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COMPARISON OF THE VOLATILE COMPONENTS RELEASED BY CALLING MALES OF *CERATITIS CAPITATA* (DIPTERA: TEPHRITIDAE) WITH THOSE EXTRACTABLE FROM THE SALIVARY GLANDS

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ABSTRACT

The volatile compounds released by calling males of *Ceratitis capitata* and those that were extracted from the salivary glands with *n*-hexane were analyzed by gas chromatography-mass spectrometry. Twelve of the 24 compounds identified in the released volatiles, namely, 2-heptanone, 2,5-dimethylpyrazine, 3-octanone, ethyl hexanoate, methyl heptanoate, 2-ethyl-1-hexanol, limonene, indene, ethyl heptanoate, methyl octanoate, α-*trans*-bergamotene and (*E,E*)-α-farnesene, also were detected in the glandular extract. The similarities found in the chemical profiles of the released volatiles and of the salivary gland suggest that the latter is the storage site, and also perhaps the site of synthesis, of some of the pheromone components in this species of fruit fly.

Key Words: *Ceratitis capitata*, sex attractant, pheromone, aeration extract; salivary gland extract, gas chromatography-mass spectrometry

RESUMEN

Os compostos voláteis liberados por machos de *Ceratitis capitata* em chamamento e os de suas glândulas salivares foram extraídos e analisados qualitativamente por cromatografia gasosa acoplada à espectrometria de massas. Comparando a composição dos extratos de aeração com a dos extratos de glândulas salivares observou-se que 12 dos 24 compostos identificados entre os constituintes voláteis liberados foram também encontrados nestas glândulas. Os compostos coincidentes foram: 2-heptanona, 2,5-dimetilpirazina, 3-octanona, hexanoato de etila, heptanoato de metila, 2-etil-1-hexanol, limoneno, 1-H-indeno, heptanoato de etila, octanoato de metila, α-*trans*-bergamoteno e (*E,E*)-α-farneseno. As similaridades encontradas na composição dos dois tipos de extratos sugerem que estas glândulas são os locais de armazenamento, e talvez de síntese, dos compostos coincidentes na espécie.

Translation by the authors.

The Mediterranean fruit fly, *Ceratitis capitata* Wiedemann (1824), is a polyphagous insect (Light et al. 1988; Liquido et al. 1991; Jang et al. 1989). It is the most invasive of all members of the Tephritidae (Zucchi 2001), and causes extensive damage to fruit worldwide. It was reported nearly 50 years ago that the volatiles released by *C. capitata* males attract females (Feron 1959). Since that time various studies have been conducted in order to identify the components of the pheromone mixture for possible control of field populations (Jacobson et al. 1973; Ohinata et al. 1977; Baker et al. 1985; Jang et al. 1989; Cossé et al. 1995). While the exact composition of the complex pheromone still remains unknown, numerous volatile components released by males have been identified (Jacobson et al. 1973; Ohinata et al. 1977; Baker et al. 1985; Jang et al. 1989; Cossé et al. 1995).

Although males of *C. capitata* release the putative pheromone components from the proboscis, the anus and from the cuticular surface, the volatile components may be synthesized in, and/or stored in, different body structures including the salivary and pleural abdominal glands, the crop, the gut and the anal gland (Nation 1981, 1989, 1990; Teal et al. 1999; Lu & Teal 2001). Some of the components of the pheromone mixture are produced and released from salivary glands in some *Anastrepha* species (Nation 1989, 1990; Teles 1987; Lima et al. 1996). The salivary glands may perform the same function in males of *C. capitata* (Nation 1989, 1990; Teles, 1987; Lima et al., 1996; Ibañes-López & Cruz-López, 2001), but

there are no reports concerning the composition of the salivary glands. The aim of the present study was to compare the chemical profiles of the salivary glands with the mixture of volatiles released by calling males of *C. capitata* in an attempt to elucidate the pheromone composition of this commercially important pest.

Materials and Methods

Insect Population

The wild population of male *C. capitata* employed in the present study were obtained from larvae collected from infested starfruit harvested from a domestic orchard located in the town of Rio Largo (09°28'42"S / 35°51'12"W; altitude 39 m), in the state of Alagoas, Brazil. Larvae were placed for pupation in boxes (44 × 35 × 25 cm) constructed of expanded polystyrene and containing vermiculite. After 13 to 15 days, adult male and female flies emerged and were separated into glass tanks (30 × 20 × 15 cm) that were maintained in the Chemical Entomology Laboratory at the Federal University of Alagoas (Maceió-AL, Brazil). Flies were held at a temperature of 25 ± 1°C with 60% relative humidity under a 14 h photoperiod, and were fed a mixture of sucrose and brewer's yeast (2:1, w/w).

