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PHEROMONE COMMUNICATION IN *ANASTREPHA OBLIQUA* (DIPTERA: TEPHRITIDAE): A COMPARISON OF THE VOLATILES AND SALIVARY GLAND EXTRACTS OF TWO WILD POPULATIONS

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ABSTRACT

The West Indian fruit fly *Anastrepha obliqua* Macquart (Diptera: Tephritidae) is one of the major pests on mango (*Mangifera indica* L.; Sapindales: Anacardiaceae) and starfruit (*Averrhoa carambola* L.; Oxalidales: Oxalidaceae) crop plantations in Brazil. Pheromone communication inter alia plays an important role in fruit-fly courtship behavior. In order to highlight the site of pheromone synthesis, we identified and compared the volatiles from the aeration extracts of calling males with the volatiles produced by their salivary glands in 2 wild populations of *A. obliqua* collected from mangoes and starfruits. In addition, we performed a series of bioassays to compare the biological significance of both extracts. In total, 36 volatile compounds were identified, with 8 of them being shared by the 2 populations and the 2 extract types. Linalool and α-copaene were exclusively found in the aeration extract while ethyl heptanoate, methyl octanoate, and 1-nonanol were detected only in the salivary-gland extracts. The chemical profiles of the volatiles from the aeration extracts and from the salivary-gland extracts differed significantly between the 2 populations as well as the chemical profiles of both extracts within each population. The quantities of the 8 shared compounds generated a variability of more than 60% in the mango population and 80% in the starfruit population. The similarities observed between the chemical profiles of the aeration extracts and the salivary-gland extracts suggest that the latter could be the storage site and probably also the production site of some pheromone components in this fruit-fly species. This hypothesis is supported by the comparable biological activities of both extracts in terms of their attractiveness for conspecific females.

Key Words: salivary glands, volatile compounds, sex attractant, wild population, gas chromatography – mass spectrometry

RESUMO

A mosca das frutas do oeste indiano Macquart (Diptera: Tephritidae) é uma das maiores pragas da cultura de manga (*Mangifera indica* L.; Sapindales: Anacardiaceae) e carambola (*Averrhoa carambola* L.; Oxalidales: Oxalidaceae) no Brasil. A comunicação através de feromônios, entre outras coisas, possui uma importante função no comportamento de côrte em moscas das frutas. A fim de elucidar o local de síntese de feromônios, identificamos e comparamos os voláteis obtidos através de extratos de aeração de machos em chamamento com os voláteis produzidos por suas glândulas salivares em duas populações selvagens de *A. obliqua*, coletadas a partir de mangas e carambolas. Além disso, realizamos uma série de bioensaios para comparar a significância biológica de ambos os extratos. No total, 36 compostos voláteis foram identificados, com 8 deles sendo comuns para as duas populações e os dois tipos de extratos. Linalol e α-copaeno foram exclusivamente encontrados nos extratos de aeração enquanto o heptanoato de etila, octanoato de metila e 1-nonanol foram detectados somente nos extratos de glândulas salivares. O perfil químico dos voláteis obtidos por aeração e a partir dos extratos de glândulas salivares diferiram significativamente

entre as duas populações bem como o perfil químico de ambos os extratos dentro de cada população. As quantidades dos 8 compostos compartilhados geraram uma variabilidade de mais de 60% na população de manga e 80% na população de carambola. As similaridades observadas entre o perfil químico dos extratos de aeração e extratos de glândulas salivares sugerem que este último pode ser o local de armazenamento e provavelmente também o local de produção de alguns componentes do feromônio desta espécie de mosca das frutas. Esta hipótese é suportada pelas atividades biológicas comparadas de ambos os extratos em termos de sua atratividade para fêmeas coespecíficas.

Palavras Chave: glândulas salivares, compostos voláteis, atraente sexual, população selvagem, Cromatografia gasosa - espectrometria de massas

Anastrepha is the largest genus of tephritid fruit flies and the most common pest in both the domestic and commercial plantations of mango, guava and starfruit in the northeast region of Brazil. Three *Anastrepha* species, *A. fraterculus* Wiedemann, *A. obliqua* Macquart and *A. sororcula* Zucchi, are of particular concern as pests of production and exports (Malavasi et al. 2000).

