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An Ectopic Expression Screen Reveals the Protective and Toxic Effects of Drosophila Seminal Fluid Proteins

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ABSTRACT

In *Drosophila melanogaster*, seminal fluid regulates the reproductive and immune responses of mated females. Some seminal fluid proteins may provide protective functions to mated females, such as antimicrobial activity and/or stimulation of antimicrobial gene expression levels, while others appear to have negative effects, contributing to a "cost of mating." To identify seminal proteins that could participate in these phenomena, we used a systemic ectopic expression screen to test the effects on unmated females of proteins normally produced by the male accessory gland (Acps). Of the 21 ectopically expressed Acps that we tested for ability to assist in the clearance of a bacterial infection with *Serratia marcescens*, 3 Acps significantly reduced the bacterial counts of infected females, suggesting a protective role. Of the 23 Acps that we tested for toxicity, 3 were toxic, including one that has been implicated in the cost of mating in another study. We also tested ectopic expression females for other Acp-induced effects, but found no additional Acps that affected egg laying or receptivity upon ectopic expression.

DURING mating in *Drosophila melanogaster*, seminal fluid proteins transferred to the female modulate her behavior, physiology, and reproduction. Approximately 70–106 seminal fluid *ac*cessory gland *p*roteins (Acps) are produced in the male's accessory gland. Acps increase the egg-laying rate of mated females, decrease their receptivity to remating, induce them to store sperm, induce them to eat (Carvalho *et al.* 2006), and decrease their life span (reviewed in Kubli 2003; Chapman and Davies 2004; Wolfner *et al.* 2005). Acps also have the potential to provide immune-related functions to females: Acps upregulate antimicrobial gene transcript levels in mated females (McGraw *et al.* 2004; Peng *et al.* 2005), and at least three Acps have antimicrobial activity *in vitro* (Lung *et al.* 2001). Although these findings suggest that Acps might perform a protective role in improving a mated female's ability to clear an infection, the ability of Acps to affect a female's immune capacity has yet to be assessed directly *in vivo*.

The female reproductive tract is constantly in contact with the external environment, making it potentially susceptible to microbial infection. Transfer of protective Acps to females could lower a female's chance of infection, thus increasing her and her mate's reproductive success. Two extracellular protein classes play decisive roles in the innate immunity of mammals and insects: proteolysis regulators and antimicrobial peptides (reviewed in Leclerc and Reichhart 2004). For example, a *D. melanogaster* serine protease, *spätzle-processing enzyme* (Jang *et al.* 2006), and a protease inhibitor, *necrotic* (Levashina *et al.* 1999), regulate the proteolytic processing of a critical immunity protein, *spätzle*. Activation of *spätzle*, via its processing in the hemolymph, triggers the rapid upregulation of the expression of antifungal peptide genes (Lemaitre *et al.* 1996). Of the 63 known *D. melanogaster* Acps (Holloway and Begun 2004; Mueller *et al.* 2005; Walker *et al.* 2006), 13 are predicted or known proteolysis regulators and 18 are peptides. Some have sequence/structural similarities with immunity proteolysis regulators and peptides (Swanson *et al.* 2001; Lung *et al.* 2002; Mueller *et al.* 2004).

Ectopic expression of antimicrobial peptides alone can combat a pathogenic infection (Tzou *et al.* 2002). We examined the immunity-promoting properties of 21 individual Acps (peptides and proteolysis regulators) for their ability to clear an infection of *Serratia marcescens* in females. We found that ectopic expression of three Acps with predicted proteolysis-regulating functions can assist in *S. marcescens* clearance.

Despite such potential beneficial effects of Acps on females, Acps have also been shown to have negative consequences, in particular by decreasing female viability postmating (Chapman *et al.* 1995; Wigby and Chapman 2005). As the ultimate outcome of mating is likely to result from a balance of "positive" and "negative" aspects, and since one Acp protease inhibitor is already known to be toxic (Lung *et al.* 2002), we tested whether any additional Acps are toxic. We found three

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additional toxic Acps, including an additional proteolysis regulator, and a peptide that was implicated among contributors to the cost of mating using a mutational assay (Wigby and Chapman 2005).

