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Are the Wild and Laboratory Insect Populations Different in Semiochemical Emission? The Case of the Medfly Sex Pheromone

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Supporting Information

ABSTRACT: The medfly (*Ceratitis capitata*) is one of the major agricultural pests controlled through sterile insect technique (SIT) programs. We studied the chemical composition of the volatiles released by calling males from one laboratory and two wild *C. capitata* populations using two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GC × GC/TOFMS) and gas chromatography with electroantennographic detection (GC-EAD). Multivariate data analyses revealed significant differences in the quantitative and qualitative composition of male chemical emanations between the three populations. The GC-EAD analyses of the male emanation of three *C. capitata* populations revealed 14 antenally active compounds. The volatiles isomenthone, *β*-pinene, ethyl octanoate, indole, geraniol, bornyl acetate, geranyl acetone, and (*E*)-caryophyllene are newly reported EAD active constituents of the male pheromone. GC-EAD analyses of the laboratory population indicated that the males and females of *C. capitata* possess comparable sensitivity to male-produced volatiles. Our results are relevant to the development of a pheromone-based monitoring system and also to the SIT control program.

KEYWORDS: *Ceratitis capitata, male sex pheromone, two-dimensional gas chromatography with time-of-flight mass spectrometric detection, gas chromatography with electroantennographic detection, principal component analysis*

INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata* Wiedemann (1824) (medfly), is a major pest in temperate climates almost all around the world. The species is among the principal targets controlled by the sterile insect technique (SIT).[1] The SIT relies on the release of mass-reared sterile males into wild populations (WPs) aiming to reach a high proportion of matings between sterile males and wild females to reduce viable offspring.[2] The success of the SIT depends on the sexual competitiveness of the mass-reared males released into the target populations.[1]

Mating in *C. capitata* is initiated by males that aggregate in small leks on trees or bushes.[3−5] Lekking males produce a sex pheromone to attract females.[6] Once the female approaches, the male performs a complex courtship ritual involving chemical, visual, and acoustic communication.[4,7] The mating success is highly variable among lekking males suggesting that female choice is an important component of the lek-mating system. The process of how females choose mates and which cues are important in this choice is not fully understood.

Food is one of the most important factors affecting reproductive success in many insect species. The nutritional needs of *C. capitata* have been quite thoroughly investigated. Examples include studies focused on larval diet selection,[8−11] the effects of larval diets upon adult biology, reproductive capacity, and behavior,[12−14] the effect of adult diets on sexual behavior and performance,[15,16] and the effect of protein and lipid uptake upon the development and reserve dynamics.[17,18]

Another factor affecting medfly male competitiveness is the mass-rearing process. Different selection pressures in nature and in the laboratory result in the development of complex behavioral differences in courtship that can lead to the development of reproduction barriers between laboratory populations (LPs) and WPs.[19] These laboratory-rearing conditions induce changes in the flies, which include earlier maturation,[20,21] diminishing of lekking behavior,[22] shifts in timing and duration of male calling periods,[23] reduction of male courtship variability and duration,[24] reduction of the variability of male traits, and reduction of female selectiveness.[25] It is also believed that the importance of chemical communication is reduced or modified under mass-rearing conditions.[26]

To clarify the effect of food and rearing conditions on medfly male pheromone emanations, we performed experiments aiming (1) to compare the pheromone composition of males originating from a laboratory colony and from two WPs originating from two different hosts (*Malus sylvestris* and *Ficus*

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Table 1. Chemical Compounds (Average ± RSD %) Identified by GC × GC/TOFMS in the Aeration Extracts of the Calling Males of *C. capitata* from Three Populations (LP, Laboratory Population; FP, Wild Fig Population; and AP, Wild Apple Population)

