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ATTRACTION OF FEMALE MEDITERRANEAN FRUIT FLIES TO THE FIVE MAJOR COMPONENTS OF MALE-PRODUCED PHEROMONE IN A LABORATORY FLIGHT TUNNEL

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Abstract—Attraction and pheromonal activity of five major identified components of the male-produced sex pheromone of the Mediterranean fruit fly *Ceratitis capitata* to virgin laboratory-reared females was assessed in a laboratory flight tunnel. Dual-choice competitive assays were run to establish a baseline response of virgin females to live male pheromone, individual components, and an ensemble of all five compounds alone (air control) and competitively against one another. Approximately 50% of the females released in the tunnel were captured on leaf models emitting pheromonal odors from five live males. Over 37% of released females responded to an ensemble of five major identified components presented in individual capillaries. Response of females to individual components was less than 10%. Competitive assays showed the live male-produced pheromone to be more attractive than either the five major component ensemble (FMCE) or individual components. Further research is likely to identify other male-produced compounds with pheromonal activity that could improve development of a pheromone-based trap for monitoring Mediterranean fruit fly populations.

Key Words—*Ceratitis capitata*, Mediterranean fruit fly, Diptera, Tephritidae, pheromone, 1-pyrroline, attractant, flight tunnel.

INTRODUCTION

The Mediterranean fruit fly (Medfly), *Ceratitis capitata* (Wiedemann) is an economically important tephritid pest whose host range extends to over 250

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different types of fruits and vegetables. Due to its wide host range, this insect represents a significant threat to agriculture in states such as California, Florida, and Texas where much of the U.S. commercial fruits and vegetables are grown. Presently, the Mediterranean fruit fly is not known to be established in these areas; however, each year the threat of its introduction increases.

One important method used in detecting foreign introductions of Medflies into the continental United States is the use of olfactory-based detection traps placed around likely introduction points such as borders, airports, and seaports. The use of chemical attractants in detection, control, and eradication of fruit fly infestations has had a long history, primarily through the use of protein-based food baits (McPhail, 1939; Steiner, 1952; Gow, 1954) and empirically identified synthetic attractants such as trimedlure (Gertler et al., 1958; Beroza et al., 1961). Interestingly, for this species, the use of pheromone-based attractants has not generated much interest, possibly due to the difficulty in identification and formulation of the pheromonal components of the male-produced odor and/or the relatively poor performance of identified pheromonal components compared to proteinaceous or synthetic attractants.

The presence of a male-emitted pheromone attractive to virgin Medfly females was first reported by Feron (1959, 1962). Subsequently, several researchers reported on identification or attractancy of the pheromonal components from calling male Medflies (Jacobson et al., 1973; Ohinata et al., 1977; Baker et al., 1985, 1990; Jang et al., 1989; Heath et al., 1991); however, detailed information on the biological activity of individual components or blends of the pheromonal components is sparse or lacking in most cases. Baker et al. (1985) identified nine components of male Medfly odor, of which 1-pyrroline was reported as the most biologically active singly, although no data were provided. Jang et al. (1989) identified 54 components from male-produced pheromonal odors including eight of the nine reported by Baker et al. (1985) and tested activity to five individual components and a synthetic blend using a close-range cage bioassay. Baker et al. (1990) conducted field tests in Mexico of various combinations of two identified male-odor components (geranyl acetate and linalool) and five additional volatiles (three pyrazines, ammonia, and trimedlure), but they made no attempt to mimic or reproduce the male odor in their study.

Heath et al. (1991) reported on the identification, natural release rates, and formulation of three of the five major male-odor components previously identified by Jang et al. (1989), and they found limited attraction of Medfly females to three components in the field. Recently, Landolt et al. (1992) reported on flight behavior of females in a wind tunnel to the three major components of the male-odor tested by Heath et al. (1991), as well as to the odor of calling males. Flath et al. (1993) investigated the effects of fly age and time of day on the composition and complexity of volatile pheromonal emissions of Hawaiian

Medfly males and reconfirmed the identity of over 30 compounds, five of which constitute the major quantitative emissions. These chemical analyses indicate a need for more detailed research on the attractiveness of identified compounds to virgin female Medflies.