MATERIALS AND METHODS

Cloning of Acp genes into a UAS *P*-element vector: We utilized the original pUAST vector [containing UAS sites, inverted *P*-element repeats, and the marker mini-*white*$^+$ (w^+)] (Brand and Perrimon 1993) to construct a Gateway (Invitrogen, San Diego) compatible (pUASTGW) plasmid by inserting the gateway cassette into the pUAST vector's multiple cloning site. The full-length coding sequences, including signal sequences, of Acp53Ea, Acp76A, CG4847, CG4986, CG5016, CG6069, CG6168, CG6289, CG6555, CG8137, CG9029, CG9074, CG9334, CG9997, CG10284, CG10433, CG10956, CG11664, CG11864, CG13309, CG14560, 1a8, and 4h1, were moved from Gateway entry clones (Mueller *et al.* 2005) to the *P*-element pUASTGW vector using the Gateway cloning technology (Invitrogen) to generate UAS-Acp transgenic fly lines.

Fly stocks: Each of the 23 pUASTGW-Acp *P*-element vectors were transformed into w^{1118} embryos using standard germline transformation protocols (Rubin and Spradling 1982). Transgenic lines were identified by using the mini-w^+ eye-color marker. Homozygous lines were established via standard crosses. The X chromosome *vs.* autosome locations of the UAS-Acp transgenes (supplemental Table 1 at http://www.genetics.org/supplemental/) were determined by segregation patterns.

UAS-Acp homozygous males were crossed to *tubulin*-GAL4/*TM3, Sb* females (Lee and Luo 1999) to drive ubiquitous expression in UAS-Acp; *tubulin*-GAL4 progeny. Because *tubulin*-GAL4-driven expression of Acp70A, CG8137, and CG10433 was lethal to preadults, we used the *hsp70*-GAL4 transgene (Brand and Perrimon 1993) to drive expression of these Acps via heat shock. Heat-shocked flies carrying both the *hsp70*-GAL4 and a UAS-Acp transgene should express the given Acp at high levels in almost all cells 24 hr after heat-shock induction (Lis *et al.* 1983).

Although 23 UAS-Acps were cloned and available for ectopic expression analyses, the number of UAS-Acps examined in each assay differs due to issues of toxicity and previous analysis of UAS-Acp lines (Lung *et al.* 2002). Twenty-one instead of 23 Acps were tested for immunity because CG8137 and CG10433 are toxic when ectopically expressed with the *tubulin*-GAL4 driver. Twenty-two instead of 23 Acps were tested for receptivity to mating because Acp53Ea had been previously tested (Kalb 1994; Lung *et al.* 2002). CG8137 and CG10433 were driven via the *hsp70*-GAL4 transgene for the receptivity assays. Twenty lines were tested for egg laying because Acp53Ea had been previously tested. Because of their toxicity when driven by *tubulin*-GAL4, CG8137 and CG10433 were not assayed because heat shock, by itself, can sometimes induce egg laying in virgin females (Y. Heifetz and M. F. Wolfner, unpublished data). Twenty-two lines were assayed for toxicity because Acp53Ea had been previously tested (Kalb 1994; Lung *et al.* 2002).

Western blot and RT–PCR analysis to confirm transgene expression: To ensure that the UAS-Acp lines being assayed indeed produce the given Acp, Western blot analysis was performed on lines for Acps for which purified antibodies were available. Extracts of whole adult male or virgin females of the genotypes UAS-Acp; *TM3, Sb* or UAS-Acp; *tubulin*-GAL4 were made as in Monsma and Wolfner (1988), and one fly equivalent of protein was loaded per lane on a 15% polyacrylamide gel. Blots were made using standard Western blot techniques (Park and Wolfner 1995). For example, affinity-purified antibodies against CG8137 (Ravi Ram *et al.* 2005) were used as primary antibodies at a 1:1000 dilution and goat anti-rabbit secondary with conjugated horseradish peroxidase (Amersham, Buckinghamshire, UK) was used at a dilution of 1:2000. Secondary antibody was visualized using enhanced chemiluminescence (Amersham) and exposure to X-ray film.

