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On the evolutionary origins of insect seminal fluid proteins☆

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ABSTRACT

In most cases, proteins affect the phenotype of the individual in which they are produced. However, in some cases, proteins have evolved in such a way that they are able to influence the phenotype of another individual of the same or of a different species ("influential proteins"). Examples of interspecific influential proteins include venom proteins and proteins produced by parasites that influence their hosts' physiology or behavior. Examples of intraspecific influential proteins include those produced by both mothers and fetuses that mitigate maternal resource allocation and proteins transferred to females in the seminal fluid during mating that change female physiology and behavior. Although there has been much interest in the functions and evolutionary dynamics of these influential proteins, less is known about the origin of these proteins. Where does the DNA that encodes the proteins that can impact another individual's phenotype come from and how do the proteins acquire their influential abilities? In this mini-review, I use insect seminal fluid proteins as a case study to consider the origin of intraspecific influential proteins. The existing data suggest that influential insect seminal fluid proteins arise both through co-option of existing genes (both single copy genes and gene duplicates) and *de novo* evolution. Other mechanisms for the origin of new insect seminal fluid proteins (e.g., retrotransoposition and horizontal gene transfer) are plausible but have not yet been demonstrated. Additional gaps in our understanding of the origin of insect seminal fluid proteins include an understanding of the cis-regulatory elements that designate expression in the male reproductive tract and of the evolutionary steps by which individual proteins come to depend on other seminal fluid proteins for their activity within the mated female.

In rare, but fascinating, instances, proteins of two individuals of the same species have the opportunity to interact to influence the phenotype of one of the interacting individuals. Examples of such molecular social interactions include protein-based pheromones and their receptors (Houck and Reagan, 1990), proteins involved in maternal-fetal interactions (Stewart and Allen, 1995), and seminal fluid proteins (Avila et al., 2011; Poiani, 2006). These interactions generally involve a "donor" individual whose proteins are transferred via either internal (e.g., copulation or puncture wounds) or external (e.g., topical application) mechanisms to the "receiver". Upon receipt, some of these proteins can change behavioral and physiological phenotypes of the receiver (Avila et al., 2011; Houck and Reagan, 1990; Stewart and Allen, 1995). Based on these induced phenotypic changes in another organism, such proteins transferred from donor to receiver can be considered "influential proteins" (Sirot et al., 2014). Although many such influential proteins have been identified, the evolutionary origins and mechanisms of action of most of these proteins are unknown.

Seminal fluid proteins (SFPs) provide an exciting opportunity to explore the evolutionary origins of influential proteins. SFPs can interact with female-derived molecules to influence a range of behavioral, morphological, and physiological phenotypes in the female (Avila et al., 2011; Poiani, 2006). The identity and function of SFPs are more readily studied than some other categories of influential proteins because their site of origin (i.e., male reproductive tissues) can be isolated for gene expression and protein identification analyses and their time of transfer (i.e., during copulation) can be pinpointed and manipulated. SFPs have been especially well-characterized in some insects through studies on the development, morphology, and biochemistry of the tissues that produce the proteins and expression and functional analyses of the proteins themselves (Avila et al., 2011; Gillott, 1988, 2003). Within the insects, our knowledge of the identity and function of individual SFPs of *Drosophila melanogaster* is the most comprehensive to date. Over 150 distinct proteins have been identified as produced in the male reproductive tissue and transferred to females during mating (Avila et al., 2011, 2015a; Findlay et al., 2008, 2009; Yamamoto and Takemori, 2010). Functions of several of these proteins have been identified and, among these, the most thoroughly understood is a small protein called "sex peptide" (reviewed in Chapman, 2001; Kubli, 2003). Transfer of sex peptide in *D. melanogaster* influences a range of female phenotypes including stimulating egg production and aggression, inhibiting re-