Anastrepha obliqua is found in many countries of the Americas (Saldanha & Silva 1999). Its occurrence has been recorded from the Caribbean, southern Texas, USA, and south through Mexico to Panama and from most South American countries, but not Chile and southern Argentina. In Brazil, it is the only species found in the state of Maranhão and is the second most important *Anastrepha* species present in the states of the southern, southeastern and northeastern regions. As a polyphagous species, *A. obliqua* attacks fruits from diverse cultures, but its main fruit hosts in Brazil are *Mangifera indica* L. (Sapindales: Anacardiaceae), *Spondias purpurea* L. (Sapindales: Anacardiaceae), *S. dulcis* L., *S. venulosa* (Engl.) Mart. ex Engl., *S. cytherea* Sonn. and *S. tuberosa* Arruda ex Koster (Malavasi et al. 2000). The direct damage done by these insects to the host fruits stems from the deposition of their eggs inside the fruit, which serves as food reservoir for the larvae. Indirect damage is also caused by the microorganisms which gain access to the fruit's interior through the perforation made by the female fly's ovipositor. These damages degrade the quality of the host fruit (Nascimento et al. 1992). *Anastrepha* flies use chemical cues to search for and select appropriate food sources, partners for reproduction and oviposition sites. Among these chemical signals, the male-produced sex pheromones have major importance. They consist of a mixture of volatile compounds, produced and released from different structures of the male's body, such as rectum, cuticle pores, abdominal pleural glands, the intestine, the anal pouch and salivary glands (Nation 1981, 1989, 1990; Teal et al. 1999; Lu & Teal, 2001). The chemistry of the volatile compounds released by calling males have been studied in 4 *Anastrepha* species: *A. suspensa*, *A. ludens, A. obliqua* and *A. fraterculus* (Nation 1983, 1990; Battiste et al. 1983; Rocca et al. 1992; Heath et al. 2000; Lima et al. 2001; López-Guillen et al. 2011). However, only in 2 of these species, *A. fraterculus* and *A. obliqua*, were the male's salivary-gland secretions and attractiveness to females investigated (Lima et al. 2001; Ibañéz-López & Cruz-López 2001). Moreover, the *A. fraterculus* and *A. obliqua* individuals previously studied came from laboratory strains reared on an artificial diet.

We have recently demonstrated that the chemistry of the volatile mixture released by the calling males of the laboratory populations of *C. capitata* or *A. fraterculus* differ from the corresponding mixtures released by conspecific males of wild populations of these 2 species (Vaníčková et al. 2012a; Vaníčková et al. 2012b). Therefore, the present study has been carried out to compare the chemistry of the volatile mixture released by *A. obliqua* calling males from 2 wild populations and also to investigate the composition of the salivary-gland secretions, which are regarded as one of the main sources of pheromone components in tephritids (Nation 1981, 1989, 1990). The final goal of this research is to find a suitable design for a selective bait for the control of *A. obliqua* populations in the field.

Materials and Methods

Insect Populations

Larvae of 2 wild populations of *A. obliqua* were collected during the summer season from infested fruits that had been harvested from a domestic mango 'rosa' variety orchard located in the town of Maceió (S 9° 39' 39" W 35° 44' 6"; 5 m asl) and from a starfruit plantation located in the town of Rio Largo (S 9° 28' 42" W 35° 51' 12"; 39 m asl), both in the state of Alagoas, Brazil. The larvae, collected from the fruits, were placed for pupation in separate boxes (44 × 35 × 25 cm) constructed of expanded polystyrene and containing a mixture of washed sand and vermiculite. After 13-15 days, male and female adult flies emerged and were separated into

glass tanks (30 × 20 × 15 cm), which were kept in the Laboratory of Chemical Ecology at the Federal University of Alagoas (Maceió-AL, Brazil) at 14:10 h L:D, 25 ± 1 °C and 60% RH. The flies were fed a diet consisting of a mixture of muscovado sugar and brewer's yeast (3:1, w/w), along with water in a separate container. The identification of the species was based on the morphological characteristics of the female ovipositor. The specimens were identified and authenticated by Dr. Roberto Zucchi (taxonomist; ESALQ, Piracicaba, SP, Brazil).

