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Seminal fluid protein depletion and replenishment in the fruit fly, *Drosophila melanogaster*: an ELISA-based method for tracking individual ejaculates

Laura K. Siro,

Department of Molecular Biology and Genetics, Cornell University, 421 Biotechnology Building, Ithaca, NY 14853, USA

Norene A. Buehner[#],

Department of Molecular Biology and Genetics, Cornell University, 421 Biotechnology Building, Ithaca, NY 14853, USA

Anthony C. Fiumera[#], and

Biological Sciences Department, Binghamton University, Vestal Parkway East, P.O. Box 6000, Binghamton, NY 13902, USA

Mariana F. Wolfner

Department of Molecular Biology and Genetics, Cornell University, 421 Biotechnology Building, Ithaca, NY 14853, USA

[#] These authors contributed equally to this work.

Abstract

In many species, seminal fluid proteins (SFPs) affect female post-mating behavioral patterns, including sperm storage, egg laying, feeding, and remating. Yet, few studies have investigated the patterns of allocation, depletion, and replenishment of SFPs in male animals, despite the importance of these proteins to male and female reproductive success. To investigate such SFP dynamics, it is necessary to have a sensitive method for quantifying SFP levels in males and mated females. We developed such a method by adapting the enzyme-linked immunosorbent assay (ELISA) using anti-SFP antibodies. Here, we first use two *Drosophila melanogaster* SFPs (ovulin and sex peptide) to demonstrate that ELISAs provide accurate measures of SFP levels. We find that, consistent with previous data from Western blotting or immunofluorescence studies, levels of both ovulin and sex peptide decline in the mated female with time since mating, but they do so at different rates. We then use ELISAs to show that males become depleted of SFPs with repeated matings, but that previously mated males are able to transfer “virgin” levels of SFPs after 3 days of sexual inactivity. Finally, we demonstrate that ELISAs can detect SFPs from wild-caught *D. melanogaster* males and, thus, potentially can be used to track mating patterns in the wild. This method of measuring SFP dynamics can be used in a wide range of species to address questions related to male reproductive investment, female mating history, and variation in female post-mating behavioral changes.

Keywords

Reproductive investment; Ejaculate quantification; Sex peptide; Ovulin; *Drosophila melanogaster*

Introduction

Proteins transferred in the ejaculate from males to females during mating (seminal fluid proteins (SFPs)) can profoundly influence both male and female reproductive success in many species. SFP functions include (a) regulating the consistency of the ejaculate (e.g., providing structural support, making the ejaculate more or less viscous, thus affecting the movement/retention of sperm and/or physically blocking remating; Lung and Wolfner 2001; Veveris-Lowe et al. 2007); (b) facilitating sperm storage, retention, and release (Bloch Qazi and Wolfner 2003; Ignatz et al. 2007; Neubaum and Wolfner 1999; Ravi Ram and Wolfner 2007); (c) modifying female physiology and behaviors, including egg production and latency until remating (Gillott 2003; Poiani 2006; Ravi Ram and Wolfner 2007; Robertson 2005, 2007); and (d) protecting the female or her offspring from predation (e.g., Gonzalez et al. 1999). As a result, SFPs can influence both male success in sperm competition and female fecundity, fertility, lifespan, and offspring survival. Despite the importance of these proteins, little is known about the dynamics of specific SFPs in the male or in the mated female in many taxa.

Evidence from several species suggests that, like sperm (Wedell et al. 2002), SFPs are a limited resource (Andersson et al. 2004; Bissoondath and Wiklund 1996; Hihara 1981; Linklater et al. 2007; Perez-Staples et al. 2008; Radhakrishnan and Taylor 2008; Savalli and Fox 1999; Smith et al. 1990; Torres-Vila and Jennions 2005; Vahed 2007; Wigby et al. 2009). In some species, repeated matings by males reduces post-mating responses of their successive mates, suggesting that SFPs become depleted (e.g., *Drosophila melanogaster*: Hihara 1981; *Anastrepha obliqua*: Perez-Staples et al. 2008). For example, a recent study of the tephritid fly, *A. obliqua*, showed that females were more likely to remate after mating with a recently mated male than after mating with a previously unmated male (Perez-Staples et al. 2008). Results of other recent studies suggest that males tailor the SFP component of their ejaculate in response to social environment or female mating status (Bretman et al. 2009; Friberg 2006; Linklater et al. 2007; Wigby et al. 2009). Yet, depletion of SFPs in males has never been directly demonstrated because it has not previously been possible to obtain sensitive quantitative measures of SFP levels in individual animals. Here, we demonstrate that enzyme-linked immunosorbent assays (ELISAs) can provide such measures.