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mating, depressing immune response, decreasing longevity, and changing diel activity patterns, rates of feeding, and digestion (Apger-McGlaughon and Wolfner, 2013; Bath et al., 2017; Carvalho et al., 2006; Chapman, 2001; Chapman et al., 2003; Kubli, 2003; Liu and Kubli, 2003; Short et al., 2012). These effects are induced through interactions between sex peptide and receptor proteins in the sperm storage organs (the spermathecae) and in neurons innervating the female reproductive tract (Avila et al., 2015b; Häsemeyer et al., 2009; Haussmann et al., 2013; Rezával et al., 2012, 2014; Yang et al., 2009). Functions have been identified for several other *D. melanogaster* SFPs and include roles in stimulating ovulation and managing sperm storage and release from storage in the mated female (Adams and Wolfner, 2007; Chapman, 2001; Qazi and Wolfner, 2003; Wolfner, 1997, 2002). Similarly, the phenotypes influenced by SFPs in other species of insects include female mate attraction behavior mating and feeding patterns, egg production and longevity (Avila et al., 2011; Gillott, 2003; Xu et al., 2013). These phenotypic changes in female behavior and physiology can positively impact male fitness but have potential detrimental effects on female fitness (Wigby and Chapman, 2005). This apparent sexual conflict might, in part, explain the relatively rapid rate of sequence evolution and turnover (loss and gain), as well as the diversity and apparent functional redundancy, of some SFP-encoding genes (Begun et al., 2006; Chapman, 2018; Findlay et al., 2008, 2009; Haerty et al., 2007; Panhuis et al., 2006; Swanson and Vacquier, 2002; Swanson et al., 2001). Despite extensive studies on the rate of SFP evolution, relatively little is known about the evolutionary origins of these influential proteins. How do some individuals in a population evolve new proteins that can be transferred to another individual and modify the recipient's behavior and physiology? Where do the DNA sequences encoding these proteins come from?

The goal of this mini-review is to address what is known about the answers to these questions on the origin of new influential SFPs and to highlight areas in need of further research. In Section 1, I identify criteria for becoming an influential SFP. In Section 2, I describe and provide examples of the different mechanisms by which new influential SFPs arise. In Section 3, I provide suggestions for future research that will advance our understanding of the origin of these fascinating proteins. Based on the availability of relevant studies, most of the examples used throughout this review come from *Drosophila* and mosquitoes. As more information is gathered about the genomes, transcriptomes, and seminal fluid proteomes of other insect species, we are likely to discover both similarities and unique patterns of evolution of SFPs. Such research is critical for a broader understanding of the origin of influential SFPs.

1. Criteria for becoming an influential seminal fluid protein

To qualify as an influential SFP, a protein must be able to enter the seminal fluid, be transferred to females, and induce phenotypic changes in females.

1.1. Pathways into the seminal fluid

To enter the seminal fluid, the protein must be produced or sequestered by tissues that are in contact with and have a mode of secretion into the seminal fluid. To date, reported insect SFPs are produced within tissues of the male reproductive tract, although many of these proteins are also produced in other tissues (e.g., Bayram et al. 2017; Boes et al., 2014; Bonilla et al. 2015; Gillott, 1988). The tissues within the male reproductive tract that produce SFPs vary between insect groups and can include specialized reproductive glands, testes, ejaculatory ducts, ejaculatory bulbs, and seminal vesicles (Gillott, 1988). Across a wide range of insects, the development and secretory activity of the specialized reproductive glands are sensitive to hormones including juvenile hormones and ecdysteroids (Gillott, 1988, 1996). The regulatory processes responsible for expression of particular genes within the male reproductive tissues of insects are not yet known for most insects but have been investigated in *D. melanogaster*. In *D. melanogaster,* trans regulatory factors that affect (either directly or indirectly) the expression of genes in the male reproductive accessory glands (where most SFPs are produced) include the transcription factor paired (prd), juvenile hormone, and several proteins in the sex determination pathway (Arbeitman, 2004; Chapman and Wolfner, 1988; DiBenedetto et al., 1987; Herndon et al., 1997; Luo et al., 2011; Wolfner, 1988; Xue and Noll, 2002). SFP-encoding genes expressed in other male *D. melanogaster* reproductive tissues (e.g., the ejaculatory bulb) are not all regulated by these same genes (Ludwig et al., 1991). Cis regulatory elements that influence accessory gland expression may include DNA motifs upstream of the coding sequences that could serve as transcription factor binding sites (Simmerl et al., 1995). Mutations in the upstream sequence of existing genes could result in the newly-derived expression of the genes such that they are expressed in male reproductive glands.

A protein could also acquire male reproductive gland expression through changes in translational regulation. For example, in *D. melanogaster*, translation of groups of SFPs has been proposed to be controlled by specific microRNAs that bind to a shared sequence in the 3′ untranslated region (UTR) of the proteins within the group (Mohorianu et al., 2018). Mohorianu et al. (2018) proposed that this mechanism of translational control could be responsible, in part, for the ability of males to rapidly adjust the absolute quantity of individual SFPs transferred to the female (Sirot et al., 2011a; Wigby et al., 2009, 2016). More broadly, this mechanism could potentially control whether the protein product of a gene expressed in the male reproductive tract was translated at all, thus controlling entry into the seminal fluid post-transcriptionally. The ability of a microRNA to regulate translation of a gene transcribed in the male reproductive tract could change if a mutation occurred in the DNA encoding the 3′ UTR. For example, since microRNAs generally repress translation (Oliveto et al., 2017), a mutation that prevented a microRNA from binding to the 3′ UTR of a transcript could result in the protein product being newly produced in the male reproductive tract.