no.	compd	$I_{R\text{-}EAD}$[a]	$I_{R\text{-}GC}$[b]	ref[c]	LP	FP	AP
1	*n*-heptanal		901	30, 47, 48, 50	0.81 ± 0.25	1.16 ± 0.39	2.00 ± 0.63
2	α-thujene		929		0.03 ± 0.01	0.02 ± 0.01	0.09 ± 0.02
3	α-pinene		939		0.79 ± 0.43	1.35 ± 0.89	2.94 ± 1.49
4	dihydro-3-methyl-2(3*H*)-furanone[d]	951	949	30, 47, 48, 50	7.84 ± 1.99	2.39 ± 0.40	1.33 ± 0.91
5	dihydro-5-methyl-2(3*H*)-furanone		956			0.83 ± 0.62	0.45 ± 0.44
6	camphene		955		0.05 ± 0.02	0.08 ± 0.02	0.21 ± 0.08
7	2-ethyl hexanal		955		0.03 ± 0.01	0.91 ± 1.25	0.17 ± 0.06
8	sabinene		976		0.09 ± 0.05	0.05 ± 0.02	0.25 ± 0.10
9	1-octen-3-ol		980		0.18 ± 0.07	0.45 ± 0.35	0.66 ± 0.27
10	β-pinene[d]	990	983		0.16 ± 0.05	0.40 ± 0.31	0.52 ± 0.14
11	6-methyl-5-hepten-2-one[d]	990	985	47−49	5.06 ± 2.06	4.71 ± 2.44	3.52 ± 2.79
12	3-octanone		986	33	0.06 ± 0.1	0.11 ± 0.10	0.43 ± 0.36
13	myrcene		991	30, 47−49, 51	3.09 ± 0.63	0.99 ± 0.11	0.94 ± 0.25
14	2-octanone		991		0.12 ± 0.04	0.78 ± 0.79	1.01 ± 0.53
15	*n*-octanal		1004	30, 47, 50	0.71 ± 0.23	0.63 ± 0.29	2.06 ± 0.39
16	ethyl (*E*)-3-hexenoate		1004	30, 47, 49	0.87 ± 0.41	0.37 ± 0.12	0.14 ± 0.11
17	*n*-hexyl acetate		1014	30	0.04 ± 0.01	0.65 ± 0.43	0.95 ± 0.56
18	Δ3-carene		1014		0.20 ± 0.06	0.25 ± 0.10	0.76 ± 0.08
19	α-terpinene		1022		0.04 ± 0.01	0.11 ± 0.17	0.05 ± 0.04
20	2-ethyl hexan-1-ol		1030	33	1.73 ± 0.48	1.22 ± 0.49	5.70 ± 1.68
21	limonene		1035	30, 33, 47, 48	0.70 ± 0.64	1.10 ± 1.05	5.21 ± 0.57
22	(*Z*)-β-ocimene		1034	30, 47−49, 51	4.06 ± 1.99	2.14 ± 1.27	1.44 ± 0.56
23	(*E*)-β-ocimene		1060	30, 33, 47−49, 51	5.81 ± 0.96	1.85 ± 0.37	1.38 ± 0.56
24	indene		1053	33	0.04 ± 0.02	14.83 ± 9.71	18.34 ± 4.53
25	γ-terpinene		1063	30, 47	0.06 ± 0.07	0.05 ± 0.02	0.10 ± 0.03
26	(*Z*)-linalool oxide		1074		0.21 ± 0.11	0.09 ± 0.01	0.14 ± 0.05
27	3-ethyl-2,5-dimethylpyrazine		1080	30, 33, 47, 51	3.41 ± 1.40	1.57 ± 0.61	1.83 ± 0.66
28	γ-terpinolene		1090	30, 47	0.12 ± 0.07	0.09 ± 0.05	0.10 ± 0.04
29	linalool[d]	1100	1100	30, 33, 47−51	7.38 ± 1.45	4.17 ± 0.91	2.83 ± 1.33
30	*n*-nonanal		1106	30, 47, 48, 50, 51	0.75 ± 0.24	1.04 ± 0.43	2.99 ± 0.85
31	*allo*-ocimene		1130		0.51 ± 0.17	0.08 ± 0.02	0.14 ± 0.10
32	neo-*allo*-ocimene		1144		0.16 ± 0.13	0.14 ± 0.13	0.21 ± 0.12
33	2-ethylhexyl acetate		1147	30	0.07 ± 0.02	0.03 ± 0.01	0.10 ± 0.03
34	isomenthone[d]	1165	1164		0.02 ± 0.01	0.06 ± 0.04	0.44 ± 0.36
35	isomenthol		1186		0.05 ± 0.01	0.11 ± 0.02	0.32 ± 0.14
36	ethyl octanoate[d]	1195	1194	33	0.07 ± 0.05	0.07 ± 0.07	0.05 ± 0.03
37	ethyl (*E*)-3-octenoate[d]	1198	1197	30, 33, 40, 45, 47, 48, 51	20.36 ± 7.98	28.24 ± 4.89	12.98 ± 4.72
38	α-terpineol		1203	30	0.15 ± 0.06	0.05 ± 0.02	0.20 ± 0.06
39	*n*-decanal		1208	50, 51	0.76 ± 1.17	0.96 ± 0.46	1.81 ± 0.55
40	ethyl (*E*)-2-octenoate		1246	30, 47−49	0.26 ± 0.28	0.12 ± 0.09	0.23 ± 0.25
41	linalool acetate		1252	30, 48, 49	0.39 ± 0.17	0.26 ± 0.22	0.32 ± 0.19
42	geraniol[d]	1252	1255	50, 51	0.80 ± 0.41	0.12 ± 0.08	0.23 ± 0.29
43	2-decenal		1266		0.07 ± 0.02	0.06 ± 0.01	0.20 ± 0.10
44	geranial		1272		0.14 ± 0.08	0.08 ± 0.03	0.15 ± 0.20
45	2-octenoic acid		1290		0.17 ± 0.07	0.07 ± 0.03	0.06 ± 0.06
46	bornyl acetate[d]	1294	1293		0.11 ± 0.04	0.05 ± 0.04	0.23 ± 0.14
47	indole[d]	1300	1306	30, 33, 47, 48, 50, 51	0.91 ± 0.15	0.14 ± 0.07	0.09 ± 0.06
48	*n*-undecanal		1311		0.04 ± 0.01	0.06 ± 0.02	0.15 ± 0.05
49	methyl geranate		1323	30, 47−49, 51	1.50 ± 0.50	0.08 ± 0.02	0.11 ± 0.05
50	α-terpinyl acetate		1355		0.06 ± 0.06	0.11 ± 0.04	0.18 ± 0.05
51	neryl acetate		1358	30, 47, 48	0.23 ± 0.08	0.06 ± 0.01	0.10 ± 0.02
52	α-copaene		1367	33, 50	0.05 ± 0.03	0.07 ± 0.02	0.11 ± 0.06
53	2-undecenal		1370	30, 33, 40, 45, 47, 48, 51	0.06 ± 0.07	6.68 ± 7.46	1.42 ± 2.60
54	geranyl acetate[d]	1385	1380	33, 50	13.73 ± 5.32	11.63 ± 3.87	12.62 ± 4.97
55	*n*-dodecanal		1411	50	0.35 ± 0.07	0.11 ± 0.04	0.34 ± 0.15
56	1. unknown (longifolene?)		1418	51	0.21 ± 0.07	0.12 ± 0.02	0.32 ± 0.18
57	2. unknown (α-gurjunene?)		1432		0.64 ± 0.24	0.39 ± 0.10	1.19 ± 0.27
58	(*E*)-caryophyllene[d]	1445	1438	33	0.19 ± 0.14	0.20 ± 0.06	0.41 ± 0.15
59	geranyl acetone[d]	1451	1452		0.12 ± 0.08	0.07 ± 0.01	0.51 ± 0.35