In contrast to the lack of knowledge of SFP dynamics in males, several aspects of SFP dynamics in mated *D. melanogaster* females have been explored. Notably, SFPs differ in their fates once they enter the female reproductive tract. For example, some SFPs move out of the reproductive tract and into circulation (Lung and Wolfner 1999; Monsma et al. 1990; Pilpel et al. 2008; Ravi Ram et al. 2005), whereas others move to specific locations within the reproductive tract such as the sperm storage organs or the base of the ovaries (Bertram et al. 1996; Heifetz et al. 2000; Ravi Ram et al. 2005). All SFPs studied to date become undetectable in the mated female within a few hours after mating (Monsma et al. 1990; Ravi Ram et al. 2005), with one exception: Sex peptide is found bound to sperm in the female's sperm storage organs for up to 4 days after mating and is gradually released over that time period (Peng et al. 2005). This difference in the "half-life" of different SFPs within the mated female suggests that SFP levels in females could be used to derive information about mating patterns of females in the wild. By measuring the amount of different SFPs in an individual female, one could potentially determine whether or not she had mated in the last few hours or in the last few days. This would be particularly useful for documenting mating patterns of species for which it is difficult to track individual mating behavior in the wild.

In this paper, we track the dynamics of two key *D. melanogaster* SFPs, ovulin (OV) and sex peptide (SP) in males and mated females using ELISAs. Ovulin increases ovulation (Heifetz

et al. 2000; Herndon and Wolfner 1995), whereas sex peptide increases both female egg production and feeding and decreases both female remating rate and lifespan (Carvalho et al. 2006; Chapman et al. 2003; Liu and Kubli 2003). Because we are using a novel method for tracking SFPs, we began by demonstrating that this method provides accurate results by showing that we find similar patterns of OV and SP depletion in mated females to those reported by other methods, in previous studies (Monsma et al. 1990; Peng et al. 2005). Having verified the accuracy of the method, we then use it to demonstrate that male *D. melanogaster* mated in rapid succession to multiple females transfer less OV and SP to each of their successive mates, but that mated males appear to fully replenish their OV and SP levels after 3 days of sexual inactivity. Finally, we demonstrate that this method can be used to track SFPs from wild-caught male *D. melanogaster* and, thus, can potentially be used to track mating patterns in the wild. The method we describe in this paper can be used to track SFP dynamics in any species in which ejaculate can be collected and an SFP-specific antibody is available. In the “Discussion” section, we present several avenues for obtaining such antibodies for a wide range of species and compare the ELISA method to previously used methods for tracking SFP dynamics.

Methods

Matings

We used virgin flies from the Canton S line of *D. melanogaster* for all lab studies. We maintained all flies at room temperature ($22\pm 1^\circ\text{C}$) and 12:12 h light/dark cycle. We separated male and female flies within 8 h of eclosion by light anesthesia with CO_2 to ensure that they were unmated and then maintained them for 4–6 days on yeast–glucose medium sprinkled with active dry yeast, before we used the flies for experiments. We conducted matings by placing one male and one female together in a glass vial (25 mm $\times$ 95 mm) with yeast–glucose medium (McGraw et al. 2007). We observed all pairs and recorded the time of the start and end of mating. We flash froze females in liquid nitrogen at several time points after the start of mating (ASM; mating duration is usually ~ 20 min) and stored them at -80°C until dissection. For the 1-, 2-, 4-, and 24-h ASM time points, we gently removed the male from the vial by aspiration immediately after the pair separated.

SFP depletion in females with time since mating

To track the change in SFP level in females with time since mating, we used ELISAs (see below) to measure the amount of SP and OV in the reproductive tracts of females flash frozen in liquid nitrogen at 25-min ($N=16$), 1-h ($N=16$), 2-h ($N=15$), 4-h ($N=15$), or 24-h ($N=11$) ASM and compared the signals to background detected in virgin females ($N=13$).