The purpose of this study was to assess the attraction of virgin female Medflies to the five major male-produced odor components identified by the present authors (Jang et al., 1989; Flath et al., 1993). The specific objectives of this study were to establish and compare the inherent attraction of virgin female Medflies in a laboratory flight tunnel to: (1) the natural male-emitted pheromone, (2) each of the five major individual components and a synthetic five-component ensemble, (3) each of the five major individual components vs. the five-component ensemble, and (4) the five-component ensemble or individual major components vs. the natural male-emitted pheromone. These competitive choice tests measured the preference in attraction (of groups) of virgin female Medflies to pairings of individual major components of the male odor, an ensemble of the five major components, or the natural-male-emitted pheromone.

METHODS AND MATERIALS

Insects. Laboratory-reared Mediterranean fruit fly pupae were obtained from the USDA-ARS, Tropical Fruit and Vegetable Research Laboratory in Honolulu, Hawaii. Males and females were segregated by sex at the late pupal stage (Cunningham et al., 1966) and placed separately by sex into groups of 50 in plastic containers (11.5 cm diameter × 7.5 cm deep) with nylon mesh covers. The flies were supplied with water, sugar, and hydrolyzed protein and tested at five to seven days postemergence. Females were held in separate rooms from males under common environmental conditions (12 : 12 hr light–dark at 23°C and 60% relative humidity) prior to testing.

Chemicals. The five identified major compounds tested were ethyl acetate, geranyl acetate, ethyl (*E*)-3-octenoate, (*E*, *E*)-α-farnesene, and 1-pyrroline. Ethyl acetate and geranyl acetate were obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). Ethyl (*E*)-3-octenoate was synthesized in the following manner: (*E*)-3-octenoic acid was synthesized from a mixture of hexanal, triethanolamine, and malonic acid by the method of Linstead et al. (1933). The acid was esterified with ethanol and the ester distilled to ca. 97% purity. 1-Pyrroline was generated by combining 5 μl of a concentrated aqueous solution of *N*-chloropyrrolidine hydrochloride and 5 μl of 4 N KOH solution and 500 μl of distilled water. Fifty microliters of the resulting solution was placed in a glass tube measuring 2.4 × 14 mm for presentation. (*E*, *E*)-α-Farnesene (97.2%) was isolated from ylang-ylang oil by fractional distillation, followed by liquid chromatography on 15% silver nitrate–silica.

All compounds except 1-pyrroline were formulated individually by placing each neat compound in individual glass capillary tubings of different sizes (1- and 5-μl microcaps, Drummond Scientific, Broomall, Pennsylvania) and different headspace levels (i.e., empty tubing space above the filling level), which allowed for different release rates for each of the five compounds (Weatherston et al., 1985; Heath et al., 1991). Individual compounds were tested by taping its capillary tube to the inside wall of the emission container. All five of the individual tubes containing compounds were taped to the sides of the emission container to produce, through simultaneous evaporation, the five major component ensemble (FMCE). Estimates of release rates for all compounds except 1-pyrroline were calculated from measurements of evaporation (drop in meniscus) in the capillaries over time in the flight tunnel. The capillary tube formulations had evaporation rates correlated to previous quantitative studies on the natural emission rates and ratios of the five major components released from calling males (Flath et al., 1993). The release rate of 1-pyrroline was estimated after adding a concentrated HCl solution to the tube containing residual 1-pyrroline at the completion of the test in order to re-form the stable *N*-chloropyrrolidine hydrochloride, which was subsequently converted to 2,3-trimethylene-4-quinazolone for quantification (Sakamoto and Samejima, 1979). The five major compounds, their purity, formulations, estimated release rates, and relationship to quantitative analysis of the five major components present in the male odor are listed in Table 1.