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Table 1. continued

no.	compd	$I_{R\text{-}EAD}$[a]	$I_{R\text{-}GC}$[b]	ref[c]	LP	FP	AP
60	(*Z*)-*β*-farnesene		1455	30, 47, 51	0.09 ± 0.02	0.03 ± 0.01	0.22 ± 0.21
61	*α*-humulene		1475	33	0.09 ± 0.06	0.06 ± 0.02	0.22 ± 0.13
62	(*Z*,*E*)-*α*-farnesene		1495	48, 51	1.23 ± 0.24	0.25 ± 0.10	0.42 ± 0.17
63	(*E*,*E*)-*α*-farnesene[d]	1504	1509	30, 33, 40, 45, 47, 48, 50, 51	11.99 ± 1.53	5.04 ± 0.93	5.36 ± 1.87

[a]The retention indices of the antennally active compounds identified by the GC-EAD analyses. [b]The retention indices of the compounds identified in the male-pheromone emanation identified by GC × GC/TOFMS. [c]The literature references in which the compound was described in the medfly. [d]The antennally active compounds identified by GC-EAD and GC × GC/TOFMS.

carica), (2) to study the biological activity of individual components within the pheromone blend using medfly antennae as biological detectors, (3) to determine the sex-specific antennal sensitivities to antennally active pheromone components, and (4) to compare population-specific antennal sensitivities to antennally active pheromone components.

MATERIALS AND METHODS

Insects. The pupae of *C. capitata* WPs from apple (*M. sylvestris*, Red Delicious and Granny Smith varieties) and fig (*F. carica*) were collected at the same time (August−September 2008) near Thessaloniki, Greece. The pupae of *C. capitata* laboratory populations (originated from a colony in Argentina and reared on standard diet) were obtained from the entomological laboratory of Food and Agriculture Organization/International Atomic Energy Agency (FAO/IAEA, Seibersdorf, Austria). The pupae of wild and laboratory fruit flies were allowed to emerge in the rearing room, the temperature of which was 25 ± 1 °C and the relative humidity of which was 60%, and the photoperiod was set to a 12:12 light:dark cycle. After emergence, the flies of all populations were separated by sex, placed into glass chambers (30 cm × 20.5 cm × 16 cm), and provided with water and a mixture of cane sugar and brewer's yeast 3:1.

Headspace Sampling Procedure. The volatiles emitted by virgin-calling male flies (21st posteclosion day) were collected using a modified technique of an air-collection apparatus as described by Nation.[27] In this technique, a purified airstream was blown over living male flies enclosed in a glass chamber (43 cm length × 6 cm o.d.). The volatiles were collected onto traps made of 150 mg of SuperQ adsorbent (Chrompack) packed in glass Pasteur-like cartridges. The air flow directed through the apparatus was set at 1 L/min (using an ELE 503-070 air flow meter; ELE International Ltd., Loveland, CO). A cohort of 30 virgin male flies was transferred into the collection chamber 1 day before aeration at the end of photophase. The collection lasted for 24 h. Ten independent repetitions for each population were performed. After aeration, the flies were removed, and the glass containers were washed with hot water containing a detergent, rinsed with distilled water and ethanol, and heated up to 150 °C to avoid cross-contamination. Prior to the volatile collection, the traps were washed with hexane and ethanol. Subsequently, the traps were dried at room temperature and conditioned for 3 h under a stream of nitrogen at 120 °C. After collection, the trapped volatile compounds were extracted with 500 μL of freshly redistilled trace analysis grade hexane (Sigma-Aldrich). The extracts were concentrated to approximately 100 μL under a gentle stream of nitrogen and stored in a freezer until analysis.

Two-Dimensional Gas Chromatography with Time-of-Flight Mass Spectrometric Detection (GC × GC/TOFMS) Analysis. The GC × GC/TOFMS analysis was carried out on a LECO Pegasus 4D instrument (LECO Corp., St. Joseph, MI) equipped with a nonmoving quad-jet cryomodulator. A DB-5 column (J&W Scientific, Folsom, CA; 30 m × 250 μm i.d. × 0.25 μm film) and a BPX-50 column (SGE Inc., Austin, TX; 2 m × 100 μm i.d. × 0.1 μm film) were used for GC in the first and second dimensions, respectively. Helium was used as a carrier gas at a constant flow of 1 mL/min. Sample injection was done with the HP 7683 autosampler, and 1 μL of sample was injected in the splitless mode. The temperatures of the GC × GC/TOFMS instrument were set at 220 °C at the injector, 260 °C at the transfer line, and 250 °C at the ion source. The temperature program on the primary GC oven was as follows: 40 °C for 2 min, then 40−190 at 5 °C/min, and finally 190−320 at 20 °C/min with a hold for 2 min at 320 °C. The program in the secondary oven was 10 °C higher than in the primary one and was operated in an iso-ramping mode. The modulation period, the hot-pulse duration, and the cool time between the stages were set at 5, 0.8, and 1.7 s, respectively. The mass spectrometer was operated in the electron impact mode (EI, 70 eV). The data-acquisition rate was 100 Hz (scans/s) for the mass range of 29−400 amu. The detector voltage was 1750 V. The purge time was 60 s at a flow of 60 mL/min. The data were processed and consecutively visualized on 2D and 3D chromatograms using LECO ChromaTOF software. A series of *n*-alkanes (C_8−C_{22}; Sigma-Aldrich) was coinjected with authentic samples to determine their retention indices ($I_{R\text{-}GC}$, retention indices for gas chromatography with time-of-flight mass spectrometric detection). The chemicals were identified by a comparison of their mass-spectra fragmentation patterns, retention times, and retention indices with previously published data and authentic standards. In the absence of standards, identifications were carried out by a comparison with the reference spectra NIST library, the Wiley/NBS registry of mass spectral data,[28] and published retention indices.[29,30]