SFP depletion and replenishment in males

To track the depletion of SFPs in males, we randomly assigned males to mate with either one or three virgin females (hereafter, called “1-female” and “3-female” treatments; $N=10$ males for each treatment). We chose the 3-female treatment because a previous study had shown that males transfer up to approximately a third of their SFP stores to females in their first mating (Ravi Ram et al. 2005), suggesting that males should be depleted of SFPs after three matings. We recorded mating duration and measured the amount of SP and OV transferred to each female. After a male and his mate separated, we gently removed the female by aspiration and, for the males assigned to the 3-female treatment, added a new virgin female to the male's vial. The males remained in their original vial. Males were removed from the study if they did not mate with the new female within 2 h of their previous mating or if they did not mate their assigned number of times. Three days later, we gave the males from both treatments the opportunity to mate again with a virgin female and measured the amount of OV and SP transferred, to test for replenishment of SFPs.

Detecting SFPs from wild-caught *D. melanogaster* males

We conducted this study in a fruit orchard in Newfield, New York (Tompkins County). We gently aspirated virgin lab-reared Canton S females that were 2 to 6 days post-eclosion into individual vials (25 mm×95 mm) with yeast–glucose medium. We caught wild males using a sweep net, and then we gently aspirated them into the vials (one to three males with each female; number of males was arbitrary with respect to treatment group). We observed the flies for mating and recorded the time that mating started. We flash froze females in liquid nitrogen immediately after the mating ended ($N=20$), or at 1- ($N=16$) or 2-h ASM ($N=15$), transported them back to the lab on ice, and stored them at -80°C until we conducted ELISAs to measure OV levels. We only measured OV because the purpose of this study was to verify that SFPs could be detected from wild males and since OV is detectable for a much shorter period of time in the mated female than SP, measuring OV provides a conservative test. We used virgin Canton S females as negative controls. We used lab-reared females rather than wild-caught females to ensure that the females were previously unmated and to minimize genetically and environmentally induced variation between females.

ELISAs

We placed frozen female flies on ice and dissected them in 10% Dulbecco's phosphate buffering solution (DPBS; 14 mM NaCl; 0.2 mM KCl; 0.1 mM KH_2PO_4 ; 0.7 mM Na_2HPO_4) with protease inhibitors (PI; Roche Complete protease inhibitor cocktail tablets). For females mated to lab-reared males, we isolated the female's lower reproductive tract (including the uterus, sperm storage organs, oviducts, and parovaria). We removed non-reproductive tissues and ovaries and placed the lower reproductive tract in a microcentrifuge tube containing 20 μl of 10% DPBS with PI. We then homogenized the reproductive tract for 30 s with a pestle, after which we rinsed the pestle into the sample with 200 μl of 10% DPBS with PI (final sample volume 220 μl). We stored the homogenized samples on ice until all dissections were complete. For females mated to wild-caught males, we followed the above protocol, except that reproductive tracts were ground in 220 μl of 10% DPBS with PI, and the pestle was wiped off against the side of the tube into the extract instead of being rinsed.

We ran two replicate ELISA plates (BD Falcon™ 96-well Flat-bottom Microplates; Cat. No. 353279; BD Biosciences, San Jose, CA, USA) each for both OV and SP. We loaded 50 μl of each sample into a well of each replicate ELISA plate and incubated the plates overnight at 4°C with shaking. The following day, we blocked the wells with 100 μl of $1\times$ DPBS containing 0.05% Tween 20 and 5% nonfat milk for 1 h with shaking at room temperature. We removed the blocking solution and then incubated the wells with 50 μl of primary antibody diluted in blocking solution for 1 h with shaking at room temperature. For OV, we used affinity-purified anti-OV antibody (Monsma et al. 1990) at 1:500 antibody/blocking solution; for SP, we used antiserum generously provided by E. Kubli (Peng et al. 2005) at 1:750 antibody/blocking solution. These antibodies are specific to their respective protein; they do not cross-react with any other proteins in male or female reproductive tracts on Western blots (Monsma et al. 1990; Peng et al. 2005).

After incubation with the primary antibody, we washed the wells with 0.05% Tween 20-DPBS three times (150 μl /wash) and then incubated them with 50 μl horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody at 1:2,000 antibody/blocking solution (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h with shaking at room temperature. We then repeated the wash step and detected the presence of OV or SP through a colorimetric reaction of the HRP with 100 μl of 3,3',5,5'-tetramethylbenzidine substrate (KPL, Gaithersburg, MD, USA) and stopped the reaction within 30 min by adding 100 μl 1

M H₃PO₄. We quantified the level of OV or SP in a well by measuring optical density with a Molecular Devices kinetic microplate reader using a 450 nm filter (OD₄₅₀).

