Effect of Male Accessory Gland Extracts on Female Oviposition and Sexual Receptivity of the Caribbean Fruit Fly (Diptera: Tephritidae)

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EFFECT OF MALE ACCESSORY GLAND EXTRACTS ON FEMALE OVIPOSITION AND SEXUAL RECEPTIVITY OF THE CARIBBEAN FRUIT FLY (DIPTERA: TEPHRITIDAE)

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ABSTRACT

Anastrepha suspensa (Loew) male accessory glands do not appear to possess a sex peptide, a factor that induces oviposition or inhibits mating receptivity. Injection of accessory gland extracts from laboratory-colony males into virgin females stimulated daily deposition of only 4 eggs per female, comparable to injections of whole reproductive tract extract (5 eggs per female) and negative controls (4 to 5 eggs per female). Mated females laid significantly more (10 eggs per female per d). Studies of wild-caught males and females yielded the same information: injection of an accessory gland/testes extract or saline both elicited 8 eggs per female per d whereas normally mated females laid 16 eggs per female per d. Female receptivity to mating following injection of accessory gland or whole reproductive tract extracts was comparable to the negative control group, in which 67% to 83% of treated females remated and 63% to 89% of control females remated. In contrast, only 43% of once-mated (positive control) females remated when placed with males. Once-mated females also took significantly longer to remate after exposure to males (359 min) than females from both treatment (61 to 169 min) and negative control groups (76 to 122 min). The duration of mating was similar among all groups (24 to 37 min). These results suggest that oviposition and receptivity inhibition in *A. suspensa* are not mediated by male-derived humoral factors.

Key Words: sex peptide, mating inhibition, female mate choice, oviposition

RESUMEN

Las glándulas accesorias de machos de *Anastrepha suspensa* (Loew) no aparece poseer un péptido sexual que induce la oviposición o inhibe la receptividad del apareamiento. La inyección de extractos de la glándula accesorias de machos criados en el laboratorio a hembras vírgenes estimulo la deposición diaria de solamente 4 huevos por hembra, comparable a inyecciones de un extracto del tracto reproductivo completo (5 huevos por hembra) y el grupo de control negativo (4 a 5 huevos por hembra). Hembras apareadas pusieron significativamente más huevos (10 por hembra por día). Estudios sobre machos salvajes capturados y hembras rindieron la misma información: la inyección de un extracto de la glándula/testes accesoria o salina resulto en 8 huevos por hembra virgen por día, mientras que las hembras apareadas normalmente pusieron 16 huevos por hembra por día. La receptividad de las hembras hacia el apareamiento después de la inyección de extractos de la glándula accesoria o del completo tracto reproductivo fue comparable con el grupo de control negativo: 67% a 83% de las hembras tratadas reaparearon versus 63% a 89% de las hembras del grupo control que reaparearon. En contraste, solamente 43% de la hembras apareadas solo una vez (grupo control positivo) reaparearon cuando fueron puestas juntas con los machos. También, hembras apareadas solo una vez tomaron significativamente más tiempo para reapararse después de ser expuestas a los machos (359 min.) que las hembras en ambos tratamientos (61 a 169 minutos) y el grupo de control negativo (76 a 122 min.). La duración del apareamiento fue similar entre todos los grupos (24 a 37 min.) Estos resultados sugirieron que la oviposición y la inhibición de la receptividad en *A. suspensa* no son mediadas por factores humorales derivados del macho.

The Caribbean fruit fly, *Anastrepha suspensa* (Loew), is an important quarantine pest of citrus in Florida. Although its impact on citrus production is minor, its presence results in export restrictions. The Florida Department of Agriculture and Consumer Services, Division of Plant Industry (FDACS-DPI) has developed fly-free zones in order to ship grapefruit and other fruits to Japan without the use of quarantine treatments. Releases of sterile male Caribbean fruit flies that would compete for wild females have been proposed as a means of suppressing flies in limited