Ectopic expression was also confirmed for all 23 UAS-Acps using RT–PCR. RT–PCR of full coding regions was performed from RNA isolated from whole flies. Approximately 20 flies were homogenized in Trizol according to the manufacturer's instructions (Invitrogen), and total RNA was prepared for RT–PCR as in Carninci and Hayashizaki (1999). Full-length coding regions were amplified using primers described in Mueller *et al.* (2005), and their amounts were determined after electrophoresis on a 1% agarose gel containing ethidium bromide and after visualization under UV fluorescence.

Immune activity: To determine if ectopic expression of individual Acps affected female immune response, virgin UAS-Acp; *TM3, Sb* or UAS-Acp; *tubulin*-GAL4 females were collected and aged 3–5 days. By minor modifications of procedures by Lazzaro *et al.* (2004), we measured the clearance of gram-negative *S. marcescens* bacteria introduced into virgin females. Minutin pins, cleaned and sterilized with 95% ethanol and thoroughly dried, were dipped into *S. marcescens* cultures of an optical density of $A_{600} = 1.0$ (± 0.1). Individual CO_2-anesthetized virgin females were pricked in the thorax. All flies were returned to vials and 24 hr later were transferred to Eppendorf tubes (two flies per tube), homogenized in 500 μl of LB, and spiral plated onto LB plates. LB plates were incubated at 37° and colonies were counted using a Qcount (version 1.4) machine (Spiral Biotech, Bethesda, MD) the following day. In total, 1304 experimental plates were counted, encompassing 2608 infected female flies. To ensure that *S. marcescens* infections were robust, *relish*^{E20} homozygote (null for *relish*) females were used as a control. *Relish* is required to mount an immune response to gram-negative bacteria (Dushay *et al.* 1996). Thus, infected *relish*^{E20} females should have significantly more *S. marcescens* bacteria per fly than infected control females (Canton-S and/or UAS-Acp; *TM3, Sb*).

Preadult toxicity: Previous work has shown that ectopic expression of Acp62F in *hsp70*-GAL4; UAS-Acp62F animals is toxic at all stages of preadult development (Lung *et al.* 2002). The *tubulin*-GAL4 driver will also drive Acp expression during preadult development and can therefore also be used to screen preadults for toxic Acps. Four replicate crosses of four to five UAS-Acp/UAS-Acp males with three to four *tubulin*-GAL4/*TM3, Sb* virgin females were set up at 25° in yeast–glucose media vials. The proportion of eclosed adults with each genotype (UAS-Acp; *TM3, Sb vs.* UAS-Acp; *tubulin*-GAL4) was tabulated. Lines ectopically expressing Acp62F were used as a positive control for toxicity upon ectopic expression. Flies ectopically expressing Acp29AB, which is not toxic, were used as a negative control (Lung *et al.* 2002). The siblings of the genotype UAS-Acp; *TM3, Sb* were used as an internal negative control for toxicity of expression of a given UAS-Acp. Acps that showed significantly fewer UAS-Acp; *tubulin*-GAL4 progeny relative to UAS-Acp29AB; *tubulin*-GAL4 progeny were considered to be toxic to preadults.

Rates of receptivity and egg laying: To determine the effects of ectopic expression of an Acp on receptivity to remating, ~20 UAS-Acp; *tubulin*-GAL4 virgin females for each Acp line were assayed. The 3- to 5-day-old (peak of sexual maturity) UAS-Acp; *tubulin*-GAL4 virgin females were placed individually into vials with glucose–yeast media. A virgin 3- to 5-day-old Canton-S male was added and the pair was watched for 1 hr to