For each plate, we included standards of accessory glands (the tissue where SP and OV are synthesized and stored) from virgin Canton S males. To prepare the standards for each experiment, we homogenized the accessory glands of 30 males and then aliquoted the homogenate into equivalents of accessory glands of three males each. The aliquots were stored at −80°C until use. On each plate, the standards consisted of serial dilutions between 1/4 and 1/512 of a male equivalent. At least three points were used from each dilution series to generate the standard curve. We also included a blank well containing 50 µl of 10% DPBS on each plate. We used the blank and the standards to allow comparison of OD₄₅₀ readings between plates within an experiment. We conducted the standardization as follows. First, we subtracted the OD₄₅₀ value of the blank well from the OD₄₅₀ values of all of the other samples on the plate. For both OV and SP, the raw data from the two replicate plates measuring each SFP gave very similar relative values. For SP, the equation of the linear regression of the two replicate plates was $y=0.95x+0.01$; for OV, the equation was $y=0.97x+0.01$. The R^2 values for replicate plates of SP and OV were 0.94 and 0.88, respectively. We considered points with residuals from these two regressions greater than three standard deviations to have low repeatability, so we removed them from the analyses. We used a linear conversion to transform the OD₄₅₀ values of one of the replicate plates so that the slope of the regression of the two replicate plates was one and that the y-intercept was zero. We averaged the OD₄₅₀ values of the two replicate plates and standardized them across plates within an experiment using the male accessory gland standards. We used Grubb's test to identify outliers. We used non-parametric tests with α of 0.05 for all analyses and sequential Bonferroni corrections for all post hoc comparisons.

Results

SFP depletion in mated females

Both SP and OV levels in the female reproductive tract decreased with time since mating, but they did so at different rates (Fig. 1a). OV decreased rapidly immediately after mating (Kruskal–Wallis: $X_5^2=42.4$; $P<0.0001$; $N=11-16$ for all groups), with a 10-fold drop between the 25-min and 1-h time points. OV levels in the reproductive tracts of mated females were significantly greater than background levels in virgin females up to 1 h after the start of mating but became indistinguishable from background levels thereafter (Mann–Whitney U : 25 min— $Z=-4.3$, $P<0.0001$; 1 h— $Z=-2.6$, $P=0.01$; 2, 4, 24 h— $Z=-0.7$ to -0.1 , $P>0.4$ for all tests). SP levels decreased more slowly and were more variable (Fig. 1b; Kruskal–Wallis: $X_5^2=57.5$; $P<0.0001$; $N=11-16$ for all groups). This greater variability may result from variation in the amount of SP in different ejaculates and/or from the fact that, within the mated female, SP targets to the sperm storage organs of mated females, some of which are sclerotized, and our grinding technique may not fully homogenize these structures. SP levels in reproductive tracts of mated females were significantly greater than background levels in virgin females at all time points tested (Mann–Whitney U : 25 min— $Z=-4.5$, $P<0.0001$; 1 h— $Z=-4.3$, $P<0.0001$; 2 h— $Z=-3.2$, $P=0.001$; 4 h— $Z=-2.3$, $P=0.02$; 24 h— $Z=2.9$, $P=0.003$). These patterns of OV and SP depletion in mated females are generally consistent with those documented previously using Western blots (Monsma et al. 1990) and immunofluorescence microscopy (Peng et al. 2005), respectively, thus validating the use of ELISAs for the study of SFP dynamics.

SFP depletion and replenishment in males

The amount of both SP and OV transferred decreased with each of three successive mating by a male on the same day (Fig. 2; Friedman test: SP— $X_2^2=16$, $P=0.0003$; OV— $X_2^2=11.1$,