areas to support these zones. The most important aspect of Sterile Insect Technique (SIT) is the ability of sterile flies to mate with wild fertile females and produce a reasonably long female refractory period before remating. Studies on the reproductive behavior and physiology of *A. suspensa* have described the role and use of male pheromones (Nation 1972, 1990), lek formation on host plant leaves (Burk 1983), acoustic courtship signals (Sivinski et al. 1984; Webb et al. 1984) and copulation (Nation 1972; Mazomenos et al. 1977). There have been no studies investigating *A. suspensa* for the presence of sex peptide, a male accessory gland factor that induces oviposition and/or inhibits sexual receptivity in some Diptera such as *Aedes aegypti* (Fuchs et al. 1969), *Drosophila* (Chen et al. 1988), and *Delia antiqua* (Spencer et al. 1992, 1995), as well as other insects (Gillott 1988).

Anastrepha suspensa males have paired testes connected to a common duct through the vasa deferentia (Dodson 1978). The sex accessory glands originate where the vasa deferentia converge and consist of five or six short, tubular glands. Females typically have three spermathecae and polytrophic ovarioles (Dodson 1978; Fritz & Turner 2002); ovaries may contain more than one flush of mature eggs at a time.

Males court females, forming leks on host plant leaves in order to mate with females before dusk (Burk 1983). Stimuli used to attract females to the leks include pheromones deposited on the underside of leaves, acoustic signals produced by wing fanning, and visual cues (Nation 1972; Sivinski et al. 1984; Webb et al. 1984). When mating, flies typically remain *in copula* for 30 to 37 min (Nation 1972; Mazomenos et al. 1977), transferring male-derived substances in addition to sperm (Sivinski & Smittle, 1987). Oviposition occurs on host plant fruit the next morning following copulation (Burk 1983).

Oviposition by *A. suspensa* is strongly influenced by both environmental cues (Landolt & Sivinski 1992) and the quality of the oviposition site (Sivinski & Heath 1988). Mated females with access to artificial oviposition devices consisting of a rolled cloth impregnated with beeswax lay significantly more eggs per female than those without access. In addition, the physical features of the oviposition substrate such as shape and color are more important oviposition stimulants than chemical cues for polyphagous species like *A. suspensa* (Szentesi et al. 1979; Greany & Szentesi 1979).

Mated females with oviposition devices are more likely to remate during weekly opportunities than those without oviposition devices (Sivinski & Heath 1988). Mated females with no opportunity to oviposit are more likely to mate only once. In another study (Mazomenos et al. 1977), none of the once-mated, wild-type females re-

mated within 5 d, even though approximately 50% of the females were not fertilized. By contrast *Rhagoletis pomonella* (Walsh) females are receptive to nearby males immediately following copulation, and *R. suavis* (Loew) may alternate egg laying and copulation with several males before leaving an oviposition site (reviewed by Christenson & Foote 1960).

In support of the sterile insect release program, we studied the effect of male accessory gland secretions on oviposition and mating receptivity in virgin females. Although increasing doses of gamma irradiation have been correlated with decreased mating success of sterilized males (Hooper 1972; Calkins et al. 1988), the means by which it occurs is not known. Because gamma irradiation of male pupae may conceivably alter their physiology, including the biosynthesis of semiochemicals, we studied both laboratory-strain and wild-caught flies to explore whether *A. suspensa* uses a sex peptide.

MATERIALS AND METHODS

Anastrepha suspensa were obtained from either the mass-rearing facilities of the USDA laboratories at Gainesville, Florida, or collected as larvae in fruit from central Florida. Larvae-infested fruit were returned to the lab and emerging larvae were placed on vermiculite to pupate. Females and males were sexed as newly eclosed adults, placed into separate cages at equal density and provided with an agar-based sugar, yeast, and wheat germ diet (1:1:1). Flies were maintained at ambient temperature and humidity under artificial lighting (L:D 14:10). Because males and females of laboratory strain of *A. suspensa* become sexually mature at 10 to 11 d old (Nation 1972), flies were held at least 14 d after eclosion before being used in an experiment.