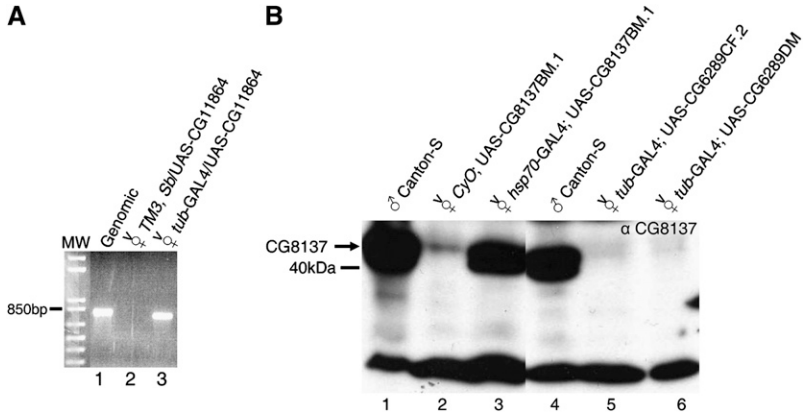

FIGURE 1.—Ectopic expression of UAS-Acps. (A) RT–PCR of ectopic CG11864 in virgin females. CG11864 RT–PCR product, which ran at ~750 bp (lane 3) near the expected size (756 bp), can be detected only in UAS-CG11864; *tubulin*-GAL4 and not in UAS-CG11864; *TM3, Sb* females. As CG11864 contains a 53-bp intron, its RT–PCR product is smaller than its genomic product (lane 1), verifying that the PCR product was only from cDNA. See supplemental Table 1 at http://www.genetics.org/supplemental/ for RT–PCR results for the remaining UAS-Acp lines. (B) Protein expression of CG8137 via ectopic expression. CG8137 protein expression (arrow) can be detected in heat-shocked female UAS-CG8137BM.1; *hsp70*-GAL4 flies (lane 3) at levels comparable to that found in males (lanes 1 and 4). A faint cross-reactive band slightly larger than CG8137 is visible in the control females (heat-shocked UAS-CG8137BM.1/*CyO*, UAS-CG6289CF.2, and UAS-CG6289DM (lanes 2, 5, and 6, respectively). The smaller cross-reactive bands serve as loading controls (RAVI RAM *et al.* 2005). Results of Western blot analysis for the remaining seven Acps for which antibodies are available can be found in supplemental Table 1 at http://www.genetics.org/supplemental/.

see if mating occurred. For the two Acps, CG8137 and CG10433, which are lethal when driven under the *tubulin*-GAL4 driver, experiments using the *hsp70*-GAL4 driver were conducted as described in HEIFETZ *et al.* (2005). Previous experiments indicated that 1 day after heat shock gives the highest level of Acp accumulation following heat shock (PARK and WOLFNER 1995). After recovery for 1 day, virgin wild-type 3- to 5-day-old Canton-S males were added and the vials were watched for 1 hr to determine if mating occurred. Canton-S females that had mated the previous day to Canton-S males and Canton-S virgin females were used as positive and negative controls, respectively.

To identify the effect of individual Acp's ectopic expression on egg-laying rate, eight *tubulin*-GAL4; UAS-Acp virgin females were collected for each line within 8 hr of eclosion, aged for 1 day in fresh vials (yeast is *ad lib*), and transferred to individual vials. The following day, the 1-day-old virgin flies were placed individually into fresh vials for 24 hr and then transferred again to fresh vials. Egg counts for the 2-day period were performed 24 hr after each transfer. Each vial was checked for first instar larvae to confirm virginity.

Virgin Canton-S females were used as a negative control for the egg-laying and receptivity experiments. Mated females were used as a positive control for the receptivity and egg-laying experiments.

Statistical analysis: All colony counts were natural log transformed and all UAS-Acp; *tubulin*-GAL4 progeny proportions were arcsine-root transformed. ANOVAs and *t*-tests (Statview version 5.0.1) were performed to compare the average number of colonies (immune activity assay) and average proportion of UAS-Acp; *tubulin*-GAL4 (toxicity assay), respectively. A Bonferroni correction for multiple tests was performed for the preadult toxicity and immune activity assays. Fisher exact tests and *t*-tests were performed to determine the significance of each UAS-Acp's effect on receptivity to mating and egg laying, respectively.

RESULTS AND DISCUSSION

Ectopically expressed Acp proteins and transcripts are detectable in virgin females: To ensure that UAS-Acp transgenes express the Acps of interest, transcript and protein levels in UAS-Acp; *tubulin*-GAL4 and control UAS-Acp; *TM3, Sb* (or in UAS-Acp; *hsp70*-GAL4 and control UAS-Acp; *CyO* females) virgin females were analyzed by RT–PCR and/or Western blot analysis. The expected RT–PCR and/or protein products were detected in all virgin females carrying both UAS-Acp and *tubulin*-GAL4 transgenes (Figure 1; supplemental Table 1 at http://www.genetics.org/supplemental/).