Trapping Volatiles

Groups of *A. obliqua* male flies of 2 wild populations, each consisting of 20 sexually mature wild virgin males aged between 15 and 20 days, were submitted separately to aeration for a period of 3 h between 06.00 and 09.00 A.M., (Malavasi et al. 1983; Gonçalves 2005). The insects were placed in a glass desiccator (180 mm high; 200 mm diam) that had been modified by the addition of an inlet tube containing activated charcoal to filter the incoming air and of an outlet tube containing Tenax® (100 mg; Chromopack) to absorb the released volatiles. Tenax® was cleaned by washing first with hexane to remove non-polar contamination and subsequently with methanol to remove polar contamination. After using each solvent, the Tenax® trap was allowed to dry at room temperature in a gas chromatograph oven with nitrogen flowing through it for a few minutes. The trap was then heated in a GC oven for 3 h at 280 °C using nitrogen. The nitrogen 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 and a mixture of muscovado sugar and brewer's yeast (3:1, w/w) were provided throughout the assay period, and a blank aeration experiment involving water and the dietary materials, but without the insects, served as the negative control. The aeration experiments were repeated 10 times for each population, and fresh flies were employed for each repetition.

Extraction of the Salivary Glands of Calling Males

Fifteen days after the emergence of adult *A. obliqua* flies, salivary glands of 10 calling males from each wild population were removed under water by using entomological forceps and 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 10 times.

Chemical Analyses

After the aeration, the Tenax® adsorbent was removed from the outlet tube of the apparatus and the volatile compounds that had been trapped were eluted with HPLC grade *n*-hexane (3 mL; Aldrich). The resulting extract was divided into 3 portions each 1 mL, and then each portion was transferred to a separate 2 mL glass ampoule and stored in a refrigerator. One portion was used in a chemical analysis and the other 2 were kept for bioassay purposes. Prior to the analysis by gas chromatography-mass spectrometry (GC-MS), the hexane extracts were 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: oven temperature initially at 30 °C for 2 min and increased to 250 °C at a rate of 8 °C/min; the injector (splitless) temperature was 200 °C; the detector temperature was 270 °C; the carrier-gas (helium) flow rate was 1 mL/min; and the MS ionization energy was 70 eV. The components were identified by a comparison of their retention times, retention indices (*RI*) and MS fragmentation patterns. A series of *n*-alkanes (C_8–C_{22}; Sigma-Aldrich) were used for calculation of the *RI* of the identified compounds. Whenever possible, the identities were confirmed by the GC-MS analyses of the standards (Sigma-Aldrich) and extracts under identical conditions. In cases where standard compounds were not available, the identifications were carried out by a comparison with the reference spectra in the Wiley database 275, the Registry of Mass Spectral Data (McLafferty & Stauffer 1989), Stokes et al. (1983), Rocca et al. (1992), and Adams (2007). (*E,E*)-α-farnesene was identified by a comparison of its retention time and MS with a synthetic mixture of farnesene isomers kindly provided by Dr. Blanka Kalinová (Institute of Organic Chemistry and Biochemistry of the ASCR, Prague, Czech Republic). Authenticated standards of the methyl and ethyl esters of hexanoic, heptanoic and octanoic acids were prepared by mixing alcohol (30 µL) with acid (30 µL) in a Keele micro-reactor and adding concentrated sulfuric 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 analyzed by GC-MS and the identities of the products confirmed.

Behavioral Assays

In order to observe the attractiveness of the virgin mature female of *A. obliqua* (25-30 days old) to conspecific male aeration and salivary-gland extracts, a polystyrene box (28 × 10 × 15 cm) was used as a bioassay arena. For each replicate, 10 virgin females were marked with odorless and non-toxic dyes of different colors and introduced into the bioassay chamber used only once per replicate. Two filter paper discs (12 mm i.d.) containing either 30 µL of the aeration extract of one male-equivalent (1ME) or 30 µL of the salivary gland extract and 30 µL of hexane (control treatment) were placed in the bioassay arena in the opposite directions and the filter paper discs were allowed to dry. For 10 min, all the attempts exhibited by the females to approach the filter papers and the regions next to them were recorded along with the behavior exhibited by them. The behavior observed was touching the odor source using their proboscides. All of the behavioral assays were performed between 9.00 a.m. to 12.00 a.m.

Statistical Analyses

All the experiments were conducted with a completely randomized design. The data obtained from the behavioral assays were checked to verify their multivariate normality and residue homogeneity using the Liliefors and Levene tests, respectively. The Post-Hoc Tukey-HSD test was used for a multiple comparison and in all the cases the level of statistic significance was set to $\alpha = 0.05$. The data from the GC-MS analysis of the male emanations and salivary-gland extracts for each population were statistically evaluated using correspondence analysis (CA). 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 recalculated to percentage ratios. The focus scaling was set for interpopulation correlation; the population scores were divided by standard deviation. The statistical significance was assessed using canonical correspondence analysis (CCA) for unimodal data or redundancy analysis (RDA) for linear data. CCA and RDA are canonical variants of canonical or principal component analysis, respectively. In addition, the Monte Carlo permutation test (unrestricted permutations, $n = 999$) was employed. In the CCA and RDA analysis, the identities of the 2 populations and the source of the extract stood as a categorical predictor. The multivariate data analysis software CANOCO 4.5 (Biometrics, Plant Research) was used for these analyses.