Extract Preparation and Injection

Reproductive tissues consisting of accessory glands, accessory gland plus testes, or whole reproductive tracts were removed from sexually mature (14- to 40-d-old) virgin males (freshly killed by freezing) and placed into a drop of cold saline (Spencer et al. 1992) in a dissecting dish. Each tissue was quickly transferred into a saline-filled microcentrifuge vial kept on ice. Tissues were processed into extracts by homogenizing them for 30 to 60 s in a bath sonicator (Heat Systems Ultrasonic), then centrifuging at 4500*g* for 10 min, and removal of the supernatant. The concentration of the supernatant (extract) was adjusted so that 0.5 mL represented 1 male gland equivalent. Extracts either were kept on ice and used within 2 h for injection or frozen before use.

To administer the extract, sexually mature virgin females were anesthetized with nitrogen and

1 male equivalent of extract was injected into the hemocoel as in earlier studies on Diptera, deep to the lateral mesothorax through a pleuron. Injection needles consisted of 3-mm glass tubing drawn into a very fine point (~40 μm at the tip). Flies were restrained for injection on a Plexiglas® plate by covering them with Parafilm®. Treatment groups not receiving injections also were anesthetized. Treated flies were placed individually into 7.5-cm diameter by 15-cm high aluminum screen cages.

Effect of Reproductive Tissue Extracts on Oviposition and Mating Receptivity of Lab-Colony Flies

Experiments were conducted as randomized block designs. Flies were randomly removed from the holding cage 1 block at a time. Treatments within blocks were assigned at random by drawing numbers from a container and all flies within a block were treated in the random order specified before proceeding to the next block. Data were statistically analyzed either with a SAS general linear models program and Student-Newman-Keul's multiple range test (SAS Institute, Cary, NC) or with Fisher's protected least squares difference test (StatView 4.0, Abacus Concepts Inc., Berkeley, CA).

Flies from the FDACS rearing facility were used for both oviposition and mating receptivity experiments. An "experimental unit" consisted of 1 female per cage containing 1 oviposition substrate, which was a 2.5-cm agar sphere surrounded with Parafilm® and suspended from the top of the cage. Experiments included up to 7 treatments with 6 to 61 experimental units per treatment. The control groups included a virgin control (no injection), female inserted with a needle only, saline injected females, and mated controls in which 3 males were placed with the female for 24 h, then removed. Extract-injected treatment groups consisted of accessory gland extract injection of 1 male equivalent, accessory gland extract injection of 5 male equivalents, and whole reproductive tract extract injection of 1 male equivalent.

Following treatment, approximately half of the females from each group were monitored for oviposition and half for mating receptivity. For the oviposition group, females were allowed to oviposit for up to 23 d on oviposition spheres replaced every few d as egg counts were performed; short term effects were not examined. For the mating-receptivity group, 3 male flies were placed with each treated female on the d following treatment and flies were observed for up to 12 h to determine the time until onset of mating and the duration of mating. At the end of 12 h, males were removed, females were divided according to whether or not they had mated, and egg output was measured as described for the oviposition group. For each treatment, the percentage of females mating was determined. Females not mating were included in the data set for the oviposition assay.

Effect of Male Reproductive Tract Extract Prepared from Wild-Caught Flies

Since mass rearing radically alters selection pressure and may cause changes in mating ability or mating effectiveness in some Diptera (Bush et al. 1976), wild Caribbean fruit flies were tested to determine whether they somehow were different than lab colony flies. Extracts were prepared from the accessory gland/testes complex of wild *A. suspensa* males as described earlier for laboratory males except that they were collected into saline containing a mixture of protease inhibitors (0.0004% w/v each of antipain, leupeptin, pepstatin A, chymostatin). These were added to prevent the possibility of rapid enzymatic degradation of extracted proteins. Tissues were sonicated for ~25 s, centrifuged at 4500*g* for 5 min, and the supernatant stored in a freezer overnight. There were 25 experimental units per treatment consisting of 3 wild-caught females and 1 oviposition substrate per experimental unit. In this test, females were given 1.5-cm diameter blue, cerasin wax domes for oviposition instead of agar balls. Females were injected with either a saline control or accessory gland/tests extract of 1 male equivalent. For the mated control, males were added to the cage at a density of 4 males per female for 24 h. Females were allowed to oviposit for up to 10 d and oviposition spheres were replaced as counts were performed. Data collected were egg counts per live female per cage.