Proteins in the seminal fluid could potentially also be produced outside of the male reproductive tract and then sequestered into the seminal fluid. To my knowledge, there are not yet any known instances of this for SFPs, although there is a precedent in dietary-derived molecules (including proteins; Silva et al., 2015) being ingested by males during development, sequestered into the adult male reproductive tract, and transferred to females during mating both in cactophilic *Drosophila* and in Lepidoptera (Dussourd et al., 1989; Markow et al., 2001; Silva et al., 2015). In sum, proteins can get into the tissues producing seminal fluid through either being produced in these tissues or, potentially, being produced outside of the reproductive tract and sequestered by male reproductive tissues.

In addition to being present in the male reproductive tract, SFPs must have a mode of entry into the seminal fluid. Direct evidence of how individual SFPs enter the seminal fluid from the cells in which they are produced is generally lacking, but bioinformatic, biochemical, and cytological studies provide several potential mechanisms. Many SFPs have predicted secretion signals which may designate them for the secretory pathway and movement out of the cell, perhaps through inclusion in exosomes (Corrigan et al., 2014; Gligorov et al., 2013; Wilson et al., 2017), and into the seminal fluid (Mueller et al., 2005; Sirot et al., 2011b). However, in some species, many SFPs lack canonical secretion signal sequences and are predicted to be intracellular or membrane-bound proteins (Baer et al., 2009a; Boes et al., 2014; Bonilla et al., 2015; Reinhardt et al., 2009; Simmons et al., 2013; Sirot et al., 2011b). Such proteins could potentially enter the seminal fluid through alternative means (Gillott, 1988). For example, in the mosquito, *Aedes aegypti*, cytological studies suggest that two modes of secretion of proteins from the male reproductive accessory glands into the seminal fluid. Cells in the anterior portion of the glands appear to use apocrine

secretion in which parts of the cells pinch off and are released into the lumen of the glands (Ramalingam, 1983). Cells in the posterior portion of the glands appear to use holocrine secretion in which proteins are transported into the seminal fluid through granules released by cell rupture (Dapples et al., 1974; Ramalingam, 1983). Thus, although identification of insect SFPs in large scale studies initially depended, in part, on the presence of predicted canonical secretion signal sequences (e.g., Mueller et al., 2005), it is now clear that, at least in some species, proteins can enter the seminal fluid through alternate mechanisms. These alternate mechanisms of secretion have not yet been carefully examined for most insect species but are important to identify because they will determine the opportunities for proteins that are present in male reproductive tissues to become influential seminal fluid proteins.

1.2. Ways to be influential

A second criterion for an influential SFP is that it must have an impact on one or more female phenotypes. Research to date from *D. melanogaster* involving male flies null or knocked-down for specific SFPs suggests that the influences of individual SFPs can vary widely in several ways including the number of phenotypes influenced, the magnitude of phenotypic change induced, and whether the influence is mediated directly through interactions with female molecules or indirectly through roles in localization or activation of other SFPs. For example, the magnitude and number of phenotypic changes induced by the aforementioned *D. melanogaster* sex peptide are both large, including: inhibiting remating by about 80%, promoting egg production by about 80%, promoting hatching success by about 60%, inhibiting sleep by about 75%, and promoting digestion by about 85% (Apger-McGlaughon and Wolfner, 2013; Bath et al., 2017; Chapman et al., 2003; Isaac et al., 2010; Liu and Kubli, 2003). However, to maintain its long-term effects on mating and egg production, sex peptide must localize and remain stored in the female sperm storage organs (Peng et al., 2005; Ravi Ram and Wolfner, 2009). Within these organs, sex peptide is bound to sperm and then is gradually released over the course of several days (Peng et al., 2005). For sex peptide to get into and remain in these storage organs, several other SFPs are required (Findlay et al., 2014; Ravi Ram and Wolfner, 2009; Singh et al., 2018). In contrast to sex peptide, the *D. melanogaster* SFPs ovulin and Acp36DE appear to have very focused and, in some cases, more subtle effects on female phenotypes. Ovulin stimulates an 8% increase in egg production by increasing the rate of release of eggs from the ovaries into the oviduct of females (Heifetz et al., 2000; Herndon and Wolfner, 1995). Acp36DE increases male success in sperm competition by about 80% through promoting sperm storage in the mated female (Chapman et al., 2000; Qazi and Wolfner, 2003). Both ovulin's and Acp36DE's influences appear to be enhanced by proteolytic processing by other SFPs in a multi-step pathway that begins while SFPs are en route out of the male reproductive tract and continues after they are transferred into the female reproductive tract (Avila and Wolfner, 2017; Heifetz et al., 2005; LaFlamme et al., 2014; Park et al., 1994; Ravi Ram et al., 2006). In addition to some SFPs playing support roles for other SFPs, there is also apparent functional overlap in SFPs in that multiple proteins influence the same phenotype (e.g., Saudan et al., 2002). Thus, although a novel SFP needs to change phenotypes in the mated female to be considered influential, the influences, if any, of individual proteins can be quite subtle.