Results

Chemical Analyses

The extracts obtained from aeration and salivary glands of the wild calling males of *A. obliqua* contained alcohols, aldehydes, esters, ketones and terpenes. In total, 36 compounds were identified in both extracts of both populations (Table 1). Linalool (19) and α-copaene (30) were found exclusively in the aeration extracts, which also contained a large amount of 1-heptanol (7), reaching up to about 50% in both populations. The salivary-gland extracts were characterized by the presence of ethyl heptanoate (20), methyl octanoate (21) and 1-nonanol (23) (Fig. 1).

Eight compounds, namely 2-heptanone (2), 1-heptanol (7), 3-octanone (8), ethyl hexanoate (10), 2-ethylhexan-1-ol (13), ethyl octanoate (27), (*E*)-α-bergamotene (34), and (*E,E*)-α-farnesene (36) were shared by the aeration extracts and salivary-gland extracts of the mango and starfruit populations and together generated 63.51% and 82.22% (for the mango population), 71.79% and 66.87% (for the starfruit population) of the total chemical variability. The CCA analysis of the complex mixtures is depicted in Fig. 1. The aeration extracts were the most chemically diversified. The CCA analysis has also revealed exclusive compounds for particular populations, emanations and gland extracts. 3-Hexanone (1), (*E*,*Z*)-3,6-nonadien-1-ol (22), δ-elemene (29), β-elemene (32), (*E*)-β-caryophyllene (33) and α-humulene (35) are characteristic for the aeration extract from the *A. obliqua* mango population. The starfruit-population emanation has also unique compounds: decane (11), (*Z*)-β-ocimene (15) and geranyl acetate (31). The salivary-gland extracts from the mango population are characterized by 1-H-indene (16), 1-octanol (17), 3-ethyl-2,5-dimethylpyrazine (18), 1-phenylpropanone (24) and decanal (28). The starfruit population is defined by 2,6-dimethylpyrazine (4), 4-methylheptan-3-ol (6) and 2-octanone (9). The differences between all 4 groups were confirmed to be significant at $P = 10^{-3}$ by the Monte-Carlo test (see the supplementary data).

For a detailed study of the population differences, RDA analyses were employed. The comparison of the aeration extracts and the salivary-gland extracts of the 2 populations is depicted in Fig. 2. It is obvious that the compounds most responsible for the population differences are those that are unique for the particular population (bold italic). However, a statistical analysis including only shared compounds also revealed significant differences between the populations (see the supplementary data). The population

TABLE 1. A LIST OF THE COMPOUNDS (AVERAGE ± RSD%) IDENTIFIED BY GC-MS IN THE AERATION EXTRACTS AND THE SALIVARY-GLAND EXTRACTS OF THE CALLING MALES OF *ANASTREPHA OBLIQUA* ORIGINATED FROM 2 WILD POPULATIONS COLLECTED IN THE FIELD AS LARVAE INFESTING STARFRUIT AND MANGO.

