

# The gifts that keep on giving: physiological functions and evolutionary dynamics of male seminal proteins in *Drosophila*

MF Wolfner

Department of Molecular Biology and Genetics, Cornell University, Ithaca NY 14853–2703, USA

During mating, males transfer seminal proteins and peptides, along with sperm, to their mates. In *Drosophila melanogaster*, seminal proteins made in the male's accessory gland stimulate females' egg production and ovulation, reduce their receptivity to mating, mediate sperm storage, cause part of the survival cost of mating to females, and may protect reproductive tracts or gametes from microbial attack. The physiological functions of these proteins indicate that males provide their mates with molecules that initiate important reproductive responses in females. A new comprehensive EST screen, in conjunction with earlier screens, has identified ~90% of the predicted secreted accessory gland proteins (Acps). Most Acps are novel proteins and many appear to be secreted peptides or prohormones. Acps also include modification enzymes such as proteases and

their inhibitors, and lipases. An apparent prohormonal Acp, ovulin (Acp26Aa) stimulates ovulation in mated *Drosophila* females. Another male-derived protein, the large glycoprotein Acp36DE, is needed for sperm storage in the mated female and through this action can also affect sperm precedence, indirectly. A third seminal protein, the protease inhibitor Acp62F, is a candidate for contributing to the survival cost of mating, given its toxicity in ectopic expression assays. That male-derived molecules manipulate females in these ways can result in a molecular conflict between the sexes that can drive the rapid evolution of Acps. Supporting this hypothesis, an unusually high fraction of Acps show signs consistent with their being targets of positive Darwinian selection.

Heredity (2002) 88, 85–93. DOI: 10.1038/sj/hdy/6800017

**Keywords:** accessory gland; reproduction; protease inhibitor; ovulation; peptide hormone; rapid evolution

## Introduction

The recognition between members or cells of the opposite sex is an important ingredient in the success of a mating. The sexes can also influence each other after mating. The focus of this article is on protein 'gifts' that a male *Drosophila* gives his mate in the seminal fluid. The proteins on which this article centers are the products of genes expressed only or primarily in the accessory glands of male flies. These genes are downstream targets of the regulatory hierarchies that determine sexual phenotype (see Zarkower (2001) for review of such hierarchies). Once transferred to a female during mating, seminal proteins influence the female's reproduction, behavior and physiology. This article will first review briefly what these seminal proteins are and how they were found, and their general functions in females. Then the focus will be on the function of two seminal fluid proteins, one that regulates ovulation and one that regulates proteolysis and is proposed to affect the mated female's lifespan. Finally, results that point to interesting evolutionary dynamics of seminal proteins will be described. These proteins provide the gift of molecular tools with which to address some hypotheses of evolutionary interest.

## What protein gifts does a male fly give his mate, and what do they do to her?

During courtship in *Drosophila*, males use chemical and visual cues to find females. Once a male has detected a female, he orients towards her, extends a wing, and vibrates it to produce a species-characteristic mating song (reviewed in Hall, 1994; Greenspan and Ferveur, 2000). A female who is receptive to mating (sexually mature and unmated or not recently mated), and who recognizes the song as from her own species, will modify her behavior, permitting the male to reach her and continue the courtship ritual. Ultimately the male will mount the female and copulate with her. After mating, several aspects of the female fly's physiology and reproductive behavior are profoundly changed (see Chen, 1996; Kubli, 1996; Wolfner, 1997 for reviews of, and references to, earlier work). First, whereas an unmated female *Drosophila melanogaster* produces and lays a couple of (unfertilized) eggs a day, after mating a female's egg-laying levels increase by an order of magnitude (with consequent increases in oogenic and ovulation rates). Second, before mating a female is receptive to mating with a male from her species, but after mating she will actively reject males by kicking them away and by extruding her ovipositor. This effect on the female's receptivity can be considered to be the result of a chemical version of the mate-guarding seen in several insects (see Krebs and Davies, 1993; Alcock 1998 for review of mate-guarding). Third, mated

females also differ from unmated females by storing sperm. Fourth, a mated female has a shorter lifespan than an unmated female (Fowler and Partridge, 1989). Almost all these changes in their full magnitude require that the female mated: it is not enough for her only to see, hear, or smell the male, no matter how enticing, graceful or charming one might think he could be. The differences between mated and unmated females indicate that copulation *per se* is required to elicit the changes in the female's reproductive behavior and physiology. Additional experiments showed that the full spectrum and magnitude of the changes requires that the female have received seminal fluid and sperm from her mate (Manning, 1962; Hihara, 1981; Kalb *et al*, 1993; Harshman and Prout, 1994; Chapman *et al*, 1995; Tram and Wolfner, 1998, 1999; Xue and Noll, 2000; Heifetz *et al*, 2001). [Males have also been reported to donate cuticular hydrocarbons, and elemental phosphorus, to their mates (for reviews, see Antony and Jallon, 1982; Jallon, 1984; also see Markow *et al*, 2001). Consideration of these, and of the contributions of the male's ejaculatory duct and bulb (eg Gilbert *et al*, 1981; Cavener and MacIntyre, 1983; Ludwig *et al*, 1991; Lung *et al*, 2001b) is beyond the scope of this review.]