If we imagine the actions of SFPs as a play, there are some SFPs with strong roles having major impacts on one or more phenotypes. Others play minor roles having subtle impacts on one or more phenotypes. Then, there is a suite of proteins that act as a supporting crew by activating or localizing the major or minor players but have no known direct impact on female phenotypes. Further, there appears to be understudies that are from similar predicted protein classes as major or minor players, but have no detectable impact themselves (Avila et al., 2011; Bayram et al., 2017; Goenaga et al., 2015; Reinhardt et al., 2009; Simmons et al., 2013; Sirot et al., 2009). The effects of individual SFPs can be difficult to identify for several reasons. Phenotypes might be missed either because researchers have not yet thought to test for them or do not have ways to assay them. Multiple proteins may have redundant functions, making it difficult to assay the function of proteins individually through techniques such as RNAi or gene knockout. Also, the sheer number of candidate SFPs in each species and post-mating phenotypic changes in females make the association of protein and function challenging. However, novel bioinformatic-based techniques examining genes that share either expression patterns or evolutionary rates are helping to identify candidate bioactive SFPs and the female-derived proteins with which they interact (Ayroles et al., 2011; Findlay et al., 2014).

To influence the phenotypes of another individual, influential proteins face additional challenges relative to "normal" proteins that remain within the individual that produce them. Influential proteins must travel through internal environments of two different individuals and reach their target cells or tissues in an active or intact form. For a protein to remain intact on this journey, it must avoid or be resistant to various degradational mechanisms (e.g., proteolytic degradation and immune responses) within both the individual that produces the protein and the individual that receives the protein (Pilpel et al., 2008; Ravi Ram et al., 2005). Protection of individual SFPs from degradation may come from effects induced by other SFPs. For example, the seminal fluids of most insects studied thus far are replete with protease inhibitors that could protect SFPs from protease-induced degradation (den Boer et al., 2009; LaFlamme and Wolfner, 2013). Protection could also come from female reproductive tract protease inhibitors (Al-Wathiqui et al., 2014; Prokupek et al., 2010; Thailayil et al., 2018). In addition to proteolytic degradation, SFPs could be degraded by female immune responses (e.g., phagocytosis). However, the transfer of SFPs reduces the female immune response, which, in turn, could potentially protect SFPs from incapacitation by the female's immune system (Short et al., 2012). Therefore, the ability of some SFPs to inhibit female immune defense and proteolytic activity may provide defense against or limit the rate of degradation for newly evolved SFPs.

In addition to avoiding degradation, to impact female phenotypes, SFPs must also be in an active state or form at the time they reach their targets. Some SFPs require post-translational processing to become active or to reach their maximal activity level. For example, in the case of the *D. melanogaster* SFP, Acp36DE, which is proteolytically processed en route to and within the female reproductive tract (see above; Ravi Ram et al., 2006), cleavage is necessary for it to have its maximal effect on sperm storage within the female (Avila and Wolfner, 2017). Another *D. melanogaster* SFP, sex peptide, is also processed within the female reproductive tract in a manner that allows its bioactive portion to reach its target. The N-terminal end of sex peptide binds to sperm. The C-terminal end of sperm-bound sex peptide is then gradually released from the sperm through proteolytic cleavage (Peng et al., 2005) and subsequently binds to receptors in neurons innervating the female reproductive tract (Häsemeyer et al., 2009; Yapici et al., 2008) and possibly the sperm storage organs (Avila et al., 2015b). In contrast to ovulin and sex peptide, the stages of post-translational processing, including steps in activation and degradation, are not known for most insect SFPs. Future research in this area will provide a more comprehensive understanding of the proximate mechanisms by which SFPs become influential as well as insights into potential conflicts between males and females over whether and for how long the SFPs remain influential.

Finally, to influence female phenotypes, SFPs must be able to reach their targets within the mated females. Once again, the study of *D. melanogaster* provides the most insight into the localization of SFPs within females. SFPs in this species appear to have protein-specific patterns of localization with some proteins remaining at or near the site of transfer, whereas others move to various parts of the female reproductive tract (e.g., ovaries and sperm storage organs) and still others

leave the female reproductive tract and enter circulation (Lung and Wolfner, 1999; Ravi Ram et al., 2005) probably through openings in the female reproductive tract that result from piercing by the male intromittent organ (Mattei et al., 2015). Presumably SFP localization patterns within the female reproductive tract correspond with each individual SFP's sites of action, although that remains to be confirmed for most SFPs. The variable patterns of localization suggest corresponding variation in the mechanisms by which SFPs exert their influence.