RESULTS

Oviposition and Mating Receptivity of Lab-Colony Flies

Injections of male accessory gland or whole reproductive tract extracts did not increase oviposition in lab colony, virgin females above that of unmated flies (Table 1). Females from the negative control treatments and the extract treatments laid a cumulative average of 4.2 eggs per d, significantly fewer than the average 9.5 eggs per female laid by normally mated females ($P < 0.05$, Student-Newman-Keul's).

Similarly, when virgin females receiving injections of male reproductive tissue extract were exposed to males, they mated like unadulterated virgins. Treated females began mating an average of 61 to 169 min after males were introduced into the cage, comparable to virgin controls that began mating 76 to 122 min following introduction. Mated females waited 6 h before mating again (Table 2). In addition, 78% of all treated virgin females mated during the 12-h bioassay in

TABLE 1. Oviposition by lab colony *Anastrepha suspensa* injected with aqueous extract of male reproductive tissue (X ± S.D.)

Treatment	n	Eggs/female/d[1]
Controls		4.8 ± 4.1
Virgin	8	4.3 ± 3.5
Needle insertion only	10	3.5 ± 4.6
Saline	10	
Male extracts		
Accessory gland	13	3.8 ± 2.6
Whole tract	9	5.2 ± 2.2
Grand mean - unmated treatment females	50	4.2 ± 3.4*
Mated females	61	9.5 ± 6.8*

[1]The 2 means indicated with an asterisk are significantly different from one another at $P < 0.05$ by SNK.

TABLE 2. Mating by lab colony *Anastrepha suspensa* injected with aqueous extract of male reproductive tissue (X ± S.D.)

Treatment	n	% Mating	Delay until mating (min)[1]	Duration of mating (min)[1]
Controls				
Virgin	9	89%	76 ± 48a	25 ± 10a
Needle insertion only	7	86%	114 ± 102a	37 ± 7a
Saline	8	63%	122 ± 69a	24 ± 7a
Male extracts				
Accessory gland 1 ME	9	67%	104 ± 66a	28 ± 19a
Accessory gland 5 MD	6	83%	169 ± 100a	35 ± 20a
Whole tract 1 ME	12	83%	61 ± 72a	27 ± 11a
Grand mean - unmated treatment females	51	78%	104 ± 82a	29 ± 13a
Mated females	7	43%	359 ± 124b	30 ± 26a

[1]Treatment means with the same letter are not significantly different from one another within columns at $P < 0.05$ by SNK.
[2]ME = male equivalent.

contrast with only 43% of previously mated females. All groups averaged about 30 min mating time suggesting that once mating began, previous treatment had no effect on duration of copulation.

Oviposition by Wild-Caught Flies

Oviposition by wild-caught virgin females injected with accessory gland/testes extract was virtually identical to that of saline-injected virgins, 8.4 and 8.3 eggs/female after 10 d (Fig. 1). Normally mated wild females laid an average of 16 eggs/female over the same period. Egg deposition was higher among feral flies than mass-reared flies, perhaps because blue wax domes were more amenable to oviposition than agar balls—both wild-caught and laboratory mated females laid twice as many eggs as unmated females regardless of treatment.

DISCUSSION

Inhibition of mating receptivity and stimulation of oviposition in *A. suspensa* females were not mediated by transfer of male accessory gland factors to the female. Sexually mature laboratory colony females injected with extracts of male reproductive tissues exhibited no observable change in egg output or mating propensity. They behaved as virgins. Protease inhibitors used to prevent or minimize digestion of extracted proteins did not change the results. Experiments with wild flies yielded virtually identical results with respect to oviposition induction, suggesting that laboratory selection had not removed a wild-type sex peptide. It is possible that delivery of extract into the hemocoel, rather than the spermathecae, failed to stimulate females, despite eliciting positive re-
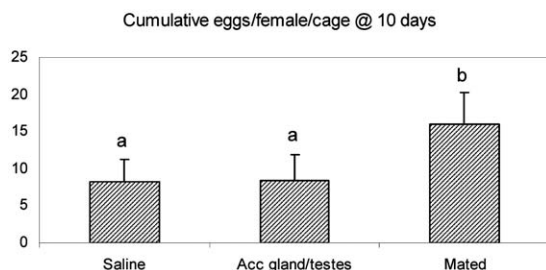
Fig. 1. Oviposition by wild-caught *A. suspensa* following injection with accessory gland/testes extract (X ± S.D.). Treated females received 1 male equivalent of extract and the negative control was saline-injected virgins. For the positive control, males were placed with females at a density of 4 males per female for 24 h then removed (n = 25). Treatment means with the same letter were not significantly different ($P < 0.05$) using Fisher's protected least squares difference test.