Sperm and seminal fluid proteins both exert post-mating influences on female insects. In *D. melanogaster*, genetic techniques can be used to tease apart their relative contributions. Flies lacking sperm can be generated because of mutations that prevent germ cell formation (eg Boswell and Mahowald, 1985); flies of the X0 chromosome constitution also lack functional sperm (Bridges, 1916). These spermless flies still make and transfer seminal proteins. Comparisons of the phenotypes of mates of spermless flies to mates of flies that make sperm have identified several general reproductive roles of sperm. First, and obviously, sperm are needed to fertilize eggs. Second, the presence of sperm in females contributes to some of the initial post-mating changes, such as increased oogenic and egg-laying rates (Xue and Noll, 2000; Heifetz *et al*, 2001). Stored sperm are also needed to maintain the mated state: long-term inhibition of mating receptivity, and continued elevated rates of oogenesis, ovulation and egg deposition all require the presence of sperm in the female (Manning, 1962, 1967; Kalb *et al*, 1993; Tram and Wolfner, 1998; Xue and Noll, 2000; Heifetz *et al*, 2001). Finally, sperm from different mates compete within a multiply-mated female for their use in fertilizing eggs (Parker, 1970). This competition between gametes from different males is likely to be of evolutionary importance.

Analogous experiments identified general roles of *Drosophila* seminal proteins, particularly those produced in the accessory gland of the male fly (these proteins are called 'Acps' for accessory gland proteins). Flies that fail to make proteins in the main cells of their accessory glands can be generated by forcing those cells to produce an intracellular toxic protein; these flies lack seminal proteins but, for technical reasons unrelated to seminal proteins, also lack sperm (Kalb *et al*, 1993). Recently, a fly line was reported to produce sperm in the absence of development of accessory glands (Xue and Noll, 2000). This line is mutated in the paired gene, whose functions include specifying accessory gland development (Xue and Noll, 2000). Results from the two types of fly strains just mentioned have identified roles for Acps in the post-mating changes in female flies. Short-term action of Acps is needed to increase egg production/laying process

(including increased rates of oogenesis, ovulation and egg deposition) and decrease receptivity to re-mating. Changes induced by Acps last for up to a day post-mating; continuation of the changes beyond this time requires the presence of stored sperm in the female. Rapid-acting, but temporary, Acps could be advantageous to both male and female. It takes at least 1 h to store sperm to maximal levels (Gilbert, 1981; Tram and Wolfner, 1999) and potentially longer for the presence of stored sperm to be manifested in behavioral or physiological changes in the female. Seminal proteins quickly change the female's physiology/behavior after mating, even while sperm are being stored. However, it would seem disadvantageous for females to produce high numbers of eggs and to avoid mating if they did not receive sperm from the mating, or after their stored sperm have been depleted. From this perspective, it seems advantageous to tie long-term persistence of these changes to the presence of stored sperm in the female. Seminal proteins are also necessary for the efficient storage of sperm by females (Tram and Wolfner, 1999). At least in part as a consequence of this (Chapman *et al*, 2000), they are likely to play roles in sperm competition. Indeed, a role for Acps in sperm competition has been reported (Harshman and Prout, 1994), and a correlation has been observed between alleles at four Acp loci and levels of sperm displacement in lines carrying chromosomes isolated from the wild (Clark *et al*, 1995). Seminal proteins also decrease the lifespan of the mated female (Chapman *et al*, 1995). Acps have roles in addition to those determined by the genetic studies: Acps include proteins with antibacterial activity (Lung *et al*, 2001a), suggesting a role in protecting the reproductive tracts (of either sex), or sperm, or the first egg laid after mating, from microbial attack. In addition, at least one Acp is a component of the gelatinous mating plug that forms in the reproductive tract of the mated female (Lung *et al*, 2001b). This structure is thought to assist in the movement of sperm into the female and into storage (Bairati, 1968).

In sum, the male provides his mate with the gifts of sperm and seminal proteins, which have profound effects on female reproductive physiology and behavior. It should be made clear that females are by no means passive players in the reproductive exchange between the sexes (Eberhard, 1996). There is evidence, for example, that *D. melanogaster* females play a role in sperm competition (Clark and Begun, 1998; Clark *et al*, 1999). Nevertheless, most molecular studies of effectors of post-mating changes in *Drosophila* females have focused on male-derived proteins. Technical considerations largely explain this apparent bias. It is easier to identify the products of a single male tissue, the accessory gland, than it is to isolate effectors of reproductive behavior/physiology among the greater complexity of all the female's tissues. In addition, it is easier to identify factors that cause a change beginning at a defined time-point of introduction (in this case, introduction from the male during mating) as compared with the effects of regulatory molecules that are continually present in female flies.