$P=0.004$); this decrease was statistically significant for each additional mating except for the third mating for OV (Wilcoxon paired test, one-tailed, first vs. second: SP— $Z=-18$, $P=0.004$; OV— $Z=-14$, $P=0.008$; second vs. third: SP— $Z=-22.5$, $P=0.002$; OV— $Z=-11$, $P=0.07$; $N=7-9$ for all groups). The amount of both SP and OV transferred to females was significantly above background levels in virgin females irrespective of mating order (Mann–Whitney U , one-tailed, first: SP— $Z=3.8$, $P=0.0001$; OV— $Z=3.8$, $P=0.0001$; second: SP— $Z=2.9$, $P=0.004$; OV— $Z=3.7$, $P=0.0002$; third: SP— $Z=3.8$, $P=0.0001$; OV— $Z=3.7$, $P=0.0001$; $N=9-12$ for all groups). Mating duration decreased, though not significantly, with each successive mating (Friedman test: $X_2^2=0.6$, $P=0.74$; data not shown). Males from both treatment groups were able to replenish their SFPs: We found no difference between the amount of SFPs transferred in the first mating and the amount transferred in the mating after 3 days of sexual inactivity (Fig. 2; Wilcoxon paired test, 1-female: SP— $Z=-7.5$, $P=0.5$; OV— $Z=-0.5$; $P=1.0$; 3-female: SP— $Z=1.0$, $P=0.9$; OV— $Z=-12$; $P=0.1$; $N=10$ for all groups).

Detecting SFPs from wild-caught *D. melanogaster* males

OV levels of Canton S lab-reared females mated to wild-caught males differed significantly from the background in virgin females at all three time points tested (Mann–Whitney U : immediately after mating— $Z=-4.6$, $P<0.0001$; 1 h— $Z=-3.9$, $P<0.0001$; 2 h— $Z=-3.2$, $P=0.002$; $N=15-20$ for all groups). We also found a significant negative relationship between OV level and time since mating (Fig. 3; Kruskal–Wallis: $X_2^2=9.8$; $P=0.007$). The decline in OV levels with time was slower and more variable than that found in females mated to laboratory stock males (Fig. 1a vs. Fig. 3), which could be due to one or more differences in experimental conditions (since the matings to wild-caught males were conducted in the field) or to biological differences in kinetics of OV from wild-caught and lab-reared males. In order to be of use for tracking mating patterns in the wild, one would need to be able to use the OV level to distinguish between recently mated females and those that had not mated recently. In this study, the OV levels of 85% of the mated females (all time points combined) were greater than the 95% confidence interval for OV levels of virgin females.

Discussion

In this study, we developed a new approach for tracking SFP dynamics by adapting the ELISA technique using anti-SFP antibodies. We demonstrated here the reliability of the ELISA results by their consistency with patterns found in previous studies using Western blots, immunofluorescence microscopy, and mRNA quantitation (Monsma et al. 1990; Herndon et al. 1997; Peng et al. 2005). Consistent with results of the previous studies that used these other methods, we found that both OV and SP levels decrease in females with time since mating, but that this decrease is more rapid for OV than for SP. Through the use of ELISAs, we also documented two new patterns of SFP dynamics in *D. melanogaster*. First, we found that the amount of both OV and SP transferred decreased with each successive mating of a male, as predicted from studies of *D. melanogaster* and other insects showing lower magnitude of post-mating changes in females mated to recently mated males than in those mated to virgin males (Hihara 1981; Smith et al. 1990; Savalli and Fox 1999; Vahed 2007; Linklater et al. 2007; Perez-Staples et al. 2008). We then showed that mated males transfer “virgin” levels of both OV and SP after 3 days of sexual inactivity. Finally, we demonstrated that, using the ELISA method, we can detect OV in females mated to wild males and that the patterns of OV depletion in females with time since mating with wild males is similar to those seen for matings with laboratory stock males.

Previous studies have found results consistent with SFP depletion by multiple mating in male *D. melanogaster*. For example, females mated to previously unmated males laid more

eggs after mating than females mated to males that had recently mated (Hihara 1981; Linklater et al. 2007). Although SFP depletion was suggested to account for these patterns, depletion of specific SFPs involved in inducing egg-laying in females had not been demonstrated before our study. Our results showed that males allocated sequentially less SP and OV to each successive mating during the course of a day. Yet, males transferred detectable amounts of both proteins even during their third consecutive mating. Extending the findings from previous research, our study suggests that male *D. melanogaster* do not transfer their full load of SFPs to their first mates and that, as a result, they are able to transfer some, albeit less, SFPs to other females on the same day. The depletion patterns we observed for *D. melanogaster* SFPs are consistent with those found for total ejaculate quantity of other *Drosophila* species (Pitnick and Markow 1994). This pattern of allocation may be an evolved strategy that maximizes male reproductive success when faced with uncertainty about future mating prospects. One would predict that male allocation strategies should change in response to males' perception of both their own future mating prospects and those of their mates. Indeed, a recent study demonstrated that the allocation of SFPs by male *D. melanogaster* varies with potential sperm competition levels (Wigby et al. 2009). Furthermore, there is evidence suggesting that patterns of SFP allocation to successive matings evolve in response to variation in operational sex ratio (Linklater et al. 2007). Future studies of SFP dynamics in *D. melanogaster* and other species will reveal how generalizable findings of plastic and evolutionary changes in SFP allocation may be.