TABLE 1. FIVE MAJOR IDENTIFIED COMPONENTS FROM MALE ODOR OF MEDITERRANEAN FRUIT FLIES: PURITY, EVAPORATIVE RELEASE RATES, FORMULATION, AND PERCENTAGE RATIOS OF COMPONENTS USED IN FLIGHT-TUNNEL STUDIES

Compound	Purity (%)	Estimated release rate (ng/hr)	Formulation	% of total measured release rate	% of total from natural male odor[a]
(*E*,*E*)-α-Farnesene	97.2	1660–2300	2 1-μl caps, 8 mm HS[b]	10.6	6.9
Geranyl acetate	98	1472	1 1-μl cap, plugged 8 mm HS	8	12.5
Ethyl (*E*)-3-octenoate	97	5144	1 5-μl cap, 8 mm HS	27	20.4
Ethyl acetate	99.9	10,000–12,000	1 1-μl cap plugged 24 mm HS	53	46.4
1-Pyrroline	100	168	1 tube (14 × 2.5 mm)	0.9	13.6

[a]Calculated from Table 1 of Flath et al. (1993).
[b]HS = headspace, capillaries were open on both ends unless indicated as plugged at one end.

The five major components of the pheromonal odor (Jang et al., 1989; Flath et al., 1993), which together constitute greater than 90% of the total male odor, were tested individually and together as a FMCE for their attractiveness to virgin sexually mature females in a series of competitive dual-choice bioassays. Initial flight tunnel assays compared the inherent attractancy of each of the five compounds individually, the FMCE, or the odor of five sexually mature males against a clean air control. A second series of assays compared the individual compounds or the FMCE against the odor of five males. A third series of tests compared the individual components to the FMCE. Additionally, we tested the effect of doubling the emission rate (i.e., adding additional capillaries) of each individual component in the FMCE (keeping the remaining four the same) against air.

Flight-Tunnel Assay. Laboratory flight-tunnel bioassays were conducted in a rectangular glass flight tunnel as described by Jang and Light (1991). The tunnel measured 0.9 m × 0.9 m × 2.8 m and contained a measured airflow of 0.15 m/sec. Lighting inside the tunnel was maintained at ca. 2000 lux using 60-W fluorescent lights. Experiments were carried out during the morning hours (0800–1200 hr).

For each dual-choice assay, two artificial "leaf models" were placed inside the flight tunnel at a height of 40 cm above the floor of the tunnel and equidistant from each other and the sides of the tunnel. An emission container was constructed using a 250-ml plastic cylindrical bottle (Nalgene), which had the bottom third (4.5 cm) removed. A 9-mm hole was drilled into the cap of each resulting cylinder and fitted to tygon tubing. Two emission containers were connected via a T fitting to a cylinder of breathing-quality compressed air. Air was passed through each tubing at a flow rate of 50 ml/min into the plastic cylinder, which mixed and flushed odors from either calling males or synthetic components into the laminar-flow flight tunnel. The open end of the cylinder was covered with dark nylon mesh to prevent flies from landing on the emission tubes or viewing enclosed live males. Two artificial leaves (15 × 23 cm), made from dark green construction paper and covered on one side with Tanglefoot sticky glue (Tanglefoot Co., Grand Rapids, Michigan) were vertically aligned and taped above and below the emission container to form the artificial leaf model with the sticky side facing down wind. Emission tubes were taped to the inner wall of the emission container. For tests using live males, five males were placed inside the emission container portion of a similarly constructed leaf model. Clean air controls emitted only air through the leaf models.

For each assay, 50 females were released at the downwind end of the tunnel from a platform situated at midheight of the tunnel (40.5 cm). The released females were allowed to fly within the tunnel for 30 min, during which time they were observed for upwind flight and capture on the artificial leaves. At the end of the 30-min assay, the flies captured on the sticky surface of the leaf

models were counted and removed from both leaf models. After each assay, all noncaptured flies were removed (by vacuum suction) from the tunnel. New flies were then introduced at the start of subsequent tests. The tunnel's glass walls were cleaned with ethanol and allowed to dry completely whenever a change in the dual-choice tests were made. All tests were replicated at least four times using new flies for each replication. Location of the leaf models relative to each other in the flight tunnel were rotated from one side of the tunnel to the other (left or right) to avoid directional bias. Analysis for significant differences in fly capture on each of the two leaf models were compared using PROC TTEST (two-sample test) (SAS Institute, 1988).