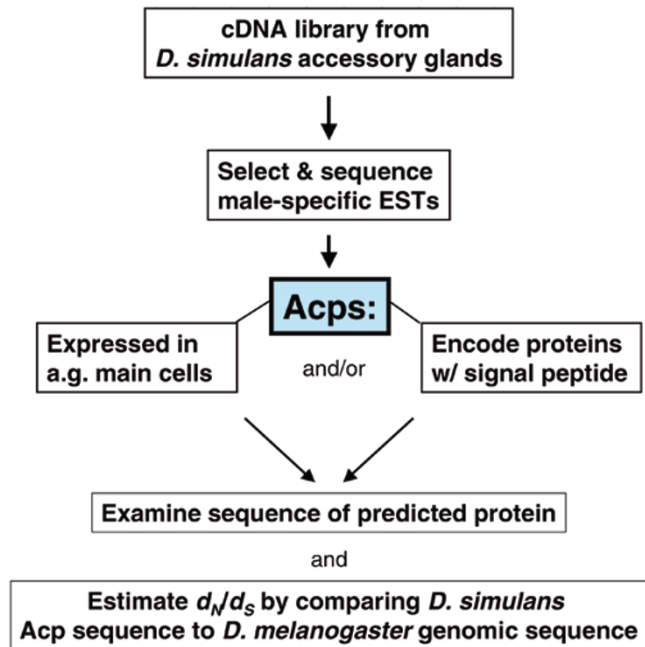
## How are *Drosophila* seminal fluid proteins identified, what are their features and how many are there?

The rest of this article focuses on individual constituents of the powerful chemical mixture provided by the *Droso-*

*phila* male in his seminal fluid. Elucidation of the action of individual Acps permits a molecular understanding of reproductive interactions between females and males, including a model system for investigating the action of hormones or neuromodulators and an examination of the 'ecology' of fertilization in its normal setting. The study of Acps also can provide molecular probes with which to address evolutionary models for competition among individuals within a species.

Before the functions of individual seminal proteins can be identified, a comprehensive picture of the proteins secreted by male accessory glands is needed. Early studies involving differential cDNA hybridization screens to identify male-specific RNAs expressed in accessory glands (Schäfer, 1986; DiBenedetto *et al*, 1987; Monsma and Wolfner, 1988; Simmerl *et al*, 1995; Wolfner *et al*, 1997), or (in one case) identification of an accessory gland peptide by a functional assay (Chen *et al*, 1988), identified 18 Acp genes; according to *Drosophila* convention, these genes are named 'Acp' followed by the designation of their chromosome position. SDS-PAGE analysis of accessory gland proteins coupled with statistical tests based on the frequency of 'multiple hits' in the cDNA screens suggested that there were about 50–100 different Acps (Chen, 1991; Wolfner *et al*, 1997). Several characteristics of the 18 initial Acp genes (see Wolfner (1997) for review) were helpful in designing a comprehensive screen to identify all Acps. First, all 18 Acps have predicted signal sequences at their N-termini. Second, Acps expressed in the predominant cell type of the accessory gland (main cells, 96% of the secretory cells in the gland; Bertram *et al*, 1992) are not expressed in flies whose main cells are ablated with an intracellular toxin (Kalb *et al*, 1993). Finally, many Acp genes encode novel proteins or novel short peptides, and the sequences of several of these genes appear to be evolving rapidly apparently as a result of positive selection.

To identify the complete spectrum of Acp genes, a comprehensive EST screen was carried out by Swanson *et al* (2001) (Figure 1). ESTs made from male accessory gland RNA were selected initially for non-expression in females. The genes thus identified include a large number that encode novel proteins: 47% do not have relatives in non-*Drosophila* sequences in GenBank, suggesting that this group of ESTs is a rich source for new protein coding sequences. Swanson *et al* (2001) applied two additional criteria to focus on those ESTs most likely to encode Acps. First, genes that were not expressed when accessory gland main cells were ablated (Kalb *et al*, 1993) were considered Acps. Second, since Acps made in accessory-gland cells other than main cells (Bertram *et al*, 1992) would be missed by this procedure, ESTs encoding proteins with predicted signal sequences were included in the group considered to be Acp genes. As with the 18 previously known Acp genes, the 57 genes selected by these criteria encode a range of molecules (Figure 2). They include predicted secreted small peptides or larger molecules that could be cleaved to yield multiple peptide hormones, predicted glycoproteins, and proteins with sequence motifs predictive of biochemical function. The latter class includes proteins predicted to be proteases, protease inhibitors or lipases. Based on multiple-hits in the EST screen, Swanson *et al* (2001) calculated that there are approximately 83Acp genes; this estimate is consist-



**Figure 1** The 'evolutionary EST screen' that identified Acp genes, and identified candidate rapidly-evolving molecules. cDNA clones made from mRNA isolated from *D. simulans* accessory glands were screened for male-specific expression by hybridization to cDNA derived from *D. simulans* females. Clones that did not hybridize to female-derived cDNA were sequenced, and further screened for presence of a predicted signal sequence (von Heijne, 1983) and/or loss of expression in males lacking the primary cell type (main cells) of the accessory gland (Kalb *et al*, 1993). These putative Acps were examined for sequence features and, by comparison to the *D. melanogaster* genomic sequence (Adams *et al*, 2000), for signs of rapid evolutionary change. See Swanson *et al* (2001) for details.