Gas Chromatography with Electroantennographic Detection (GC-EAD) Experiments. Male headspace samples were injected splitless into a 5890A Hewlet-Packard gas chromatograph equipped with an Rxi-5Sil MS (Restek, Bellefonte, PA; 30 m × 0.25 μm i.d. × 0.25 μm film) column. The column was split at the end by a Graphpack 3D/2 four-arm splitter, allowing the division of the eluate to the flame ionization detection (FID) and EAD detectors. The GC was operated at an initial temperature of 40 °C for 2 min, then ramped up at a rate of 10 °C/min to 270 °C with a 10 min hold. The temperature of the GC inlet and detector was set to 200 and 260 °C, respectively. About 30 GC-EAD analyses were performed for each population and sex. The time scales of both the GC-EAD and the GC × GC/TOFMS analyses are similar to allow a direct comparison of the major antennal activity (EAD response) with individual compounds. The EAD activities that were consistently observed in at least five independent GC-EAD recordings were further evaluated. These EAD responses were characterized by retention indices ($I_{R\text{-}EAD}$, retention indices for gas chromatography with FID and electroantennographic detection). A series of saturated C_8−C_{22} *n*-alkanes was coinjected with the analyzed samples to allow for the calculation of the linear retention indices ($I_{R\text{-}EAD}$) of the EAD active peaks and to check the correspondence of the retention behavior and antennal activity of authentic compounds with synthetic standards. The identification of the compounds started by delimitations of the GC × GC/TOFMS areas with similar retention parameters ($I_{R\text{-}GC}$) as retained EAD active areas ($I_{R\text{-}EAD}$). All of the compounds within the delimited areas were identified, and their antennal activity was tested in GC-EAD experiments.

The male pheromone of each population was analyzed using antennae of flies originating from each respective population (e.g., antennae of flies from apples for the pheromone of apple males etc.). Both males and females were used to allow comparison between the sexes. The comparison was based on a calculation of the FID/EAD ratio using the FID and EAD peak areas from the GC-EAD experiments. The FID/EAD mean values were calculated and compared between the sexes and the populations.

Statistical Analysis. The data obtained from the GC × GC/TOFMS analysis of the male emanations for each population were statistically evaluated using principal component analysis (PCA). The compounds included in the statistical analyses are listed in Table 1. For each component, the relative peak areas of the particular compounds were calculated in the deconvoluted total ion chromatogram mode. The relative peak areas were subjected to logarithmic transformation. The focus scaling was set for interpopulation correlation, the population scores were divided by standard deviation, and the data were centered by population. The statistical significance was assessed using redundancy analysis (RDA), a canonical variant of PCA, and the Monte Carlo permutation test (unrestricted permutations, n = 999). In the RDA analysis, the identities of the three populations stood as a categorical predictor. The multivariate data analysis software CANOCO 4.5 (Biometris, Plant Research International, Wageningen UR, The Netherlands) was used for both the PCA and the RDA analyses. For the data obtained by the GC-EAD analyses, the Student t test was used to determine the statistical differences of antennal sensitivity to the respective EAD active compounds.

RESULTS

GC × GC/TOFMS. The males originating from the laboratory population (LP) produced almost three times more volatiles than the males from the two WPs, whose volatile production was almost equal. The mean of the GC × GC/TOFMS total peak areas of the identified male volatile components of the LP reached $1.67 \times 10^9 \pm 0.68 \times 10^9$ total ion counts (TIC), whereas the total peak areas of the WPs reached $0.6 \times 10^9 \pm 0.33 \times 10^9$ TIC in the apple population (AP) and $0.63 \times 10^9 \pm 0.23 \times 10^9$ TIC in the population from figs (FP) (N = 8, 9, and 10 for LP, AP, and FP, respectively). The medfly male emanations represent a complex mixture of diverse chemical structures including nitrogen-containing compounds, terpenoids, alcohols, ketones, and esters (Table 1 and Figure 1). In total, we detected 63 compounds (Table 1). All of the compounds, except for dihydro-5-methyl-2(3*H*)-furanone, were present in the samples of all three populations, but their respective ratios significantly differed. The major components present in all of the populations (>8%) were ethyl (*E*)-3-octenoate (**37**), geranyl acetate (**54**), and (*E*,*E*)-α-farnesene (**63**). In the LP, ethyl (*E*)-3-octenoate (**37**), geranyl acetate (**54**), and (*E*,*E*)-α-farnesene (**63**) were the major components, and together, they comprised 46.1% of the volatile content. The WPs were characterized by a relatively higher amount of indene (**24**) and a lower amount of (*E*,*E*)-α-farnesene (**63**) related to LP. In the FP and the AP, indene (**24**), ethyl (*E*)-3-octenoate (**37**), geranyl acetate (**54**), and (*E*,*E*)-α-farnesene (**63**) accounted for 59.7% and 49.3% of the total volatile content, respectively. Minor components (1−8%) in all of the populations included dihydro-3-methyl-2(3*H*)-furanone (**4**), 6-methyl-5-hepten-2-one (**11**), 2-ethyl hexan-1-ol (**20**), (*Z*)- and (*E*)-β-ocimene (**22** and **23**), 3-ethyl-2,5-dimethylpyrazine (**27**), and linalool (**29**). The remaining compounds (listed in Table 1) were present in trace amounts (<1%). 2D chromatograms for each respective population are depicted in Figure 1A−C.