RESULTS

Inherent Attractancy Tests. Females released into the flight tunnel in the absence of live male odor, FMCE, or individual components did not show any directed upwind anemotaxis toward the leaf model. Without odor stimuli, females congregated in the release or downwind end of the tunnel, and most did not pass the halfway point towards the upwind area of the tunnel during the course of the 30-min assay. Resulting capture of flies on the air (control) leaf model was always low (overall mean of 0.4 flies).

When live males were placed in the emission container, females exhibited a directed upwind anemotaxis to the male odor and significantly chose the male odors (pheromone) over the air control in all assays performed. Flights consisted of either a general movement upwind towards the odor source or a directed, upwind flight. Oriented upwind flights were either straight-line or a side-to-side zigzag movement characteristic of the counterturning motion of insect flight in flight tunnels to pheromonal odors. In general movement upwind, females would often take flight from the platform, alight on the sides of the glass tunnel and exhibit cleaning of antennae and legs prior to resuming their upwind flight. A mean of 24.3 $\pm$ 2.0 (48.6%) of released flies (50/test) were captured on leaf models emitting male pheromone compared to a mean of 1.0 $\pm$ 0.6 captured on the leaf model emitting air (control). This represents nearly a 50% behavioral response from which we designed further experiments.

Individual pheromonal components [except for ethyl (*E*)-3 octenoate] were significantly more attractive to virgin females than air alone (Table 2). However, the response to the individual components was less than the females' response to the male-emitted pheromone. Responses of virgin females to the FMCE approached that of live males when either was paired to air alone. A mean of 18.8 females (37.6% of released flies) were captured on the artificial leaves emitting the FMCE compared to a mean of 0.2 females captured on the leaf models emitting air alone. The response was significantly greater to both the

TABLE 2. INHERENT ATTRACTION OF VIRGIN FEMALE MEDITERRANEAN FRUIT FLIES TO NATURAL LIVE MALE PHEROMONE, FIVE MAJOR COMPONENT ENSEMBLE (FMCE), OR INDIVIDUAL COMPONENTS VERSUS AIR-ALONE CONTROL IN LABORATORY FLIGHT TUNNEL[a]

Test components	N	Mean No. of females captured ± SEM	
		Treatment	Air control
Live males	4	24.3 ± 2.0	1.0 ± 0.6**
Five components (FMCE)	24	18.8 ± 0.8	0.2 ± 0.1**
(*E*,*E*)-α-Farnesene	4	4.0 ± 1.6	0.0 ± 0.0*
1-Pyrroline	4	4.5 ± 0.9	0.0 ± 0.0**
Geranyl acetate	4	1.5 ± 0.3	0.0 ± 0.0**
Ethyl acetate	4	4.8 ± 1.8	0.0 ± 0.0*
Ethyl (*E*)-3-octenoate	4	4.0 ± 1.1	2.0 ± 0.9

[a]Five major component ensemble and individual pheromone components presented as in text at release rates specified in Table 1. Mean number of females captured were significantly different at the indicated (**$P \leq 0.01$, *$P \leq 0.05$) level of significance based on paired t tests of the means (SAS). Each replicate contained 50 females.

live male odor and the FMCE than to air. The responses to live male odor or FMCE were greater than those to the major individual components tested against air.

Competitive Attractancy Tests. Response of virgin females to individual pheromonal components compared to live male pheromone was low (Table 3). Mean responses to individual major components ranged from 0 to 2.2 females captured compared to a mean range of 16.5 to 25.8 females captured on leaf models emitting male pheromone. When evaluating the competitive efficacy relative to natural pheromone (or percentage of catch) for each of the five individual major components, ethyl acetate > 1-pyrroline > (*E*, *E*)-α-farnesene as single component attractants of females. Females showed a significantly greater preference for natural male pheromone ($\bar{X} = 16.7$) over the FMCE ($\bar{X} = 5.7$) when tested competitively. However, the FMCE did show some competitive effect in preferentially capturing 34% of the total number of females captured by the natural pheromone. This competitive activity reduced the predicted number of females expected to be captured on the leaf model emitting natural pheromone ($X = 24.3$) based on previous tests using live males.