Extraction of Salivary Glands of Calling Males

Eleven days after the emergence of adult flies, salivary glands of 10 calling males were removed under water by using entomological forceps and the aid of a stereoscopic microscope. The glands were placed into 2-mL vials, each containing 1 mL of HPLC grade *n*-hexane (Aldrich). The vials were sealed and stored in a freezer until required for analysis. This procedure was replicated 8 times.

Trapping of Volatiles Released by Calling Males

A group of 20 calling males (11 days post-emergent) was submitted to aeration for a period of 3 h between 06.00 and 09.00 h when *C. capitata* is known to be sexually active (Gonçalves 2005). Insects were placed in a glass desiccator (180 mm high, 200 mm diameter) that had been modified by the addition of an inlet tube containing activated charcoal to filter the incoming air, and an outlet tube containing Tenax® (100 mg; Chrompack) to adsorb the released volatiles. Tenax was initially washed with hexane to remove non-polar contaminants and subsequently with methanol to remove polar contamination. After each solvent, the Tenax trap was allowed to dry at room temperature in a modified electric oven (Walita, Microchef Luxo) with nitrogen flowing through it for a few minutes. The trap was then heated for 3 h at 280°C. The N_2 flow rate was 1 L/min, flowing constantly and measured with an airflow meter (ELE International Ltd ELE 503-070). An air flow of 0.5 L/min was induced through the desiccator containing the flies by connecting a water vacuum pump to the outlet of the tube containing the adsorbent. Water, sucrose, and brewer's yeast (2:1 w/w), were supplied throughout the assay period. Following aeration, the Tenax adsorbent was removed from the outlet tube of the apparatus and the trapped volatiles were eluted with 1 mL of HPLC-grade n-hexane. Eluates were transferred to individual 2-mL glass ampoules, which were sealed and stored in a freezer until required for analysis. A blank aeration experiment, in which water and the dietary materials (but no insects) were placed in the desiccator, was carried out in order to determine if any of the trapped volatile materials derived from the food. This experiment was replicated 8 times and, for each replicate, the insects were replaced by new ones of the same age.

Gas Chromatographic-Mass Spectrometric (GC-MS) Analyses

Prior to the analysis of hexane extracts, each sample was concentrated under a gentle stream of nitrogen at room temperature to a final volume of 150 µL. Aliquots (1 µL) were injected into a Shimadzu model 17A gas chromatograph, equipped with a Shimadzu non-polar capillary column (30 m × 0.25 mm i.d.; 0.5 µm polydimethylsiloxane film) and coupled to a Shimadzu model QP 5050A mass selective detector. The chromatographic conditions were as follows: oven temperature - initially 30°C and increased to 250°C at a rate of 8°C/min; injector (splitless) temperature - 200°C; detector temperature - 270°C; carrier gas (helium) flow rate - 1 mL/min; MS ionization energy - 70 eV.

Components were identified by comparison of their retention times and MS fragmentation patterns with those of authentic standards (obtained commercially or laboratory-prepared). Identities were confirmed by GC analyses of standards and extracts under identical conditions. A solution containing an isomeric mixture of α-farnesene, a gift from Prof. E. D. Morgan (Keele University, Keele, UK), was used to confirm the identity of (*E,E*)-α-farnesene. An authentic sample of 2-methyl-4-heptanone was kindly provided by Dr. N. Oldham (Oxford University, Oxford, UK) for comparison purposes.

In cases where standard compounds were not available, identifications were carried out by comparison with reference spectra in the Wiley database 275, the Registry of Mass Spectral Data (McLafferty & Stauffer 1989), Stokes et al. (1983), and Rocca et al. (1992).

Preparation of Esters

Authenticate standards of the methyl and the ethyl esters of hexanoic, heptanoic, and octanoic

acids were prepared by mixing alcohol (30 µL) with acetic acid (30 µL) in a Keele micro-reactor and adding concentrated sulphuric acid (10 µL) (Attygalle & Morgan 1986). The resulting mixture was heated for 12 h at 120°C, neutralized with the minimum quantity of sodium bicarbonate and extracted with *n*-hexane (500 µL). The extracts were analyses by GC-MS and the identities of the products confirmed.