Females expressing CG6168, CG9334, or CG10284 resisted *S. marcescens* infection better than control females or experimental females that ectopically expressed other Acps: Virgin females of 21 independent ectopically expressing Acp lines (UAS-Acp; *tubulin*-GAL4) were compared to sibling controls (UAS-Acp; *TM3, Sb*) for their ability to clear a bacterial infection. Females ectopically expressing CG6168, CG9334, or CG10284 cleared *S. marcescens* significantly better than their sibling control females (Figure 2; supplemental Table 2 at http://www.genetics.org/supplemental/). The natural log mean number of bacteria per fly expressing CG6168, CG9334, and CG10284 were 7.50 (SE ± 0.314, $N = 39$), 7.37 (SE ± 0.277, $N = 42$), and 7.17 (SE ± 0.302, $N = 39$), respectively (supplemental Table 2 at http://www.genetics.org/supplemental/). These values are significantly lower (ANOVA, $P < 0.0001$; Bonferroni correction at the 5% level is 0.0024) than the mean of control females not expressing any Acps (UAS-Acp; *TM3, Sb,* mean = 8.80; supplemental Table 2 at http://www.genetics.org/supplemental/). On the other end of the distribution, females expressing CG5016 appear more susceptible to *S. marcescens* infection, although this is not significant after a Bonferroni correction.

To ensure that the *S. marcescens* clearance results were not due to the UAS-Acp transgene insertion or to sampling errors, a second set of independent UAS-Acp lines was tested for five Acps: CG6168 and CG10284, which cleared *S. marcescens* significantly better than non-UAS-Acp expressing females, and CG5016, CG9029, and CG11864, which did not (supplemental Table 2 at http://www.genetics.org/supplemental/).

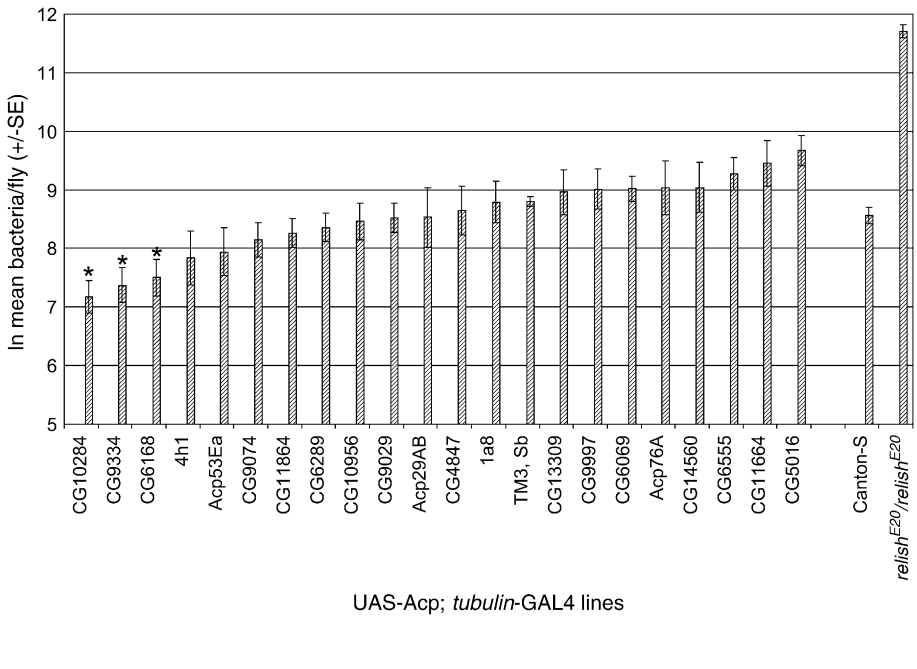

FIGURE 2.—Females ectopically expressing certain Acps can suppress *S. marcescens* growth. Three Acps (CG6168, CG9334, CG10284), when driven under the *tubulin*-GAL4 promoter, can clear *S. marcescens* bacteria significantly better (ANOVA, $P < 0.0001$, indicated by an asterisk) than controls (*TM3, Sb*). Homozygous *relish*^{E20} female flies have significantly higher bacterial counts per fly than infected virgin Canton-S females (supplemental Table 2 at http://www.genetics.org/supplemental/), indicating that *S. marcescens* can induce an immune response and is capable of rapid growth in the hemolymph. Additionally, virgin females carrying the *TM3, Sb* balancer could clear an infection similar to Canton-S virgin females, indicating that the *TM3, Sb* balancer does not significantly affect the immune response of UAS-Acp; *TM3, Sb* females (supplemental Table 2 at http://www.genetics.org/supplemental/). SE, standard error.