## The 83 predicted Acps include:

- **Peptides (25) or prohormone precursors**  
e.g. the ovulation hormone Acp26Aa (ovulin)  
the "sex peptide" Acp70A
- **Glycoproteins**  
e.g. the sperm storage protein Acp36DE
- **Modifying enzymes**  
**Proteases (9)**  
**Protease inhibitors (8)**  
e.g. the trypsin inhibitor Acp62F  
**Lipases (6)**
- **Many novel proteins**
- **~ 11% with signs of rapid evolution**

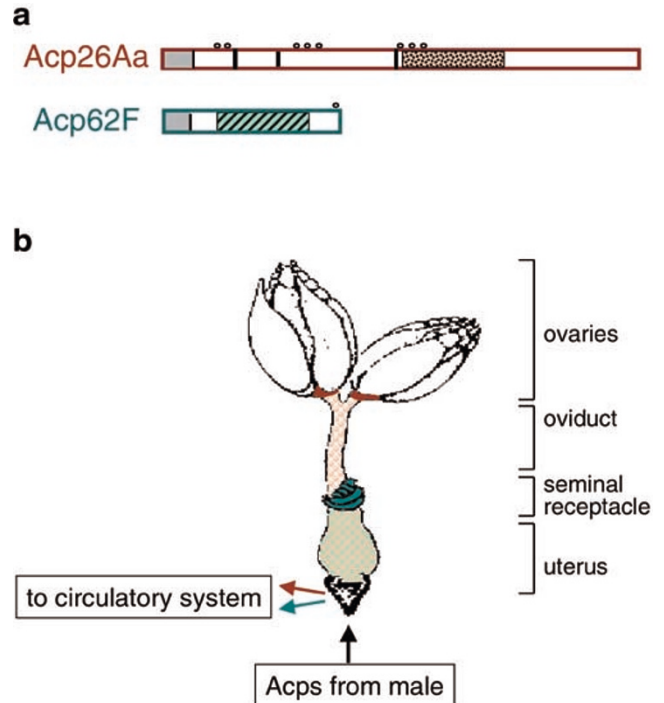
**Figure 2** Sequence classes of the 83 predicted Acps, isolated in several screens (Schäfer, 1986; DiBenedetto *et al*, 1987; Chen *et al*, 1988; Monsma and Wolfner, 1988; Simmerl *et al*, 1995; Wolfner *et al*, 1997; Swanson *et al*, 2001). The sequence and function of the four example Acps are described in: Acp26Aa (Monsma and Wolfner, 1988; Herndon and Wolfner, 1995; Heifetz *et al*, 2000; Chapman *et al*, 2001), Acp70A (Chen *et al*, 1988; Aigaki *et al*, 1991; Kubli, 1996; Moshitzky *et al*, 1996; Soller *et al*, 1997), Acp36DE (Wolfner *et al*, 1997; Neubaum and Wolfner, 1999; Chapman *et al*, 2000) and Acp62F (Wolfner *et al*, 1997; Lung *et al*, 2002).

ent with those from the earlier studies. Ninety percent of these genes (75 predicted genes) are in hand.

### What are the functions of individual seminal fluid proteins?

Now that predicted Acps have been identified, the function(s) of each Acp, and the way in which this function(s) is carried out, needs to be defined. Two types of genetic assay can be used to assign functions to individual Acps. The most conclusive is a knockout approach, in which an Acp is chosen for examination, often because of some interesting feature of its sequence or targeting. This Acp gene is knocked out, and the post-mating response(s) that fails to occur in the absence of this particular Acp can be determined. This approach was successful for two Acps: the ovulation hormone Acp26Aa, which is discussed further below, and the sperm storage protein Acp36DE (Herndon and Wolfner, 1995; Neubaum and Wolfner, 1999; Chapman *et al*, 2000). Until recently, use of this method was limited owing to the difficulty in generating mutants in a gene defined only by sequence and without a phenotype that could be predicted in advance. A recently-reported technique for homologous recombination shows great potential for making it possible to generate future Acp knockouts more routinely (Rong and Golic, 2000, 2001). Given the difficulty of the knockout approach, other assays have also been used to identify potential functions of Acps. These assays usually scan several Acps at once for effects on a specific phenotype. For example, it can be determined whether ectopic expression of an Acp in unmated females causes them to display phenotypes that resemble any seen in mated female flies. This approach has led to the assignment of function to two Acps. Ectopic expression of the protease inhibitor Acp62F suggests a role in the survival cost of mating (Lung *et al*, 2002; discussed below). Ectopic expression and injection assays have also been used by the Kubli, Chen and Aigaki labs to show that Acp70A (sex peptide) can induce rejection behavior and increase egg-laying in females (Chen *et al*, 1988; Aigaki *et al*, 1991; Nakayama *et al*, 1997). Data from these labs and their collaborators are consistent with a model in which Acp70A stimulates the production of juvenile hormone BIII, which in turn stimulates oogenic progression (Moshitzky *et al*, 1996; Soller *et al*, 1997, 1999). Non-genetic assays also have helped to dissect Acp function. Immunofluorescence or GFP fusions have identified target tissues of Acps, which in turn provides insight into the mode of Acp action (Bertram *et al*, 1996; Lung and Wolfner, 1999; Heifetz *et al*, 2000; Ottiger *et al*, 2000). Direct biochemical or physiological assays, including some discussed below, have also provided functional information (Chen *et al*, 1988; Schmidt *et al*, 1989; Lung *et al*, 2001a; Lung *et al*, 2002), and correlations of allelic variation have in some cases suggested potential roles to consider for Acps (Clark *et al*, 1995).