2. Origins of new influential seminal fluid proteins

Given that influential SFPs must be able to get into the seminal fluid, reach their targets, and be in a state able to influence female phenotypes, how do new influential SFPs arise? Genes encoding new influential SFPs could potentially arise through any of several mechanisms that have been proposed for the birth of new genes including: gene duplication followed by mutations, recombination resulting in the shuffling of DNA sequences, retrotransposition, horizontal gene transfer, and *de novo* evolution where non-coding DNA changes to become coding sequence (Chen et al., 2013; Gubala et al., 2017; Long et al., 2003; Reinhardt et al., 2013; Zhao et al., 2014; Zhou et al., 2008). Alternatively, new influential SFPs also could arise through changes in temporal or spatial expression of pre-existing genes (McIntyre et al., 2006). Expression changes could come about through mutations of cis regulatory elements or trans-acting factors of the gene (Yang and Wittkopp, 2017). Such changes could result in the protein being produced in such a way that allows it to be transferred to a conspecific. The evolution of novel functions of pre-existing genes and the evolution of new genes through gene duplication or retrotransposition are all forms of intraspecific co-option in which genes with an existing function may be used for a novel function (e.g., Martinson et al., 2017; Meslin et al., 2015; Sirot et al., 2014). Of these potential mechanisms, there is strong evidence for evolution of new SFPs through gene duplication, expression changes in existing genes, and *de novo* evolution (Fig. 1; Begun et al., 2006; Boes et al., 2014; Findlay et al., 2008, 2009; Mueller et al., 2005; Sirot et al., 2014).

Duplication of existing SFP-encoding genes followed by divergence appears to be a common mechanism by which new SFPs originate (Begun and Lindfors, 2005; Findlay et al., 2008; Holloway, 2004; Mancini et al., 2011; Mueller et al., 2005; Wagstaff and Begun, 2007). For example, two of the *D. melanogaster* SFPs involved in the long-term sex peptide network described above (section 1.2; CG1652 and CG1656) are the products of apparent tandem gene duplicates (Mueller et al., 2005). Both proteins are required for long-term persistence of sex peptide in the female sperm storage organs and have roles that partially overlap (Ravi Ram and Wolfner, 2009). Since both genes are highly expressed predominately in the male accessory glands but the proteins they encode only share ~50% similarity (Mueller et al., 2005), it is likely that the cis-regulatory elements were also duplicated and remained relatively stable while the coding sequence mutated or that the two genes share an enhancer region. One of the proteins necessary for the proteolytic processing of ovulin and Acp36DE (CG10586; 'seminase') also shows evidence of arising through tandem gene duplication of pre-existing SFPs followed by divergence, although the order of appearance has not been established (LaFlamme et al., 2012). Similar patterns of apparent SFP origin through duplications of genes encoding existing SFPs occur in other insects. For example, in the mosquito *Anopheles gambiae* a cluster of three genes occurring close together and encoding highly similar SFPs (with > 75% sequence identity) appear to have resulted from gene duplication (Mancini et al., 2011). Thus, one effective way of generating new influential SFPs is through duplication and modification of existing influential SFPs.

In addition to origin through duplication of other SFP-encoding genes, new influential SFPs could also arise from duplicates of genes encoding proteins not found in the seminal fluid (Mueller et al., 2005). One likely source for such genes would be those encoding influential female reproductive tract proteins since such proteins may already have secretion signals and are already able to exert effects on female phenotypes (Baer et al., 2009b; Findlay et al., 2014; Prokupek et al., 2010). A change in the regulation of female reproductive tract genes resulting in expression in males could provide the immediate origin of new influential SFPs. Evidence consistent with this scenario comes from a set of three *D. melanogaster* paralogs, two of which are expressed exclusively in the female sperm storage organs and the third of which is expressed exclusively in the male reproductive accessory glands (Sirot et al., 2014). Sequence and expression analyses of the orthologs of these genes across several Drosophila species suggest that the original gene was female-expressed, with two subsequent gene duplications resulting in a second female-expressed gene and a novel SFP (Sirot et al., 2014). Interestingly, the female paralogs promote remating whereas the male paralog shows a non-significant trend for inhibiting remating (Sirot et al., 2014). Therefore, this SFP (CG32833) might be in the process of evolving a novel effect on the same phenotype impacted by its female-expressed paralogs. In contrast to CG32833, the expression patterns of the putative original duplicated gene have not been identified for most insect SFPs that appear to have originated through gene duplication.