For each of the five Acps, its two independent lines did not differ significantly in bacterial clearance ability (data not shown). This indicated that the initial assay is robust and that the ability of CG6168 and CG10284 to clear an infection is not line specific or due to differences in sample sizes or sampling errors.

The phenotypes that we observed for ectopic expression of the predicted protease inhibitors CG9334 (serpin) and CG10284 (Kunitz protease inhibitor; KRESS *et al.* 2004; as well as a cysteine-rich secretory protein; MUELLER *et al.* 2004) and a predicted protease, CG6168, suggest that Acp proteolysis regulators play important functions in the immunity of mated females. Two previous studies of *D. melanogaster* immune genes support our results. IRVING *et al.* (2001) found that CG9334 and CG10284 are transcriptionally upregulated upon gram-negative infection, supporting the hypothesis that these proteins might participate in immune defense, either directly via antimicrobial functions or indirectly via activation of immune cascades. KAMBRIS *et al.* (2006) performed an *in vivo* RNA interference screen for serine proteases with immune functions. Both CG9997 and CG11664 were negative for immune activity to gram-negative bacteria in the KAMBRIS *et al.* (2006) assay, as they were for *S. marcescens* clearance in our screen, suggesting that they do not have immune functions.

One Acp has previously been tested for an interaction with the immune system. This molecule (Acp70A, the sex peptide, reviewed in KUBLI 2003) stimulates antimicrobial peptide RNA levels in mated females (PENG *et al.* 2005); it is not known whether it causes a consequent improvement of the female's ability to fight infection. Acp70A's entry into the mated female's hemolymph has been suggested as permitting its upregulation of antimicrobial gene expression. CG9334, one of the three proteolysis regulators identified here as assisting in immune defense, also enters the mated female's hemolymph (RAVI RAM *et al.* 2005), suggesting that CG9334 might exert its immunoprotective effects by participating in Acp-mediated upregulation of immune defense genes (MCGRAW *et al.* 2004). Antibodies are not available with which to test whether CG6168 and CG10284 can also enter the hemolymph.

The effects of Acps on bacterial clearance have a continuous distribution with a negative skew, which suggests that other Acps (*e.g.*, 4h1 and Acp53Ea, significant at the nominal 5% level) might also affect bacterial clearance ability. Support of other Acps playing roles in mated-female immunity comes from the finding that Acp76A, CG6069, and CG8137 have been discovered in genomewide microarray screens for genes upregulated postinfection (DE GREGORIO *et al.* 2001; IRVING *et al.* 2001).

Acp70A, CG8137, and CG10433 are toxic when ectopically expressed in preadults: In prior phenotypic tests on eight Acps, one (Acp62F) was found to be toxic to Drosophila upon ectopic expression (LUNG *et al.* 2002). This suggested that toxicity upon ectopic expression could potentially provide a bioassay for categorizing Acps and perhaps a basis for assays to determine their molecular targets, even though the levels and the spatiotemporal parameters of ectopic Acp expression differ from those for Acps encountered by mated females. We therefore tested for toxicity 22 new Acps plus Acp70A, which had not been tested for this phenotype. We determined whether each UAS-Acp line produced the expected 1:1 ratio of experimental UAS-Acp; *tubulin*-GAL4 to control UAS-Acp; *TM3, Sb* progeny. Three of