GC-EAD. A typical GC-EAD record is depicted in Figure 1D. In total, 13 EAD responses (corresponding to 14 antennally active compounds) were determined. Out of these, 10 responses ($I_{R\text{-}EAD}$ 990, 1100, 1195, 1198, 1252, 1294, 1300, 1385, 1451, and 1504) were present in all of the populations, two responses ($I_{R\text{-}EAD}$ 951 and 1165) were detected only in the AP (Figure 1D), and one response ($I_{R\text{-}EAD}$ 1445) was present in the LP and FP but not in the AP. The following 14 compounds

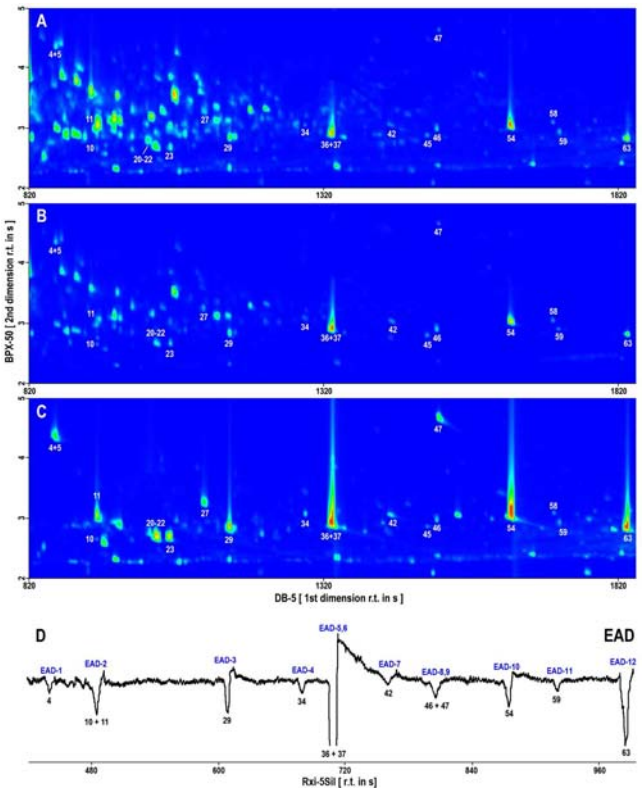

Figure 1. GC × GC/TOFMS analysis of the *C. capitata* male pheromone. (A) Wild FP, (B) wild AP, and (C) LP. Each dot represents one compound. The numbering corresponds to Table 1. The intensity of the signals is color-coded from blue (zero) to red (maximum). (D) The GC-EAD analysis of AP sex pheromone using AP female antenna as an EAD detector. Symbols EAD-1−12 depict EAD activity areas.

were identified as antennally active: dihydro-3-methyl-2(3*H*)-furanone (**4**), 6-methyl-5-hepten-2-one/β-pinene (**10**/**11**), linalool (**29**), isomenthone (**34**), ethyl octanoate (**36**), ethyl (*E*)-3-octenoate (**37**), geraniol (**42**), bornyl acetate (**46**), indole (**47**), geranyl acetate (**54**), (*E*)-caryophyllene (**58**), geranyl acetone (**59**), and (*E*,*E*)-α-farnesene (**63**). Ethyl octanoate and ethyl (*E*)-3-octenoate eluted in the GC × GC/TOFMS analyses almost simultaneously. However, the GC-EAD analysis detected two distinct responses corresponding to the two compounds (Figure 2).

Sex-Specific Differences in Antennal Sensitivity. A sex-specific analysis of antennal sensitivity was performed only for LP. We did not observe any sex specificity in the perception of the major EAD-active compounds (t test at p = 0.05, N = 10; Figure 3). In both sexes of LP, the highest EAD responses were observed at $I_{R\text{-}EAD}$ 1195 and 1198, corresponding to ethyl octanoate and ethyl (*E*)-3-octenoate. Linalool ($I_{R\text{-}EAD}$ 1100) and (*E*,*E*)-α-farnesene ($I_{R\text{-}EAD}$ 1504) showed moderate EAD activities, while geraniol ($I_{R\text{-}EAD}$ 1252) and geranyl acetate ($I_{R\text{-}EAD}$ 1385) elicited relatively small EAD activity.

Because of the limited amount of biological material, we do not have enough data to provide the same comparison for males and females of FP and AP. The comparison of population specificity in the perception of the major EAD-active compounds is available for females. On the basis of these data, we did observe interpopulation differences in the female perception of (*E*,*E*)-α-farnesene (Figure 4).

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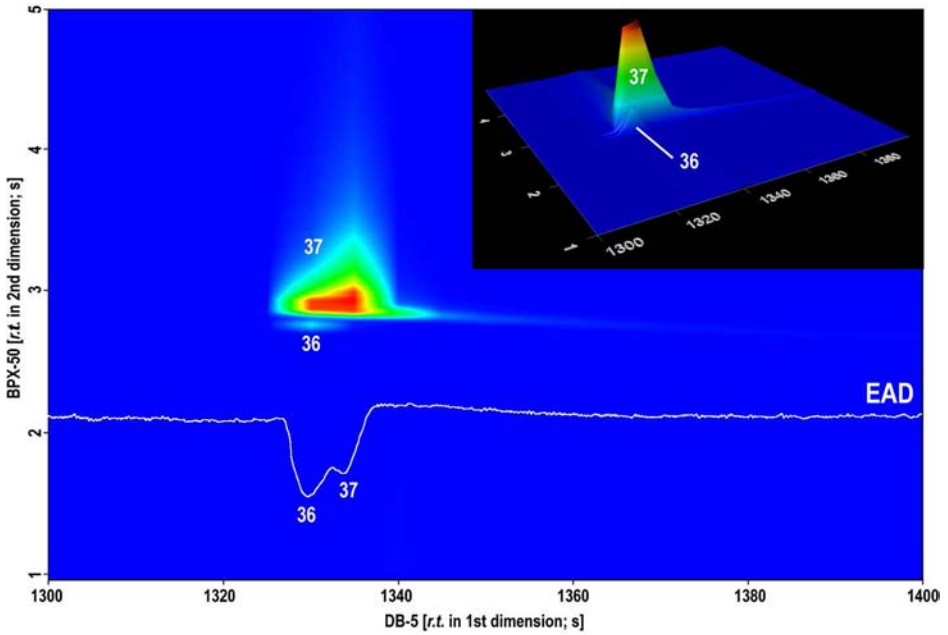