The functions of four Acps are particularly well understood. Here, the focus is on two of them, the ovulation hormone Acp26Aa, and the protease inhibitor Acp62F (schematically shown in Figure 3a). The other two are the receptivity/egg-laying modulator Acp70A (Chen *et al*, 1988; Aigaki *et al*, 1991; Kubli, 1992, 1996; Moshitzky *et al*, 1996; Nakayama *et al*, 1997; Soller *et al*, 1997, 1999; Ottiger *et al*, 2000), and the sperm storage protein



**Figure 3** Features of Acp26Aa and Acp62F. (a) Schematic diagram of the primary sequence of Acp26Aa and Acp62F. Acp26Aa is a 264 amino acid predicted polypeptide (Monsma and Wolfner, 1988). It initiates with a predicted signal sequence (gray), and contains sites at which the protein is cleaved proteolytically once it is in the reproductive tract of a mated female fly (vertical bars) (Monsma and Wolfner, 1988; Monsma *et al*, 1990; Park and Wolfner, 1995). A region of Acp26Aa (brown, dotted: Monsma and Wolfner, 1988; Heifetz *et al*, 2000) has sequence similarity to califin C and ELH of *Aplysia californica* (Scheller *et al*, 1983; Rothman *et al*, 1986; Kurosky *et al*, 1998). Acp62F is a 115 amino acid predicted polypeptide (Wolfner *et al*, 1997), that initiates with a predicted signal sequence (gray) and has a region of sequence similarity to a class of secreted protease inhibitors from *Ascaris* (Peanasky *et al*, 1984a, b; Lung *et al*, 2002). (b) Schematic of the localization of Acp26Aa (brown) and Acp62F (green), shown on a simplified diagram of the mated female reproductive tract (spermathecae and female accessory glands have been omitted from the diagram, and 'oviduct' refers to common and lateral oviducts). Acp26Aa accumulates at the base of the ovaries, and is also seen in the lumen of the oviducts and uterus, possibly simply *en route* to the base of the ovaries (Heifetz *et al*, 2000). Acp62F is found in the seminal receptacle and in the uterine lumen (again presumably *en route*) (Lung *et al*, 2002). Some Acp26Aa and some Acp62F also cross the wall of the posterior vagina to enter the circulatory system of the female fly (Monsma *et al*, 1990; Lung and Wolfner, 1999).

Acp36DE (Bertram *et al*, 1996; Neubaum and Wolfner, 1999; Chapman *et al*, 2000).

The ovulation hormone Acp26Aa ('ovulin') Acp26Aa is a 264 amino acid-long polypeptide (Monsma and Wolfner (1988); Figure 3a). It is hydrophilic after its signal sequence and contains several pairs of basic amino acids (or single basic amino acids in cleavage contexts); thus, it resembles a precursor to multiple peptide hormones. Indeed when Acp26Aa enters the female fly, it undergoes proteolytic cleavage in a process that requires components donated by both male and female (Monsma *et al*, 1990; Park and Wolfner, 1995). The proteolytic products are consistent with ordered cleavage at the predicted cleavage sites, proceeding from the more

N-terminal to the more C-terminal sites. The amino acid sequence of Acp26Aa contains an interesting similarity to regions of a family of related egg-laying hormones made by the mollusk *Aplysia californica* (and also produced by cleavage from larger precursors; Scheller *et al*, 1983; Rothman *et al*, 1986).

A knockout mutation of Acp26Aa was without consequence to males themselves (Herndon and Wolfner, 1995). However, mates of males lacking Acp26Aa were affected in a single aspect of their post-mating physiology: they laid fewer eggs than normal on the first post-mating day (Herndon and Wolfner, 1995), the time of action of Acps (Kalb *et al*, 1993). Therefore Acp26Aa was necessary to stimulate the egg-laying process in mated females. Females mated to males that lacked the protein still show some egg-laying elevation, indicating that molecules besides Acp26Aa also stimulate egg-laying. One of these molecules is likely to be Acp70A, based on the results cited above (Chen *et al*, 1988; Aigaki *et al*, 1991). To gain a full understanding of Acp26Aa's action, it was necessary to understand how it stimulated the egg-laying process, and whether its action was redundant with, or complementary to, that of other Acps such as Acp70A.