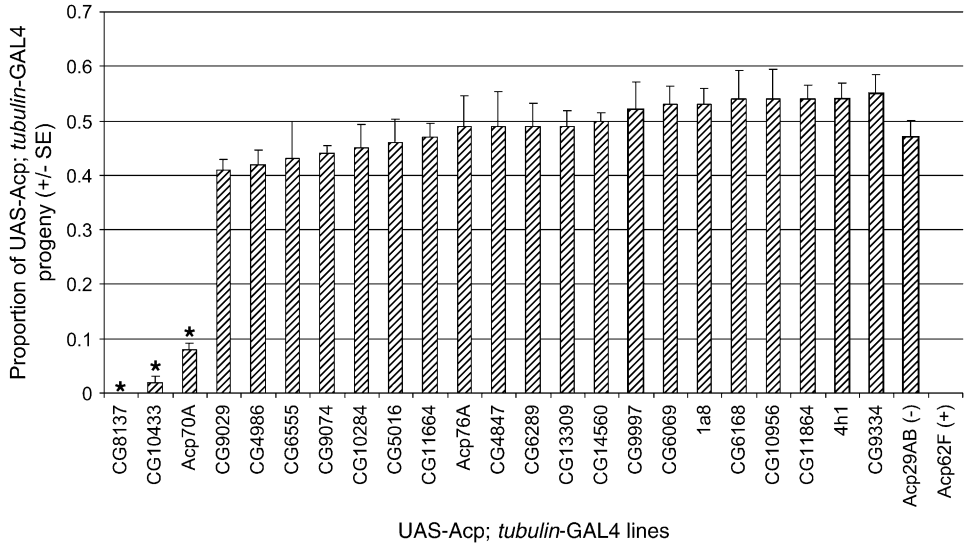

FIGURE 3.—Toxicity of 22 individual Acps when constitutively expressed in preadults. Percentage of total UAS-Acp; *tubulin*-GAL4 adult progeny that emerged from UAS-Acp/UAS-Acp males crossed to *tubulin*-GAL4/*TM3, Sb* females. Acp62F, toxic to preadults (LUNG *et al.* 2002), serves as the positive control, and Acp29AB, not toxic to preadults (LUNG *et al.* 2002), serves as the negative control. Asterisks denote Acps with significantly (*t*-test, *P*-value of <0.0001, supplemental Table 3 at http://www.genetics.org/supplemental/) toxic effects to preadults upon ectopic expression, as compared to control UAS-Acp29AB. Standard error bars are shown for each Acp, except for CG8137 and Acp62F for which no UAS-Acp; *tubulin*-GAL4 progeny were observed.

the tested Acps (Acp70A, CG8137, and CG10433) had significantly fewer UAS-Acp; *tubulin*-GAL4 progeny (8, 0, and 2% of control levels, respectively) than the control UAS-Acp29AB line (LUNG *et al.* 2002). The other 20 UAS-Acp; *tubulin*-GAL4 lines tested showed no toxicity in our assay and did not differ significantly from results with UAS-Acp29AB (Figure 3; supplemental Table 3 at http://www.genetics.org/supplemental/).

The mechanism by which these three ectopically expressed Acps, as well as Acp62F (LUNG *et al.* 2002), cause preadult toxicity is unknown. We cannot rule out that the toxicity might be due to disruption of essential pathways in preadults, unrelated to the Acps function in mated females. Yet it is also plausible that the toxicity is due to effects on pathways that these Acps normally regulate after mating, in which case the toxicity will provide a phenotype useful for defining how these Acps regulate mated-female physiology or molecular cascades. For example, since Acp70A can activate immune pathways within mated females (PENG *et al.* 2005), perhaps Acp70A's ectopic expression (and possibly that of other toxic Acps) could misregulate immunity pathways. Alternatively, since Acp70A can raise juvenile hormone B3 (JHB3) synthesis by the corpora allatae (MOSHITZKY *et al.* 1996), its ectopic expression might cause lethality by altering the normal hormonal milieu. The lethality associated with ectopic expression of toxic Acps prevents us from assaying their effects on a female immunity (or on JHB3 levels) *in vivo*. Nevertheless, examining the gene regulation of antimicrobial peptides in females mated to males lacking Acp62F, CG8137, and/or CG10433 (as was done for Acp70A; PENG *et al.* 2005) could help in determining whether any of these Acps play a role in mated-female immunity.