Figure 2. Detail of the GC × GC/TOFMS and GC-EAD (inserted white line) analyses of the *C. capitata* male pheromone. The area of ethyl octanoate (**36**) and ethyl (*E*)-3-octenoate (**37**) elution is depicted. The color coding in the GC × GC/TOFMS analysis shows the quantitative differences in the two visualized compounds. In the GC-EAD, a response with two peaks (EAD areas 5 and 6) can be consistently seen at the retention time when ethyl octanoate and ethyl (*E*)-3-octenoate elute.

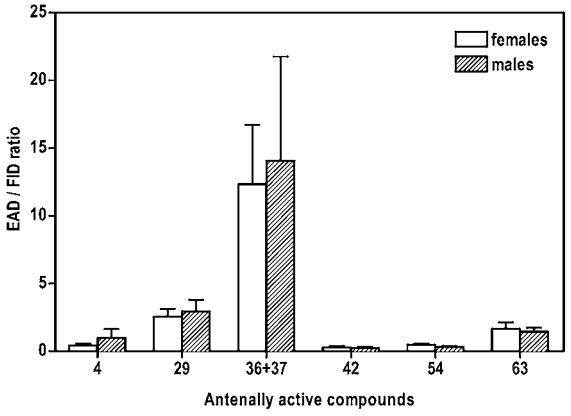

Figure 3. Comparison of male and female antennal sensitivities using the EAD/FID ratio (*y*-axis) calculated from the GC-EAD experiments for the most active components of *C. capitata* male sex pheromone (the bars represent means and SEMs of EAD/FID ratios obtained from the GC-EAD analysis of the LP). The numbers at the *x*-axis represent the respective compounds as listed in Table 1. Specifically: (**4**) dihydro-3-methyl-2-(3*H*)-furanone, $I_{R\text{-}EAD}$ = 951; (**29**) linalool, $I_{R\text{-}EAD}$ = 1100; (**36** and **37**) ethyl octanoate/ethyl (*E*)-3-octenoate, $I_{R\text{-}EAD}$ = 1195/1198; (**42**) geraniol, $I_{R\text{-}EAD}$ = 1252; (**54**) geranyl acetate, $I_{R\text{-}EAD}$ = 1385; and (**63**) (*E*,*E*)-*α*-farnesene, $I_{R\text{-}EAD}$ = 1504. The figure shows that ethyl octanoate and ethyl (*E*)-3-octenoate are the most active compounds followed by linalool and (*E*,*E*)-*α*-farnesene. No significant differences in the perception of the major antennally active compounds between the sexes were observed (*t* test, *N* = 10).

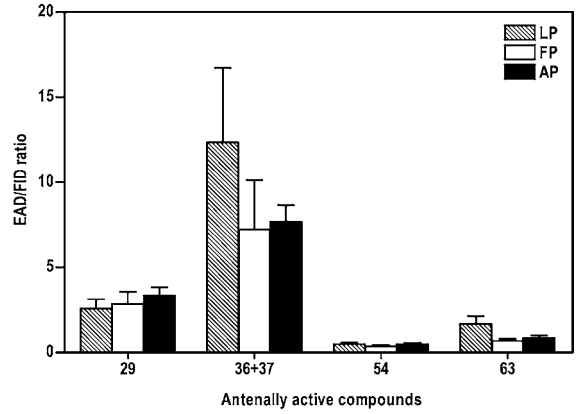

Figure 4. Comparison of female antennal sensitivities for the most active components of *C. capitata* male sex pheromone (the bars represent means and SEMs of FID/EAD ratios calculated from GC-EAD analyses of LPs and WPs). The numbers at the *x*-axis represent the respective compounds as listed in Table 1. Specifically: (**29**) linalool, $I_{R\text{-}EAD}$ = 1100; (**36** and **37**) ethyl octanoate/ethyl (*E*)-3-octenoate, $I_{R\text{-}EAD}$ = 1195/1198; (**54**) geranyl acetate, $I_{R\text{-}EAD}$ = 1385; and (**63**) (*E*,*E*)-*α*-farnesene, $I_{R\text{-}EAD}$ = 1504. The figure shows that the perception of linalool and geranyl acetate is similar in all populations tested. Interpopulation differences were observed in the perception of (*E*,*E*)-*α*-farnesene.

PCA and RDA. The results of PCA are depicted in Figure 5. The PCA shows a good separation of the LPs and WPs, indicating that the composition of the male pheromones is specific in each of the populations. The two principal components (PC1 and PC2) together accounted for 90% of the total variability. The subsequent RDA confirmed significant differences in the chemical composition between each pair of the three populations (p = 0.001). The contribution of particular compounds to overall differences is depicted in Figure 6. The compounds dihydro-5-methyl-2(3*H*)-furanone (**5**), *n*-hexyl acetate (**17**), and indene (**24**) are characteristic for WPs, while the compounds dihydro-3-methyl-2(3*H*)-furanone (**4**), sabinene (**8**), myrcene (**13**), ethyl (*E*)-3-hexenoate (**16**), 2-ethyl hexan-1-ol (**20**), (*Z*)-*β*-ocimene (**22**), (*E*)-*β*-ocimene (**23**), linalool (**29**), *allo*-ocimene (**31**), 2-ethylhexyl acetate (**33**), *α*-terpineol (**38**), bornyl acetate (**46**), indole (**47**), methyl geranate (**49**), neryl acetate (**51**), (*Z*)-*β*-farnesene (**60**), *α*-