Female responses to individual components relative to the FMCE are compared in Table 4. The FMCE caught significantly more females than any of the individual major male-odor components. Mean captures ranged from 11.8 to 19.5 to the FMCE compared to 0.3–2.3 for the individual components. In these

TABLE 3. ATTRACTION OF VIRGIN FEMALE MEDITERRANEAN FRUIT FLIES TO FIVE MAJOR COMPONENT ENSEMBLE (FMCE) OR INDIVIDUAL COMPONENTS VERSUS LIVE MALE-EMITTED PHEROMONE IN LABORATORY FLIGHT TUNNEL[a]

Test component(s)	N	Mean No. of females captured ± SEM: Treatment	Mean No. of females captured ± SEM: Live male standard	Treatment capture as a % of live male standard
Air[b]	4	1.0 ± 0.7	24.3 ± 2.0**	4.1
Five components (FMCE)	11	5.7 ± 0.7	16.7 ± 1.3**	34.1
(*E*,*E*)-α-Farnesene	4	1.0 ± 0.0	16.5 ± 2.6**	6.1
1-Pyrroline	4	1.8 ± 0.6	24.5 ± 3.4**	7.4
Geranyl acetate	4	0.0 ± 0.0	25.8 ± 1.9**	
Ethyl acetate	4	2.2 ± 1.0	21.3 ± 2.9**	10.3
Ethyl (*E*)-3-octenoate	4	0.3 ± 0.3	18.3 ± 2.2**	1.6

[a]Five major component ensemble and individual pheromone components presented as in text at release rates specified in Table 1. Mean number of females captured were significantly different at the indicated (**$P \leq 0.01$) level of significance based on a paired *t* test of the means (SAS).
[b]Data taken from Table 2.

TABLE 4. ATTRACTION OF VIRGIN FEMALE MEDITERRANEAN FRUIT FLIES TO INDIVIDUAL PHEROMONE COMPONENTS VERSUS FIVE MAJOR COMPONENT ENSEMBLE (FMCE) IN LABORATORY FLIGHT TUNNEL[a]

Test component	N	Mean No. of females captured ± SEM: Treatment	Mean No. of females captured ± SEM: Five component ensemble	Treatment capture as a % of five component ensemble
Five males[b]	11	16.7 ± 1.3	5.7 ± 0.7**	293.0
(*E*,*E*)-α-Farnesene	4	0.8 ± 0.5	16.0 ± 1.5**	5.0
1-Pyrroline	4	0.3 ± 0.3	19.5 ± 2.5**	1.5
Geranyl acetate	4	0.5 ± 0.3	11.8 ± 1.4**	4.2
Ethyl acetate	4	2.0 ± 1.2	15.3 ± 1.8**	13.1
Ethyl (*E*)-3-octenoate	4	2.3 ± 0.6	14.3 ± 2.1**	16.1
Air[c]	4	0.2 ± 0.1	18.8 ± 0.79*	1.0

[a]Five major component ensemble and individual pheromone components presented as in text at release rates specified in Table 1. Mean number of females captured were significantly different at the indicated (*$P \leq 0.05$, **$P \leq 0.01$) level of significance based on a paired *t* test of the means (SAS).
[b]Data comparing five males with the five components are from Table 3.
[c]Data on air control versus five components are from Table 2.

tests, ethyl (*E*)-3-octenoate and ethyl acetate showed the best competitive activity (16.1% and 13.1% relative capture) followed by (*E*, *E*)-α-farnesene (5.0%) and geranyl acetate (4.2%), while 1-pyrroline showed little competitive activity.

Doubling the emission rate of individual components in the FMCE one at a time resulted in mean captures ranging from 11.8 flies [ethyl (*E*)-3-octenoate] to 21.0 flies [(*E*, *E*)-α-farnesene)] (Table 5). Total capture by the augmented FMCE was, however, still significantly greater than to air alone. These data were similar to responses obtained for FMCE in earlier tests (Tables 2 and 4).