The toxicity of the three Acps shown here, as well as of Acp62F (LUNG *et al.* 2002), is also intriguing in light of another effect of Acps. Acps cause mated females to have a shorter life span than virgin females (CHAPMAN *et al.* 1995). One possible basis for such an effect could be toxicity of some Acps upon systemic exposure, for example, after Acps enter the hemolymph of mated females. The connection between the toxicity found upon ectopic expression and the cost of mating still needs to be established. Nonetheless, it is interesting that 1 of the 4 toxic Acps (of 31 tested) is Acp70A. Mutational assays have implicated this Acp in contributing to the cost of mating (WIGBY and CHAPMAN 2005). This convergence of phenotypes suggests that toxicity may help in identifying Acps that are high-priority candidates for testing for involvement in the cost of mating. We note that 3 of the 4 toxic Acps identified here and previously (see supplemental material for CG10433 at http://www.genetics.org/supplemental/) are known (Acp62F, CG8137; LUNG and WOLFNER 1999; RAVI RAM *et al.* 2005) or expected (Acp70A; AIGAKI *et al.* 1991) to access targets throughout the female via the hemolymph. Interestingly, the localization patterns of CG8137 (a predicted protease inhibitor) and Acp62F (a known protease inhibitor) are almost perfectly parallel; upon transfer, both localize to sperm storage organs and are found on oocytes and laid eggs (LUNG *et al.* 2002; RAVI RAM *et al.* 2005). Acp62F's and CG8137's toxicity, similar localization pattern, and predicted function in regulating proteolysis suggest that they may have similar biological functions.

Ectopic expression of individual Acps in virgin females did not affect receptivity to mating or egg-laying rate: The postmating increase in egg laying and decrease in receptivity are also regulated by Acps (reviewed in WOLFNER *et al.* 2005). To test the 22 Acps for roles in these processes, we compared the receptivity to mating and the egg-laying rates of virgin females

ectopically expressing a single Acp to those of control females. None of the 22 Acps tested for receptivity or the 20 Acps tested for egg laying significantly affected virgin female receptivity to mating or levels of egg laying (supplemental Table 4 at http://www.genetics.org/supplemental/). It is possible that the egg-laying sample size was not large enough to conclusively rule out small effects; if the egg-laying effect were twofold, we would have a 70.4% chance of detecting a significant difference (95% confidence interval). It is also possible that these Acps may not regulate receptivity or egg laying, or may do so only in the presence of other factors received during a normal mating, such as other seminal fluid proteins or sperm.

Conclusion: Successful reproduction often requires a balance of several factors. For example, resources must be allocated appropriately to reproductive *vs.* somatic maintenance to allow optimal levels of reproduction and survival (Zera and Harshman 2001). Seminal fluid proteins can have effects that appear beneficial to the female or effects that seem harmful. Our identification here of specific Acps that promote immunity or cause toxicity provides candidates with which to investigate how such a balance is achieved, and what its larger consequences could be.

We identified three Acps (CG6168, CG9334, and CG10284) that increase a female's ability to combat an infection of *S. marcescens in vivo*. Although prior studies have demonstrated that Acps can upregulate antimicrobial peptide transcript levels, this report is the first demonstration that Acps can affect a female's ability to fight a bacterial infection. The three immunity-promoting Acps that we identified are predicted proteolysis regulators. They may play protective functions within mated females by protecting sperm and/or the female reproductive tract from infection. Since an enormous diversity of other bacteria and fungi are capable of infecting *D. melanogaster* (reviewed in Leclerc and Reichart 2004), other Acp immunity regulators may be uncovered if infections are performed with pathogens other than *S. marcescens*. Our findings suggest that Acp peptides and proteolysis regulators play an important role in mediating a mated female's immune response, thus potentially providing a benefit to the female and the mating pair. Host–parasite interactions are known to drive the rapid evolution of immune defense proteins (*e.g.*, Richards *et al.* 2005); therefore the rapid evolution of Drosophila seminal fluid proteins may reflect not only their reproductive functions but also their roles in immunity.

We also identified three Acps (Acp70A, CG8137, and CG10433) that are toxic to preadults, suggesting the possibility of harmful effects to the mated female. Since Acp70A contributes to the cost of mating, our findings suggest that Acp70A toxicity may play a role in decreasing a female's life span. Identifying toxic components of the seminal fluid therefore might help to reveal additional candidate Acps that contribute to female mating costs.

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