
	Culcidae	*Aedes aegypti*	bioinf. candidates/RT-PCR[30] proteomic[3032]	63[30]*a*	56[32]
		Anopheles gambiae	bioinf. candidates/RT-PCR[25] proteomic[37]	46[25], 20[37]	15[37]
Lepidoptera	Nymphalidae	*Heliconius erato*	EST screen/bioinf. filter*c*/RT-PCR[22], proteomic[38]	371[22]	25[38]
		H. melpomene	EST screen/bioinf. filter/RT-PCR[22], proteomic[38]	340[22]	10[38]
Hymenoptera	Apidae	*Apis mellifera*	proteomic[29163]	69[29]	33[29], 57[163]
Coleoptera	Tenebrionidae	*Tribolium castaneum*	microarray[20]	112[20]	—
Orthoptera	Gryllidae	*Allonebrius*	EST screen[17]	183[17]	—
		Gryllus firmus	EST screen[1718]	24717 18	—
		G pennsylvannicus	EST screen[18], proteomic[39]	277[18]	22[39]
Arachnida Ixodida	Ixodidae	*Amblyomma hebraeum*	EST screen[16]	35[16]	—

*a*Candidate SFPs were based on criteria of accessory gland-specific or enriched expression and/or presence of a predicted secretion signal sequence

*b*SFPs or Acps were identified by finding orthologs of identified SFPs or Acps in other organisms

[c] Putative SFP or Acp databases were filtered by applying bioinformatic criteria