sponses in other Diptera (Fuchs et al. 1969; Chen et al. 1988; Spencer et al. 1992, 1995).

In mating competition experiments, male *A. suspensa* rendered sterile by a low dose of gamma radiation (3 krad) were equally competitive with normal males when placed with normal females as measured by egg output (Calkins et al. 1988). Therefore, sterile male flies are capable of inducing oviposition behavior in females without transferring viable sperm. Studies on the apple maggot fly, *R. pomonella*, with normal and irradiated males showed similar results (Myers et al. 1976). Remating in the melon fly, *Bactrocera cucurbitae* (Coquillett), was inhibited at the same rate in females mated to either normal or sterile males as long as copulation was not terminated prematurely (Kuba & Ito 1993). These studies suggest there is a factor(s) other than normal sperm that causes female tephritids to change from virgin to mated behavior, but we could find no evidence of a sex peptide extractable by methods amenable to proteins.

A humoral factor produced by males and transferred to females may change female behavior but in ways not measured by this study. Jang (1995) demonstrated that female olfactory-mediated behavior in the Mediterranean fruit fly, *Ceratitis capitata*, was altered when virgin females were injected with 0.2 equivalent of male accessory gland fluid. In a wind tunnel assay, injected, unmated females preferred host fruit odor (representing post-mating oviposition site selection) over male pheromone odor, similar to mated females. Virgin or saline-injected virgin females preferred male pheromone odor over fruit odor.

Perhaps mating receptivity and oviposition are controlled more by some physical aspect of copulation than by a chemical factor, or by a chemical factor not originating in the reproductive tract. Structurally, the spermathecae, associated ducts, and the ventral receptacle are innervated with muscle fibers surrounding the spermathecal capsule, indicating that females may regulate sperm location and use (Fritz 2002; Fritz & Turner 2002). Pereira et al. (2006) suggested that exposure to volatile male sex pheromones accelerates ovarian development in virgin *A. suspensa* females as preparation for mating. In *Anastrepha striata* (Schiner), females mated to virgin males lived longer than those mated to sexually experienced males, presumably because of a transferred substance (Perez-Staples & Aluja 2004). However, because *A. striata* males provide both orally derived fluid (trophallaxis) and accessory gland secretions to females during courtship, additional studies would be needed to establish the origin of the longevity substance. Although extensive trophallaxis does not occur in *A. suspensa,* males lick the female's head during copulation, suggesting another route by which females may acquire semiochemicals. In the tsetse fly, *Glossina morsitans* Westwood, ovulation appears to be induced by the physical stimulation of mating but not by the presence of a spermatophore in the uterus, or by chemical factors originating from the accessory gland or testes (Saunders & Dodd 1972; Gillott & Langley 1981). Inhibition of receptivity is regulated differently, being dependent on both chemical and physical stimuli (Gillott & Langley 1981).

Perhaps the mating system of *A. suspensa* and other lekking tephritid species is only peripherally connected to sex peptide communication or manipulation. In mating systems where pre-copulatory female choice is highly developed, as is apparently the case in *A. suspensa* (e.g., Burk 1983), the arena for post-copulatory manipulation by either sex may be limited. That is, males may not benefit by investing in manipulative chemicals if females are unlikely to remate until a male's sperm are depleted, and choosey females may not require the means to manipulate sperm from multiple males if they are likely to mate carefully and infrequently. If this is the case, one might predict a greater role for sex peptides and other forms of post-copulatory sexual conflict in resource-based mating systems with a high degree of polyandry, such as occurs in many *Rhagoletis* species.

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