The egg-laying process involves multiple steps (Soller *et al*, 1999; Heifetz *et al*, 2000). Eggs have to be produced (via oogenesis), pass a checkpoint during oogenesis, be released from ovaries (ovulated), move down the oviducts to the uterus, be released from the uterus, and be laid. In principle an Acp could act at any or all stages in the process. To determine at which step(s) Acp26Aa exerts its effect, we developed an assay to measure the progress through each step individually, by directly quantifying the number of eggs at each stage of their movement through the female reproductive tract (Heifetz *et al*, 2000). Then the progress through each stage of the egg-laying process was compared in females who had received Acp26Aa from their mates relative to females who had mated to males lacking Acp26Aa. It was discovered that just one step, the initial release of oocytes at ovulation, is dependent on the transfer of Acp26Aa; because of this function, Acp26Aa has been renamed 'ovulin' (Heifetz *et al*, 2000). Our data indicate that Acp26Aa stimulates the immediate release of eggs following mating – its effects are evident by 1.5 h post-mating and persist until about 6 h post-mating. This early time of action leads to the model that Acp26Aa causes the release of mature oocytes accumulated in the ovary before the mating. Since such action affects only eggs that are already in existence, it would not be expected to exact a further energetic cost on the female. Consistent with this expectation, Chapman *et al* (2001) showed that Acp26Aa does not impact the lifespan of mated females. Acp26Aa releases eggs while sperm are still being stored. Those eggs would therefore be expected to be fertilized less efficiently than eggs ovulated later, when sperm are fully stored and in position to be released efficiently; this too was observed (Chapman *et al*, 2001). Thus, the function of Acp26Aa appears to be to stimulate ovulation shortly after mating, to 'clear' mature eggs from the ovary. This sacrifice of a few mature eggs would relieve the pre-mating arrest of oogenesis caused by the accumulation of mature, unovulated eggs. It could potentially also allow subsequent synchronization of egg and sperm release to permit optimal rates of fertilization.

How does Acp26Aa cause this oocyte release?

Acp26Aa might act directly on targets in the reproductive tract, since it has been seen to bind to sites at the base of the ovaries (Heifetz *et al* (2000); summarized in Figure 3b). Alternatively, since Acp26Aa also enters the circulatory system of the female fly (Monisma *et al*, 1990; Lung and Wolfner, 1999), it could trigger endocrine or neural signals that stimulate ovulation. In either case, Acp26Aa action must ultimately affect reproductive tract tissues at or near the base of the ovary. To identify the molecular consequence of Acp26Aa action in the *Drosophila* female reproductive tract, we are presently examining these tissues for the role of Acps, and Acp26Aa in particular, in triggering vesicle exocytosis and/or alteration in levels of neuromodulators that cause muscle contraction and thus might be involved in inducing ovulation (Trent *et al*, 1983; Lange *et al*, 1986, 1991; Bamji and Orchard, 1995; Lange and Nykamp, 1996; Monastiriotti *et al*, 1996; Yamauchi *et al*, 1997; Waggoner *et al*, 1998).

In summary, Acp26Aa (ovulin) has been identified as a mediator of one specific step in the egg-laying process: the release of mature oocytes from the ovary. Studies in the Kubli lab indicate that the stimulation of egg-laying by another Acp, Acp70A (sex peptide), occurs by stimulating oogenesis (Soller *et al*, 1997, 1999). Thus, although at least two Acps modulate the egg-laying process, they act on different steps. This suggests that seminal fluid is a potent chemical cocktail, whose components independently regulate individual steps in multi-step processes. Having independent regulators allows each stage of the process to be tuned to maximal efficiency on its own, as well as in concert with other steps. Moreover, given the fact that many Acps appear to be evolving rapidly (see below), it may be best to have critical processes controlled at several steps, independently. This could allow essential reproductive processes to accommodate changes in individual Acps better than if the entire pathway were controlled by one Acp, or by a group of Acps acting together in a single biochemical complex, on only one physiological step.

#### The protease inhibitor Acp62F

Action of Acps contributes to the survival cost of mating. In other words, not all gifts are pleasant or benign. How could an Acp, deposited into the reproductive tract of a female (and hence, topologically on her outside) cause a systemic detrimental effect on the female? It turns out that most Acps, including Acp62F (and Acp26Aa as noted above), can cross a permeable region at the posterior of the female reproductive tract to enter the circulatory system of the female and gain access to all her other tissues, including neural and endocrine tissues (Lung and Wolfner, 1999). The basis for this entry is not apparent in Acps' sequences, and may be simply due to non-selective transport of Acps that are below a given size cutoff or that are not bound to sperm or to the reproductive tract wall. Entry of Acps into the female's circulation gives them the means to exert systemic effects on her physiology and behavior.