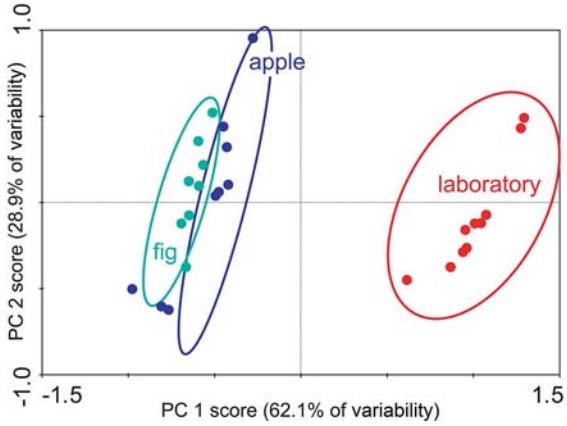

Figure 5. Results of the multivariate PCA of the sex pheromone of the males of *C. capitata* originating from three different populations (apple: wild AP; fig: wild FP; and laboratory: LP). The three populations are clearly segregated. Each symbol on the plot represents one sample (blue, AP; green, FP; and red, LP). The numbers of the analyzed samples (*N*) for the AP, FP, and LP populations were 8, 9, and 10, respectively.

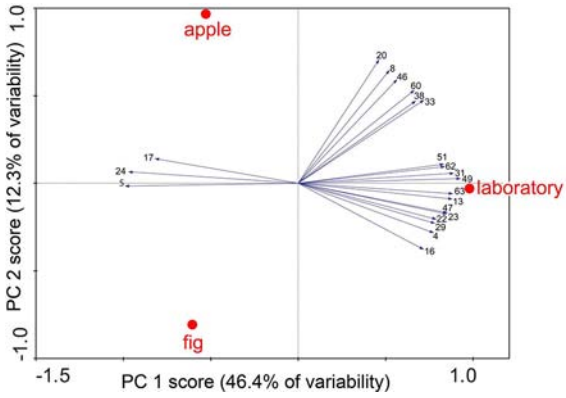

Figure 6. Results of the multivariate redundant analysis (RDA) of the sex pheromone of the males of *C. capitata* originating from three different populations (apple: wild AP; fig: wild FP; and laboratory: LP). The vertical dashed line separates the LP and WPs, and the horizontal line separates the FP and the AP. The arrows represent 21 compounds that characterize the respective populations. The numbering of the compounds corresponds to Table 1. The compounds dihydro-5-methyl-2(3*H*)-furanone (**5**), *n*-hexyl acetate (**17**), and indene (**24**) are characteristic for WPs, while the compounds dihydro-3-methyl-2(3*H*)-furanone (**4**), sabinene (**8**), myrcene (**13**), ethyl (*E*)-3-hexenoate (**16**), 2-ethyl hexan-1-ol (**20**), (*Z*)-*β*-ocimene (**22**), (*E*)-*β*-ocimene (**23**), linalool (**29**), *allo*-ocimene (**31**), 2-ethylhexyl acetate (**33**), *α*-terpineol (**38**), bornyl acetate (**46**), indole (**47**), methyl geranate (**49**), neryl acetate (**51**), (*Z*)-*β*-farnesene (**60**), *α*-humulene (**62**), and (*E,E*)-*α*-farnesene (**63**) are relatively more abundant in the LP.

humulene (**62**), and (*E,E*)-*α*-farnesene (**63**) are relatively more abundant in LPs. The differences among the WPs and LP are evident. When all three populations are depicted on one figure (Figure 5), AP and FP populations look like one cluster. However, when LP is omitted, the segregation between AP and FP is clear (Supporting Information). Subsequent PCA and RDA analysis for WPs showed separate clusters for AP and FP. The two principal components (PC1 and PC2) together accounted for 75% of the total variability. The RDA analysis confirmed significant differences between the two WPs (p = 0.001, Supporting Information).

DISCUSSION

Our data show that laboratory males produce more pheromone of different quality in comparison with WPs. Our data also suggest that the pheromone composition may be influenced by larval food.

The pheromone comparison was based on an evaluation of 63 compounds, 25 of which were reported for the first time as part of the *C. capitata* male sex pheromone. These new compounds are *α*-thujene, *α*-pinene, camphene, sabinene, *β*-pinene, Δ3-carene, *α*-terpinene, (*Z*)-linalool oxide, *allo*-ocimene, neo-*allo*-ocimene, isomenthone, isomenthol, geranial, *α*-terpinyl acetate, bornyl acetate, *α*-copaene, geranyl acetone, dihydro-5-methyl-2(3*H*)-furanone, 2-octanone, undecanal, 2-ethyl hexanal, 2-decenal, 2-undecenal, 1-octen-3-ol, and 2-octenoic acid. Of all of the identified compounds, 14 elicited significant antennal responses [these are the terpenoids *β*-pinene, linalool, geranyl acetate, (*E*)-caryophyllene, (*E,E*)-*α*-farnesene, the aliphatic compounds, ethyl (*E*)-3-octenoate, ethyl octanoate, 6-methyl-5-hepten-2-one, and aromatics dihydro-3-methyl-2(3*H*)-furanone and indole]. Linalool, ethyl (*E*)-3-octenoate, geranyl acetate, dihydro-3-methyl-2(3*H*)-furanone, 6-methyl-5-hepten-2-one, indole, and (*E,E*)-*α*-farnesene were already reported as antennally active constituents of medfly male sex pheromone in previous studies.[30,31] The volatiles *β*-pinene, ethyl octanoate, indole, and (*E*)-caryophyllene have not yet been reported as antennally active constituents of male pheromone in *C. capitata*, but they have been found as antennally active volatile constituents of ripe mango and sugar baits.[31,32] Ethyl octanoate was also found in *C. capitata* male salivary glands.[33] Isomenthone, geraniol, bornyl acetate, and geranyl acetone are newly reported EAD-active compounds for medfly. Many medfly male pheromone constituents occur among plant volatiles.[34] Some antennally active pheromone constituents found in this study are used as semiochemicals for communication purposes.[35] Thus, isomenthone functions as kairomones for *C. capitata*.[36] Ethyl octanoate is an attractant for *Anastrepha ludens* and *A. obliqua*.[37−39] Ethyl (*E*)-3-octenoate and geranyl acetate are pheromonally attractive components for females of *C. capitata*.[40] Geranyl acetate is a pheromone component in *Rhagoletis cerasi*.[41] (*E*)-caryophyllene represents an attractant of *Rhagoletis pomonella*.[42] (*E,E*)-*α*-Farnesene represents antennally and behaviorally active pheromone component in three tephritid species, namely, *C. capitata*, *A. ludens*, and *A. suspensa*.[43−45]