Results

Components Extracted from Salivary Glands

The hexane extracts obtained from salivary glands of *C. capitata* calling males were characterized by the presence of a mixture of 14 volatile compounds consisting of an aromatic hydrocarbon, alcohols, ketones, esters, nitrogen compounds, and terpenoids (Table 1). 2-Heptanone was the most abundant compound followed by (*E,E*)-α-farnesene, ethyl hexanoate, 2,5-dimethylpyrazine, and 1-nonanol: together these components accounted for 71.9% of the total extract. Limonene was the only monoterpene identified in the salivary gland extract.

Volatiles Released by Calling Males

The volatile mixture released by calling males of *C. capitata* was characterized by 24 components (Table 2) belonging to chemical classes similar to those found in the extracts of salivary glands. The major component was (*E,E*)-α-farnesene, followed by geranyl acetate and 3-ethyloctenoate: together these compounds accounted for 46.56% of the total extract. Minor components of the extract included 2-heptanone, ethyl hexanoate, 2,5-dimethylpyrazine, indol, and 3-octanone. Limonene and (*E*)-β-ocimene were the only monoterpene hydrocarbons identified in the volatiles mixture.

Discussion

Twelve of the 14 compounds identified in the *n*-hexane extracts of salivary glands of *C. capitata* calling males were present in the more complex volatile mixture released by the males. While 1-nonanol and ethyl octanoate were the only compounds exclusive to the salivary gland extracts, the proportions of 2-heptanone, 2,5-dimethylpyrazine, ethyl hexanoate, and (*E,E*)-α-farnesene varied significantly between the *n*-hexane and the aeration extracts. In contrast, the volatiles released by calling males contained 12 exclusive compounds, namely 1-heptanol, linalool, (*Z,Z*)-3,6-nonadien-1-ol, 2-phenylethyl acetate, ethyl 3-octenoate, 2,6-dimethylpyrazine, indol, 2-methyl-4-heptanone, (E)-β-ocimene, geranyl acetate, *trans*-caryophyllene, and α–copaene.

Seventeen compounds, namely, 1-heptanol, 2-heptanone, 2-phenylethyl acetate, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2-methyl-4-heptanone, 3-octanone, 1-nonanol, methyl heptanoate, 2-ethyl-1-hexanol, indene, ethyl heptanoate, (*Z,Z*)-3,6-nonadien-1-ol, ethyl octanoate, *trans*-caryophyllene, α-copaene, and α-*trans*-bergamotene, were identified in salivary gland extracts or the aeration mixture of calling males of *C. capitata* for the first time. Nine compounds previously reported present in *C. capitata* were detected in the aeration or salivary gland extracts investigated in the present study, namely, ethyl 3-octenoate, geranyl acetate, (*E,E*)-α-farnesene, and linalool (Baker et al. 1985; Jang et al. 1989),

Table 1. Content of extracts of salivary glands of calling males of *C. capitata* collected in the field as larvae infesting starfruit

Compound[a]	Retention time (min)	Percentage composition[b]
2-Heptanone	8.14	19.57 (± 3.45)
2,5-Dimethylpyrazine	8.58	12.33 (± 2.06)
3-Octanone	10.26	6.32 (± 3.16)
Ethyl hexanoate	10.60	13.93 (± 1.79)
1- Nonanol	10.71	10.84 (± 2.37)
Methyl heptanoate	11.13	2.93 (± 1.19)
2-Ethyl-1-hexanol	11.29	2.01 (± 0.65)
Limonene	11.39	3.43 (± 1.01)
Indene	11.43	2.99 (± 0.67)
Ethyl heptanoate	12.63	2.93 (± 0.79)
Methyl octanoate	13.16	2.19 (± 0.84)
Ethyl octanoate	14.56	4.49 (± 1.23)
α-*trans*-Bergamotene	19.79	0.97 (± 0.19)
E,E-α-Farnesene	19.99	15.02 (± 1.25)

[a]Peaks were identified by comparison of retention times and mass spectral data with those of authentic samples.
[b]Values shown are means (± standard error: $n = 8$).

TABLE 2. CONTENT OF AERATION EXTRACTS OF CALLING MALES OF *C. CAPITATA* COLLECTED IN THE FIELD AS LARVAE INFESTING STARFRUIT.