To identify an Acp that might be an agent for decreasing the lifespan of the mated female, we tested eight separate Acps for effects on viability (Lung *et al*, 2002). We introduced each Acp individually into *D. melanogaster* and compared the effects of ectopic expression of each Acp on viability, relative to expression of a control protein. Only one Acp, Acp62F, was toxic to preadult

*Drosophila* and, upon multiple rounds of expression, to adults.

What is the biochemical action of Acp62F, and can this explain its toxicity? The sequence of Acp62F has several intriguing similarities, but the most extensive is to a class of extracellular protease inhibitors produced by *Ascaris* worms (Peanasky *et al*, 1984a, b; schematically shown in Figure 3a). Moreover, the similarity extends to the predicted 3D structure of the proteins: the structure of the *Ascaris* inhibitors has been solved, and Acp62F's sequence threads along it very well. This threading allows the prediction of the active site of Acp62F; it has the characteristics of a trypsin inhibitory site. *In vitro* assays showed that Acp62F indeed inhibits trypsin (and not other proteases like elastase) and that mutations of its predicted active site either abolish this biochemical activity or change its specificity exactly as predicted from effects of the mutations on the protein's 3D structure. Thus Acp62F is a trypsin inhibitor in *Drosophila* seminal fluid that is transferred to females during mating (Lung *et al*, 2002).

Why might a seminal protease inhibitor be toxic? About 10% of the Acp62F transferred to a female enters her circulatory system, through the permeable region mentioned above (Lung and Wolfner (1999); summarized in Figure 3b). Thus, Acp62F could act on a cell type or process anywhere in the female. One possible model is that entry of this proteolysis regulator into the circulatory system places it at a site from which it can alter the progress of essential proteolytic cascades. These cascades include those that are needed for the fly's immunity to microbial parasites and could thus make females more susceptible to infection (eg see Imler and Hoffmann (2000) for review), thereby shortening their life. Altering the efficiency of other metabolic or endocrine proteolytic cascades could also, in theory, affect pathways essential for robust viability. Small amounts of Acp62F, introduced repeatedly upon multiple matings could therefore result in a decrease in female viability – part of the measured survival cost of mating. Further experiments are required to determine the nature of and reason for the toxicity of Acp62F, and whether it truly is an agent of the survival cost of mating. In light of this hypothesis, it is interesting to note that seven other Acps are predicted to be protease inhibitors. Their specificities may differ from that of Acp62F, and thus they could affect different proteolytic cascades. If any of them (or any of the nine predicted proteases) also enters the circulation of the mated female, they too could alter the balance of precisely-tuned proteolytic cascades. Thus, if effects on proteolytic cascades contribute to the survival cost of mating, there is the potential that this cost could reflect the aggregate effect of several Acps.

But if protease inhibitors like Acp62F decrease the lifespan of the mated female fly, why would their presence in seminal fluid (and entry into the circulatory system) be tolerated through evolution? An attractive explanation is that these seminal protease inhibitors play a positive role that is so valuable to reproductive success that it would be disadvantageous to lose these proteins. Indeed, in mammals, protease inhibitors have been suggested to play a positive role in male fertility. For example, knock-out of the mouse seminal protease inhibitor protease nexin-1 (PN-1) results in male infertility (Murer *et al*, 2001), and altered levels or function of PN-1 or of the

serpin protein C inhibitor are observed in seminal fluid of some infertile men (He *et al*, 1999; Murer *et al*, 2001). The short-term benefit of a protease inhibitor in *Drosophila* seminal fluid could outweigh long-term negative consequences, particularly if those consequences occur after many progeny have been produced. In addition, evolution could generate situations in which the negative effects of Acp62F were decreased by having proteins in circulation that sequester it or dampen its activity, or by keeping the amount transferred to a level sufficient for its positive function but below the threshold for serious or immediate negative effects. Indeed the toxicity seen in our experiments occurred at high levels of Acp62F, and multiple matings are required to detect the survival cost of mating under normal conditions. What sorts of positive roles could seminal protease inhibitors play? First, a protease inhibitory Acp may be necessary to regulate the proteolysis of other proteins, such as Acp26Aa (Monisma *et al*, 1990; Park and Wolfner, 1995). Such action might protect Acps from non-specific proteolysis in the accessory gland, keeping them intact and at high levels, or it could regulate the rate or position of Acp processing in the mated female's reproductive tract. Second, protease inhibitors might be needed to regulate the coagulation of seminal fluid once it is in the female, as has been proposed for the mammalian protein C inhibitor (Kise *et al* (1996); see Robert and Gagnon (1999) for review). Third, a protease inhibitor may be needed to protect sperm from proteolytic degradation of their surface proteins and to keep them stable in storage. In cattle, the seminal plasma inhibitor BUSI-II has been proposed to protect sperm from premature acrosome reaction (Veselsky and Cechova, 1980). Intriguingly suggestive of a sperm-protective role for Acp62F, this protein is present in *D. melanogaster* sperm storage organs along with sperm (Lung *et al*, 2002; summarized in Figure 3b). Reproductive functions such as these could require inhibition of many, or many types, of proteases; perhaps this accounts for the number and variety of protease inhibitors in seminal fluid.