Our observation of the high complexity of the medfly male sex pheromone agrees with previous studies.[30,33,40,45−51] Foremost attempts to determine the compounds responsible for pheromone attraction focused initially on the most abundant compounds.[6,30,40,45,48] Synthetic blends of major pheromone components [e.g., (*E,E*)-*α*-farnesene, 1-pyrroline, geranyl acetate, ethyl acetate, and ethyl (*E*)-3-octenoate] attracted females, but the natural male pheromone was far more powerful, indicating that other pheromone components are involved. Light et al.[49] found that less abundant components contribute to pheromone attractiveness. Using GC-EAD, Cossé et al.[31] disclosed four compounds with biological activity from male pheromone and three compounds from ripe mango headspace samples [these were the pheromone constituents ethyl (*E*)-3-octenoate, geranyl acetate, (*E,E*)-*α*-farnesene, and linalool, mango constituents (*E*)-caryophyllene, *β*-pinene, ethyl octanoate, and *α*-copaene]. In spite of all of these discoveries, the efficiency of the medfly

monitoring system based on the male sex pheromone is still far from being optimal.[50,52−54] In this view, the newly discovered pheromone component ethyl octanoate might be considered as a promising candidate to investigate.

There is a growing body of evidence showing that artificial fruit fly rearing changes reproductive behavior as compared to WPs.[19,55] Our observation that the sex pheromones of laboratory and wild males differ both qualitatively and quantitatively provides another aspect to the general consensus. Among other factors, larval food quality may be responsible for the observed differences. Although adult flies are anautogenous, nutritional reserves carried over from the larval development provide a distinct reproductive advantage.[13] Higher nutritional levels in the larval diet accelerate medfly maturation.[12,13,56,57] In agreement with the available knowledge, there is a great probability that well-fed laboratory males have more resources to produce more pheromone. Besides food, also, rearing conditions might affect adult reproduction success. Different selection pressures in the wild and in the laboratory may result in complex behavioral and physiological differences.[19] We can speculate on whether the laboratory environment can enhance a selection of males with higher pheromone production to overcome the high chemical background in a crowded environment and improve the signal-to-noise ratio of premating signaling.

Qualitative differences between the male pheromones from different populations reported in the present study may be related to differences in larval food, since adults were provided with the same treatment (water and a mixture of cane sugar and brewer's yeast 3:1). The effects of different preadult rearing environments on adult mating behavior and mate recognition signals are well assessed in cactophilic *Drosophila mojavensis*.[58]

We observed that in the LP both sexes possess similar sensitivity to major male sex pheromone components and thus an equal competence to perceive the sex pheromone. Unfortunately, because of the limited amount of available wild material, we do not have enough data to allow a comparison between the sexes in WPs. Interpopulation differences in antennal sensitivity of females to major antennally active male pheromone components were observed only for (*E*,*E*)-α-farnesene. Further experiments are needed to confirm the absence of sex-specific differences in pheromone perception in WP and to determine more precisely interpopulation differences in pheromone perception.

Using GC × GC/TOFMS, which allows better separation of coeluting compounds and significant improvement of detection sensitivity,[59] α-copaene was detected in the pheromone of both LPs and WPs. This sesquiterpene, originally found as a constituent of angelica (*Angelica archangelica*) seed oil, is highly attractive for medflies.[60] It is present in many medfly host plants[61] and represents a powerful attractant and pheromone enhancer.[62−65] We found that the concentration of α-copaene was higher in wild males than in the laboratory ones. Previously, the presence of α-copaene in the pheromone of wild males was reported by Mavraganis et al.[33] and Gonçalves et al.[50] Because the authors used wild flies captured as adults from nature, they assumed that α-copaene was acquired during post-tenereal period. Our laboratory flies could not acquire α-copaene during adulthood; thus, this compound is likely to originate from the larval food.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure displays the results of multivariate RDA of sex pheromone of the males of *C. capitata* originating from two different WPs. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

GC × GC/TOFMS, two-dimensional gas chromatography with time-of-flight mass spectrometric detection; GC-EAD, gas chromatography with electroantennographic detection; $I_{R\text{-}GC}$, retention indices for gas chromatography with time-of-flight mass spectrometric detection; $I_{R\text{-}EAD}$, retention indices for gas chromatography with flame ionization detection and electroantennographic detection; FID, flame ionization detection; PCA, principal component analysis; RDA, redundancy analysis; SIT, sterile insect technique; FAO/IAEA, Food and Agriculture Organization/International Atomic Energy Agency; RSD %, relative standard deviation; LP, laboratory population of *C. capitata*; WPs, wild populations of *C. capitata*; AP, apple population; FP, fig population.

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