Influential SFPs could also arise through changes in the expression patterns of existing genes. As mentioned previously, some genes encoding insect SFPs are also expressed in other parts of the body. Yet, the evolutionary pathway by which these SFP-encoding genes came to be expressed in multiple tissues has not yet been studied. Therefore, it is not yet known whether their expression in the male reproductive tract evolved before, concurrently, or after their expression in other tissues. In some cases, however, there is strong circumstantial evidence for evolutionary co-option into the seminal fluid of a protein that was already produced in other tissues. For example, in the mosquitoes, *Aedes albopictus* and *Ae. aegypti*, the gene encoding the adipokinetic hormone is expressed in the male reproductive tissues and transferred to females during mating (Boes et al., 2014; Kaufmann et al., 2009; Sirot, unpubl. data). Adipokinetic hormones are well-conserved, multi-functional invertebrate neuropeptides whose expression across a wide range of insects has been observed primarily or exclusively in neurosecretory cells of the *corpora cardiaca* (Caers et al., 2012; Lorenz and Gäde, 2009). Adipokinetic hormones have not been reported to be expressed in the male reproductive tract of any other species thus far, suggesting that *Aedes* males have co-opted this influential neuropeptide into their seminal fluid over evolutionary time. The evolutionary changes in the regulatory elements that resulted in male reproductive tract expression of adipokinetic hormone in *Aedes* mosquitoes are not yet known. Further research into the evolutionary origins of and underlying mechanisms controlling male reproductive tract expression will provide valuable insights into the origins of new influential SFPs.

In addition to originating from duplication or changes in expression of pre-existing genes, genes encoding SFPs in some insects also arise *de novo* from regions of previously non-coding DNA (Begun et al., 2006; Findlay et al., 2009). Begun et al. (2006) argue that SFP-encoding genes are especially likely to evolve through this mechanism for several reasons. At least in the *Drosophila* lineage, SFP-encoding genes: (i) experience a higher rate of loss and gain than other genes; (ii) have short open-reading frames which occur frequently in non-coding regions throughout the genome; and (iii) tend to evolve more rapidly than other genes which might allow a new SFP to quickly gain function (Begun et al., 2006; Findlay et al., 2009; Haerty et al., 2007). It is currently unknown whether *de novo* evolution of SFP-encoding genes is also common in other insect lineages. An interesting trend in the seminal fluid of many insect species is the occurrence of "novel" proteins which contain no similarity to existing proteins, suggesting that *de novo* evolution of SFPs may be common across insects (Al-Wathiqui et al., 2018; Bayram et al., 2017; Bonilla et al., 2015; Kelleher et al., 2009; Simmons et al., 2013). Evidence for SFPs originating from alternative mechanisms (e.g., horizontal gene transfer and retrotransposition) have not yet