Compound[a]	Retention time (min)	Percentage composition[b]
2-Heptanol	6.40	2.50 (± 0.86)
2-Heptanone	8.13	9.11 (± 2.14)
2-Phenylethyl acetate	8.17	1.61 (± 0.53)
2,5-Dimethylpyrazine	8.53	5.68 (± 1.77)
2,6-Dimethylpyrazine	8.73	0.49 (± 0.13)
2-Methyl-4-heptanone	8.91	0.48 (± 0.20)
3-Octanone	10.25	4.36 (± 1.26)
Ethyl hexanoate	10.59	6.87 (± 1.05)
Indol	10.71	5.17 (± 2.18)
Methyl heptanoate	11.12	1.21 (± 0.49)
2-Ethyl-1-hexanol	11.29	1.62 (± 0.57)
Limonene	11.39	3.58 (± 0.91)
Indene	11.43	2.04 (± 0.65)
(*E*)-β-Ocimene	11.71	0.29 (± 0.07)
Ethyl heptanoate	12.63	1.38 (± 0.49)
Linalool	12.78	1.03 (± 0.29)
(*Z,Z*)-3,6-Nonadien-1-ol	13.16	1.35 (± 0.22)
Methyl octanoate	13.75	0.87 (± 0.36)
Ethyl 3-octenoate	14.56	11.09 (± 3.08)
Geranyl acetate	17.78	13.34 (± 1.57)
trans-Caryophyllene	18.07	2.42 (± 0.54)
α-Copaene	18.45	0.95 (± 0.37)
α-*trans*-Bergamotene [c]	19.76	0.42 (± 0.12)
E,E-α-Farnesene	19.99	22.13 (± 3.03)

[a]Peaks were identified (except as indicated otherwise) by comparison of retention times and mass spectral data with those of authentic samples.
[b]Values shown are means (± standard error: $n = 8$).
[c]Peak identified by comparison of mass spectral data with literature values (see Materials and Methods).

ethyl hexanoate, limonene, (*E*)-β-ocimene, and methyl octanoate (Jang et al. 1989), and indol (Cossé et al. 1995).

Considering the 26 compounds presently identified in the aeration extracts obtained from *C. capitata* calling males with the 59 compounds previously reported by Jang et al. (1989), it is clear that the volatile mixture obtained in this study was much less complex than that described earlier. Such a difference may be due to dissimilar types of fruit from which the insects were obtained, and/or by different diet given to the insects during the larval phase (Gonçalves, 2001; Silva, 2005). Both factors may exert a direct influence on the volatiles produced and released by adult males.

By comparison with previous studies, it is apparent that (*Z,Z*)-3,6-nonadien-1-ol, *trans*-caryophyllene, α-*trans*-bergamotene, (*E,E*)-α-farnesene, limonene, and/or (*E*)-β-ocimene are common to the volatile emissions of *C. capitata*, *Anastrepha suspensa* and *A. ludens* (Rocca et al. 1992), and that limonene, (*Z*)-β-ocimene, and 2,5-dimethylpyrazine are common to *C. capitata* and *A. fraterculus* (Lima et al. 2001). Analogous to the situation for *A. obliqua* (Gonçalves 2005), linalool, (*Z,Z*)-3,6-nonadien-1-ol, (*E*)-β-ocimene, geranyl acetate, *trans*-caryophyllene, and α-copaene were identified only in the aeration extracts obtained from calling males, suggesting that these compounds are synthesized and stored in different body structures in insects of the genera *Ceratitis* and *Anastrepha*. The absence of (*Z,Z*)-3,6-nonadien-1-ol in solvent extracts of salivary glands is in agreement with the proposal of Nation (1989) that the nonenols are stored in the intestine of *A. suspensa*. According to this author, the sesquiterpenes α–*trans*-bergamotene and (*E,E*)-α-farnesene are produced by the salivary glands and released from the proboscis, while the nonenols are released from the everted anal tissue.

Considering that 12 of the 24 compounds identified in the aeration extracts from calling males of *C. capitata* were also present in extracts of the salivary glands, it is reasonable to suggest that such glands are the site of storage, and possibly also of synthesis, of these compounds in *C. capitata*. Detailed biosynthetic and histological studies involving the salivary glands would be necessary in order unambiguously to confirm this hypothesis.

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