In summary, *D. melanogaster* seminal fluid contains at least one active regulator of proteolysis, the protease inhibitor Acp62F. The predicted sequences of 16 other Acps suggest they too could modulate proteolysis (Wolfner *et al*, 1997; Swanson *et al*, 2001), though this still requires biochemical verification. Protease inhibitors in seminal fluid could play positive roles in regulating seminal protein proteolysis and/or in contributing to the functional viability of sperm. Yet, the entry of these proteins into the female's circulation, as occurs for Acp62F, could potentially interfere with the proper efficiency of essential proteolytic cascades. This could cause negative consequences to the female, including toxicity, as is seen upon ectopic expression of Acp62F.

## Acps give investigators tools with which to address evolutionary questions

Action of Acps requires interaction at the molecular level between female and male. The reproductive interests that impact on the evolution of Acps, and their receptors and response pathways can be common, such as increased progeny production, but can also be at odds between the sexes. For example, while it may be advantageous to a male that his mate avoids re-mating so that his stored

sperm are not subject to competition, it may be advantageous to the female to have multiple partners, who can provide different genetic and chemical 'donations'. Several excellent hypotheses for the evolution of male or female sexual traits have been advanced (for example: Eberhard, 1996; Rice, 1996; Holland and Rice, 1999; Arnqvist *et al*, 2000; Birkhead, 2000; Gavrillets, 2000; Chipindale *et al*, 2001; Gavrillets *et al*, 2001; Hosken *et al*, 2001). One proposes that there is an evolutionary cross-talk between the sexes that could drive rapid evolution of reproductive molecules: male molecules could 'manipulate' a female's physiology and females could evolve to become inured to this effect. This, in turn, could drive rapid evolution of the male molecules.

Among the first 18 Acps, the sequences of several, including Acp26Aa, show signs of rapid evolution consistent with positive Darwinian selection (Aguadé *et al*, 1992; Tsaur and Wu, 1997; Aguadé, 1998; Tsaur *et al*, 1998, 2001; Begun *et al*, 2000). The EST screen described above (Swanson *et al*, 2001) not only provided raw material to test systematically and in a large scale whether an unusually high fraction of Acps show signs consistent with rapid evolution, but in fact was designed to pinpoint Acps that appear to be the target of positive selection. The screen was not, in other words, an ordinary EST screen; it was an 'evolutionary EST screen'. The ESTs were made from accessory gland RNA from *Drosophila simulans*, a close relative of *D. melanogaster*. The genomes of these flies show an average of 11% base pair differences (Begun and Whitley, 2000).

Sequences of the ESTs were compared with the *D. melanogaster* genomic sequence (Adams *et al*, 2000). This allowed immediate identification of Acps whose sequences had characteristics suggesting that they might be evolving rapidly; these were Acps whose dN/dS ratios (number of nonsynonymous substitutions/nonsynonymous sites relative to synonymous substitutions/synonymous sites) were greater than 1 (the ratio for sequences changing at the neutral rate) (Hughes and Nei, 1988). Eleven percent of Acps identified in the EST screen show elevated dN/dS ratios, suggesting that they might be candidates for genes showing rapid evolution (Swanson *et al*, 2001). This frequency is much higher than that observed for ESTs from other tissues and is consistent with the idea that some Acps might be involved in molecular interchanges between the sexes according to models of sexually antagonistic coevolution (Rice, 1996). This new method can be used to identify rapidly-evolving genes in any pair of related species of which one has a fully characterized genomic sequence.

In conclusion, the male *Drosophila* gives his mate, during mating, a potent mix of seminal proteins (and sperm) that can alter the physiology, behavior and lifespan of the female. Individual Acp molecules influence the rate or occurrence of particular steps within multi-step processes and act together to regulate these processes to full efficiency. Gene knockout approaches, and ectopic expression assays have begun to identify Acps that mediate increased ovulation, decreased receptivity or storage of sperm as well as a candidate for the Acp that decreases the lifespan of the mated female fly. Future studies will identify the molecular mechanisms, receptors and pathways through which these molecules act. Acps are interesting not only for their physiological and hormonal roles, but also for their evolutionary dynamics. An

unusually high fraction of these proteins shows signs consistent with having been subjected to positive selection. The identification of Acp genes gives molecular tools for investigation of the evolutionary dynamics of reproductive proteins.

## Acknowledgements

I thank the present and former members of my laboratory, and our collaborators C Aquadro, T Chapman, A Clark, L Partridge and members of the 'Seminal Peptides Group', for many enjoyable discussions about Acps, and the NSF and NIH for funding. I also thank the organizers of the Genetics Society meeting on 'Sex' (March, 2001) for inviting me to give the talk that formed the basis for this article. S Albright, C Aquadro, M Bloch Qazi, T Chapman, Y Heifetz, L McGraw and J Mueller provided many helpful comments on the manuscript. I thank C Aquadro for the gift of this article's title. I apologize to those whose work could not be cited, or is cited only via a review article, owing to the journal's limitations on citations.

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