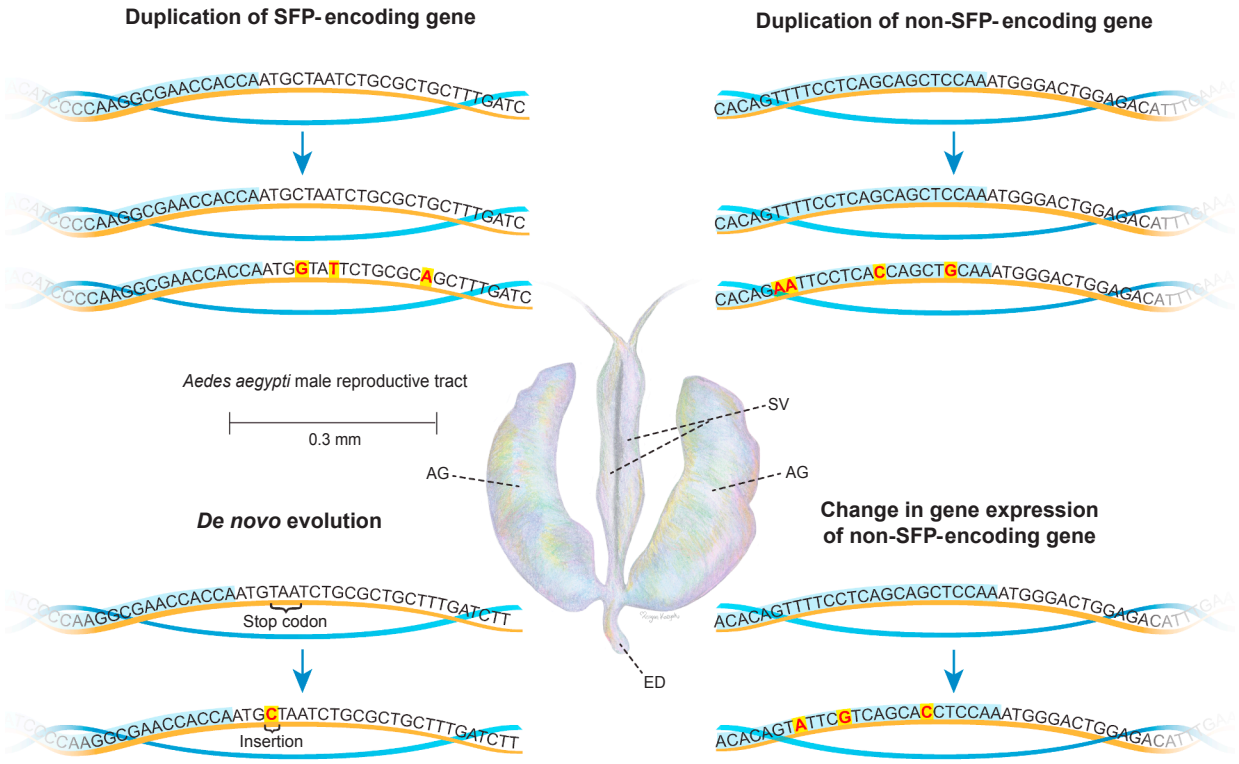

Fig. 1. Four mechanisms for the origin of seminal fluid proteins. DNA with blue highlighting symbolizes the 5′ regulatory region. DNA with no highlighting indicates the coding sequence. Nucleotides highlighted in yellow indicate mutations. Upper left: Novel seminal fluid protein (SFP) encoding genes could arise through duplication of pre-existing SFP-encoding genes followed by divergence through mutations in the coding sequence. Lower left: Novel SFP-encoding genes could evolve in a location in the genome where no gene previously existed through mutations that result in changes either from a non-regulatory region to a regulatory region (not shown) or from a non-coding sequence to a coding sequence. Lower and upper right: Novel SFP-encoding genes could also evolve from pre-existing non-SFP genes either through mutations in the regulatory region (lower right) or through duplication followed by mutations in the regulatory region (upper right). Other potential mechanisms for the origin of seminal fluid proteins are plausible, but the four highlighted in this figure are those with the most support from existing data (see text for more details). AG: Accessory glands; SV: Seminal vesicles; ED: Ejaculatory duct. Graphics by Jodi Robison. Drawing by Reagan Kazyak.

been reported but are important to explore.

3. Future directions and insights from studies of other influential molecules

Based on the information currently available, it is clear that influential SFPs can arise through multiple alternative pathways. However, there is still much that is not known about the origin of these proteins. Below, I identify important areas for future research.

3.1. What are the regulatory elements that drive expression in the male reproductive tract?

No matter what evolutionary pathway proteins took to become SFPs, one commonality is that the genes encoding them are expressed in male reproductive tissues (at least based on our current knowledge). Molecules regulating the development of the glands that SFPs are produced in have been identified in several species (Gillott, 1988; Sharma et al., 2017; Wolfner, 1988), but the cis and trans regulatory elements for specific genes have only been identified for a subset of SFPs in a few species (DiBenedetto et al., 1987; Gillott, 1996; Ismail and Gillott, 1995; Monsma and Wolfner, 1988; Wolfner, 1988). Based on this research, it has been suggested that different molecules might regulate different SFPs within a single species. For example, in the grasshopper, *Melanoplus sanguinipes*, the production of some proteins produced in the male reproductive accessory glands is regulated by juvenile hormone, whereas the production of other accessory gland proteins is regulated by 20-hydroxyecdysone (Ismail and Gillott, 1995; Gillott, 1996).

The cis elements that regulate expression in male reproductive glands have not been well-studied and are an important area for future research. Insights and inspiration for this area of research may be gained from studies on the origin of regulatory motifs that designate testes-specific expression which demonstrate both multiple pathways for the origin of tissue-specificity and multiple motifs designating tissue-specific expression (Gubala et al., 2017; Sorourian et al., 2014; Zhao et al., 2014). Further, tissue-specific control of SFP production could be regulated post-transcriptionally. As described in Section 1.1, recent evidence suggests that the amount of individual SFPs transferred by males to females in *D. melanogaster* can be regulated post-transcriptionally by microRNAs that are predicted to bind to sequences in the 3′ untranslated regions of mRNA transcripts (Fricke et al., 2014; Mohorianu et al., 2018). Further research should investigate whether post-transcriptional mechanisms regulate not only how much of each protein is transferred to female but also whether transcripts of individual genes are translated at all in the male reproductive tissues.