DISCUSSION

Female Medflies orient, fly upwind, and discriminate multicomponent male-produced pheromone odors from individual odors in a flight tunnel. While long-range attraction of females to calling males in the field has been reported previously (Ohinata et al., 1977; Nakagawa et al., 1981), the development of a five-component synthetic pheromonal blend (FMCE) attractive to females that approaches the attractancy of live calling males has not been previously reported. Ohinata et al. (1973), using a cage bioassay, reported that up to 50% of 100 released females responded to odor from filter paper that had been exposed to 2000 males or to a methylene chloride extract of a cold-trapped condensate from air passed over caged males. Compounds identified from that study were subsequently found to be unattractive to females. More recent attempts to formulate a synthetic pheromonal lure have centered around either a minimalist approach using only three components (Heath et al., 1991; Landolt et al., 1992) or an empirically derived combination of three identified (Howse, unpublished) components of male odor (Baker et al., 1990), which had little in common with the

TABLE 5. ATTRACTION OF VIRGIN FEMALE MEDITERRANEAN FRUIT FLIES TO FIVE MAJOR COMPONENT ENSEMBLE (FMCE) CONTAINING TWICE THE EMISSION OF ONE COMPONENT VERSUS AIR CONTROL IN LABORATORY FLIGHT TUNNEL[a]

FMCE plus additional	*N*	Treatment	Air control
Ethyl acetate	4	14.2 ± 2.2	0.5 ± 0.5**
(*E*,*E*)-α-Farnesene	4	21.0 ± 2.2	0.0**
1-Pyrroline	4	13.5 ± 1.9	0.0**
Ethyl (*E*)-3-octenoate	4	11.8 ± 1.6	0.2 ± 0.2**
Geranyl acetate	4	13.3 ± 2.5	0.3 ± 0.3**

[a] Five major component ensemble and individual components presented as in text at release rates specified in Table 1. Mean number of females captured were significantly different at the indicated (**$P \leq 0.01$) level of significance based on paired *t* tests of the means (SAS).

qualitative or quantitative complexities of the natural pheromonal odor. Neither the Heath et al. (1991) nor the Baker et al. (1990) studies directly compared the responses of their blends/formulations with a source of natural pheromone. The mean response of virgin females to the five-component ensemble in this study (37.6%) was far greater than that obtained by Landolt et al. (1992), who reported that only 3.3% of the individually released females contacted the source emitting a three-component blend, whereas 43.3% of individually released females responded to the odor of 15 caged males.

In almost all cases, responses of virgin females to individual pheromone components were low (overall $X = 0.4$ flies), while the male pheromone or the FMCE caught significantly more females, suggesting that qualitative differences or complexity in the number of pheromone components are key factors in the elicitation and degree of female attraction. Increasing the emission rate of individual components over the levels reported in this study did not significantly increase female capture (data not shown).

We believe that much of the observed differences in response between the FMCE and the natural male pheromone reported in this study are due more to qualitative differences (or complexity in odor composition) than quantitative differences in emission rates of the constituents tested. Increasing numbers of calling males (from 5 to 10) confined in the emission container of the leaf model did not increase attraction of females in our studies (data not shown). Ohinata et al. (1973) reported that there was little difference in attraction of females to 10, 25, or 50 live males in laboratory bioassays. Recent results of the present authors (Light et al., unpublished), showing that intermediate and minor male odor components can enhance the attraction of virgin females to natural male pheromone, suggests that further improvements in the synthetic five-component blend are possible.

Baker et al. (1985) reported that 1-pyrroline was the most active individual component for female attraction, an observation we confirmed in earlier close-range cage tests (Jang et al., 1989). However, in the flight tunnel assay, delta-1-pyrroline alone did not attract more females that other individual components. Apparently, its power lies in its ability to synergize with other components to produce the active pheromone (Jang et al., unpublished).

The identification of biologically active pheromone components attractive to female Medflies will be important additions to our knowledge of fruit fly semiochemicals and may lead to further studies on mating behaviors critical to development of sound control strategies.

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