SFP allocation strategies will depend, in part, on the ability of a male to replenish his SFP supplies and the rate at which he is able to do so. Previous studies of *D. melanogaster* demonstrated that mating induces transcription and translation of SFP genes (Bertram et al. 1992; DiBenedetto et al. 1990; Herndon et al. 1997; Monsma et al. 1990; Schmidt et al. 1985; Simmerl et al. 1995; Styger 1992), yet ours is among the first to demonstrate that males appear to fully replenish their SFP levels within 3 days of sexual inactivity (see also Coleman et al. 1995). SFP replenishment also appears to occur in other Diptera: Male stalk-eyed flies (*Cyrtodiopsis dalmanni*) and Queensland fruit flies (*Bactrocera tryoni*) restore their accessory glands (the main site of SFP synthesis) to virgin size within ~24 h after mating (Rogers et al. 2005; Radhakrishnan and Taylor 2008). Interestingly, SFP replenishment does not appear to occur in all Diptera. For example, in the Mediterranean fruit fly *Ceratitis capitata*, females produce more offspring after mating to an unmated male than to a mated male—even after the latter has experienced 5 days of sexual inactivity—a pattern that suggests his inability to replenish SFPs and/or sperm (Whittier and Kaneshiro 1991). Patterns of depletion and replenishment of ejaculate components may both shape and be shaped by mating patterns within a species and explain some of the tremendous variation in mating patterns across species (Pitnick and Markow 1994; Svärd and Wiklund 1986, 1989).

Direct observations of mating patterns under natural conditions can prove quite challenging for some species such as *D. melanogaster*. Therefore, tools that aid in determining the timing and frequency of matings provide valuable insights. A variety of molecular techniques have been developed to determine the minimum number of males a female has mated with, based on the genotypes of her offspring or the sperm in her sperm storage organs (e.g., Harshman and Clark 1998; Tripet et al. 2003). SFP levels provide another potential tool for documenting the mating patterns of insects in the wild in that they may be able to provide information about time since mating in females. For example, our results and those of previous studies show that OV and SP decay at different rates in mated females—with OV becoming undetectable within a few hours of mating (our study and Monsma et al. 1990) and SP being detectable for up to 24 h (ELISAs) or even several days (immunofluorescence microscopy; Peng et al. 2005). Furthermore, we demonstrated that at least one SFP (OV) from wild-caught male *D. melanogaster* follows the same general

pattern of detectability in mated females as that found in females mated to lab-reared males. Measuring the levels of SP and OV in a wild-caught female, which our results suggest is possible, provides the possibility to determine whether the female had mated within the last few hours (if OV and SP were detectable), within the last day but not within the last few hours (if only SP was detectable), or not within the last day (if neither OV nor SP were detectable). By measuring these SFPs in many females over the course of a day or a season, one could determine population-level mating patterns.

ELISAs are an excellent technique for studying the dynamics of specific SFPs for several reasons: (1) They provide a quick and sensitive quantification of SFP levels, (2) an SFP's levels in 96 samples can be quantified simultaneously on a single plate, and (3) levels for different SFPs and/or replicate measures for the same SFP can be determined from a single ejaculate. Although relative SFP quantity can also be estimated using Western blots (Lung and Wolfner 1999), the measurements collected by ELISAs require less protein and antibody and provide more quantitative results. For Western blots of *D. melanogaster* SFPs such as OV and SP, reproductive tracts from two females are needed to produce a clear band that can be accurately and repeatedly quantified using standard techniques (Ravi Ram et al. 2005). In contrast, using ELISAs, we can quantify OV and SP levels accurately and repeatedly using the equivalent of 1/4 of a female reproductive tract. Furthermore, SFP levels in up to 96 samples can be quantified simultaneously in ELISAs by using a plate reader, whereas SFP levels of each Western sample must be quantified individually. ELISAs also provide a more accurate measure of SFP levels in males than RNA quantification (Herndon et al. 1997) because the RNA levels for genes do not always predict protein levels (Griffin et al. 2002). Furthermore, RNA quantification cannot be used to measure the amounts of SFPs transferred to females.