3.2. Are there common tissues of expression for genes whose descendants are co-opted to become SFPs?

Some influential SFPs appear to arise through co-option of either gene duplicates or of single copy genes (Boes et al., 2014; Findlay et al., 2008; Mueller et al., 2005). For these proteins, it would be interesting to discover the ancestral expression pattern of these genes before co-option into the seminal fluid. This topic has not yet been studied for SFPs, but insights can be gained through research both on female

reproductive proteins and on venom proteins. In both *Drosophila* and in the Lepidoptera, *Pieris rapae*, many secreted proteolysis regulating proteins in the somatic tissue of the female reproductive tract appear to have arisen through gene duplication (Kelleher and Markow, 2009; Kelleher and Pennington, 2009; Kelleher et al., 2007; Meslin et al., 2015). In *P. rapae*, the original genes were expressed in the digestive tract or salivary glands and maintain a digestive function within the female reproductive tract (Plakke et al., 2015). Therefore, it is possible that influential SFPs with specific functions may originate from genes expressed in tissues which share that function. Similar studies of insect SFPs arising from gene duplication could determine whether SFPs which share phenotypes or mechanisms of action arise from genes expressed in the same tissues. Like some SFPs and female reproductive proteins, many snake venom proteins appear to have arisen through co-option of duplicates of genes originally expressed in tissues other than the venom glands, although the particular tissues vary between venom proteins (Fry, 2005; Reyes-Velasco et al., 2015). In contrast, a recent study on parasitoid wasps suggests that duplication followed by neofunctionalization is not a common source for novel venom proteins (Martinson et al., 2017). Rather, many novel parasitoid wasp venom proteins have evolved through rapid changes to the cis-regulatory elements of existing single copy genes (Martinson et al., 2017). This finding is relevant to the study of the origin of insect SFPs because of the similarity between SFPs and parasitoid venoms in that both groups of proteins manipulate the behavior and physiology of the arthropod recipient, and because co-option of single copy genes has been an understudied mechanism in the study of SFP origins. Future research comparing the origins and mechanisms of actions of SFPs and parasitoid wasp venoms will likely benefit both fields of study.

The consideration of the expression patterns of SFP-encoding genes across all body tissues will also provide opportunities to understand whether the evolutionary dynamics of SFP-encoding genes expressed exclusively in the male reproductive tract differ from those that are expressed in additional tissues. Although many SFP-encoding genes evolve relatively rapidly (e.g., Haerty et al., 2007), those which encode proteins with functions outside of the seminal fluid may be more constrained in their evolutionary rate than those that exclusively encode SFPs.

3.3. What are the evolutionary steps by which influential SFPs develop dependence on other SFPs for their activity?

Several of the well-studied SFPs require other SFPs to arrive at their site of action in an activated state. These supporting proteins may act either to protect the protein from degradation or to activate the protein through post-translational processing (see Section 1.2 above). For new SFPs arising through co-option of existing proteins, this dependence on other SFPs prompts the question of whether the dependence evolved before or after the co-option into the seminal fluid. It could be that the ancestral non-seminal fluid protein was already dependent on other proteins for its action and that all of these proteins were subsequently co-opted into the seminal fluid. A second possibility is that the ancestral protein was dependent on other proteins, but that its partner proteins did not get co-opted into the seminal fluid. In this case, the evolution of new partnerships could have evolved from the suite of pre-existing SFPs. A third possibility is that the ancestral protein was not dependent on any other proteins and the dependence evolved after the protein was co-opted into the seminal fluid. To distinguish between these possibilities, one would need to conduct a combination of comparative genomic, gene expression, and functional analysis studies to establish the relative timing of co-option into the seminal fluid and dependence on partner proteins. A promising place to start such studies is with well-studied insect hormones (juvenile hormone and 20-hydroxyecdysone) that, in some species, are produced in accessory glands and transferred in the seminal fluids to females where they can have profound effects on physiology and behavior (Baldini et al., 2013; Borovsky et al., 1994; Gabrieli et al., 2014; Gillott, 1996; Mitchell et al., 2015; Pondeville et al., 2008). Although these hormones are not themselves proteins, proteins are involved in their biosynthesis and/or activity (Noriega, 2014). In the case of juvenile hormone (JHIII), for example, which is in the seminal fluid of *Ae. aegypti* mosquitoes, one could use comparative proteomics across related species to establish the presence and order of appearance in the seminal fluid of proteins involved in its biosynthesis and activity. Similarly, for SFPs with established partnerships (e.g., the proteins involved in the long-term sex peptide network), one could use comparative proteomics and genomics to establish the order in which the various proteins became part of the network (Findlay et al., 2014; Ravi Ram and Wolfner, 2009). Such studies will provide insights not only to the origin of individual influential SFPs but also into the evolution of the complex molecular interactions in which they are involved.

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