One disadvantage of ELISAs relative to Western blots is that ELISAs require very specific antibodies that do not cross-react with any spurious proteins. In contrast, Western blots can be interpreted even if the antibodies also recognize other proteins, as long as the molecular weights of those other proteins do not overlap with that of the protein of interest. Unlike ELISAs, Western blots can also provide information about the rate and extent of processing of SFPs, which may be important for their activation and/or degradation (Ravi Ram et al. 2006). Immunofluorescence microscopy can provide information about the exact localization of a protein (Peng et al. 2005), but it is less quantitative than ELISAs. ELISAs also may be less sensitive to detecting proteins that locate to the sclerotized sperm storage organs (such as SP) than immunofluorescence microscopy because these organs are difficult to completely homogenize during sample preparation.

The utility of ELISAs for studying SFP dynamics is not restricted to model organisms such as *D. melanogaster*. ELISAs provide a powerful tool to track SFP dynamics in any species in which seminal fluids, spermatophores, and/or ejaculates can be collected, and a specific antibody can be raised to at least one SFP. The limiting step for using this technique in most species will be obtaining a specific antibody for one or more SFP. There are many avenues to obtain such antibodies. For some species, such antibodies are already available (e.g., carp: Wojtczak et al. 2007; bulls: Ignatz et al. 2007; humans: Cattini et al. 1994; and through commercial companies). Since antibodies generated against proteins of one species sometimes recognize similar proteins from another species (e.g., Carter et al. 1985), researchers studying SFPs in species that are closely related to these aforementioned species may be able to use the already existing antibodies.

In other species, antibodies to SFPs have not yet been developed but specific SFPs (and, for some, their associated functions) are known. For example, suites of SFPs have been identified in mosquitoes (Dottorini et al. 2007; Sirot et al. 2008), honeybees (Collins et al.

2006), Mediterranean fruit flies (Davies and Chapman 2006), crickets (Braswell et al. 2006), butterflies (Walters and Harrison 2008), bulls (Moura et al. 2007), and human and non-human primates (Clark and Swanson 2005; Pilch and Mann 2006), and individual SFPs are known for many more species (Gillott 2003; Poiani 2006; Robertson 2005). With the increased use of genomics and proteomics, it is likely that partial or complete seminal fluid proteomes of many more species will become available in the near future. Once these proteins are identified in a particular species, antibodies can be raised to them by commercial companies (e.g., as noted in Ravi Ram et al. 2006; Rheault et al. 2007) and used in ELISAs to study SFP dynamics. Together with studies of the dynamics of other components of the ejaculate (Wedell et al. 2002), studies of SFP dynamics will provide a more complete understanding of the intriguing intricacies of influences on male reproductive investment and female behavioral responses to mating.

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Fig. 1. Seminal fluid protein level (mean±SE) in mated females in relation to time after the start of mating for **a** ovulin (*OV*); **b** sex peptide (*SP*). $N_{25 \text{ min}}=16$ females; $N_{1 \text{ h}}=16$; $N_{2 \text{ h}}=15$; $N_{4 \text{ h}}=16$; $N_{24 \text{ h}}=11$; $N_{\text{unmated}}=13$. Relative units were based on a standard curve consisting of serial dilutions of an extract of accessory glands of Canton S males

Fig. 2.

Seminal fluid protein level (mean±SE) in mated females as a function of mating order with a particular male (day1) and in females mated to males with 3 days of sexual inactivity (day4). On day4, the data are divided into males that had mated with one or three females on day1. **a** Ovulin (OV); **b** sex peptide (SP). Each bar represents data from nine to 12 females. Relative units were based on a standard curve consisting of serial dilutions of an extract of accessory glands of Canton S males

Fig. 3.

Ovulin level (mean±SE) of Canton S females mated to wild-caught males immediately after mating ended (“immediate”) at 1 and 2 h after the start of mating and in unmated females (negative control). $N_{\text{immed}}=20$ females; $N_{1\text{ h}}=16$; $N_{2\text{ h}}=15$; $N_{\text{unmated}}=15$. Relative units were based on a standard curve consisting of serial dilutions of an extract of accessory glands of Canton S males