			Mango population		Starfruit population	
N.	Compound[a]	*RI*	Aeration extracts[c]	Salivary glands extracts[c]	Aeration extracts[c]	Salivary glands extracts[c]
1	3-Hexanone[b]	790	11.76 (± 2.31)	—	—	—
2	2-Heptanone[b]	893	1.47 (± 0.75)	21.09 (± 3.78)	4.62 (± 0.63)	20.82 (± 2.34)
3	2,5-Dimethylpyrazine[b]	912	—	4.61 (± 1.56)	1.18 (± 0.02)	9.02 (± 1.22)
4	2,6-Dimethylpyrazine[b]	914	—	—	—	3.19 (± 0.83)
5	2-Methyl-4-heptanone[b]	934	—	2.00 (± 0.89)	1.14 (± 0.21)	0.86 (± 0.31)
6	4-Methyl-3-heptanol[b]	965	—	—	—	0.57 (± 0.2)
7	1-Heptanol[b]	970	59.55 (± 5.32)	1.49 (± 0.74)	47.83 (± 1.31)	0.59 (± 0.07)
8	3-Octanone[a]	986	1.60 (± 0.16)	7.98 (± 2.45)	8.08 (± 0.94)	5.89 (± 1.12)
9	2-Octanone[a]	991	—	—	—	3.92 (± 0.98)
10	Ethyl hexanoate[a]	1004	7.65 (± 1.56)	30.34 (± 4.23)	19.46 (± 1.11)	31.05 (± 4.7)
11	Decane[b]	1005	—	—	0.55 (± 0.02)	—
12	Methyl heptanoate[b]	1015	—	3.63 (± 0.97)	1.08 (±0.01)	1.75 (± 0.12)
13	2-Ethylhexane-1-o1[b]	1030	1.13 (± 0.07)	3.34 (± 0.80)	0.39 (± 0.01)	2.21 (± 0.99)
14	Limonene[a]	1035	Tr	3.33 (± 0.03)	8.34 (± 0.05)	3.03 (± 1.15)
15	(*Z*)-β-Ocimene[b]	1034	—	—	0.32 (± 0.03)	—
16	1-H-Indene[b]	1053	Tr	0.79 (± 0.01)	—	—
17	1-Octanol[b]	1071	—	1.46 (± 0.01)	—	—
18	3-Ethyl-2,5-dimethylpyrazine[b]	1080	—	1.41 (± 0.06)	—	—
19	Linalool[a]	1100	2.41 (± 0.13)	—	0.37 (± 0.97)	—

RI – Retention index.
[a]Compounds identified by comparison with synthetic standards.
[b]Compounds identified by comparison with the Wiley database 275, the Registry of Mass Spectral Data (McLafferty & Stauffer 1989), Stokes et al. (1983), Rocca et al. (1992), and Adams (2007).
[c]Values shown are mean % of the relative abundance (±standard error; *N*=10). Tr – trace amount.

TABLE 1. (CONTINUED) A LIST OF THE COMPOUNDS (AVERAGE ± RSD%) IDENTIFIED BY GC-MS IN THE AERATION EXTRACTS AND THE SALIVARY-GLAND EXTRACTS OF THE CALLING MALES OF *ANASTREPHA OBLIQUA* ORIGINATED FROM 2 WILD POPULATIONS COLLECTED IN THE FIELD AS LARVAE INFESTING STARFRUIT AND MANGO.

			Mango population		Starfruit population	
N.	Compound[a]	*RI*	Aeration extracts[c]	Salivary glands extracts[c]	Aeration extracts[c]	Salivary glands extracts[c]
20	Ethyl heptanoate[b]	1190	—	3.00 (± 0.89)	—	2.31 2.38 (± 0.45)
21	Methyl octanoate[b]	1195	—	1.94 1.97 (± 0.07)	—	2.01 2.08 (± 0.78)
22	(*E,Z*)-3,6-Nonadien-1-ol[a]	1161	1.49 1.59 (± 0.18)	—	—	—
23	1-Nonanol[a]	1170	—	1.10 (± 0.04)	Tr	1.75 (± 0.04)
24	1-Phenylpropanone[b]	1180	—	1.02 (± 0.01)	—	—
25	3-Decanone[a]	1185	—	1.02 (± 0.02)	0.55 (± 0.07)	1.48 (± 0.10)
26	2-Decanone[a]	1193	—	1.86 (± 0.24)	0.21 (± 0.04)	1.37 (± 0.08)
27	Ethyl octanoate[a]	1195	0.42 (± 0.09)	4.74 (± 0.87)	0.78 (± 0.06)	1.37 (± 0.13)
28	Decanal[a]	1208	—	0.39 (± 0.01)	—	—
29	δ-Elemene[b]	1338	0.36 (± 0.05)	—	—	—
30	α-Copaene[b]	1367	1.19 (± 1.11)	—	3.44 (± 0.01)	—
31	Geranyl acetate[b]	1380	—	—	0.78 (± 0.09)	—
32	β-Elemene[b]	1392	0.56 (± 0.09)	—	Tr	—
33	(*E*)-β-Caryophyllene[b]	1415	7.27 (± 1.67)	—	—	—
34	(*E*)-α-Bergamotene[b]	1435	1.80 (± 0.03)	2.96 (± 0.67)	0.10 (± 0.86)	5.79 (± 1.34)
35	α-Humulene[b]	1475	0.72 (± 0.08)	—	—	—
36	(*E,E*)-α-Farnesene[a]	1509	0.51 (± 0.01)	0.49 (± 0.08)	0.78 (± 0.07)	0.87 (± 0.02)

RI – Retention index.
[a]Compounds identified by comparison with synthetic standards.
[b]Compounds identified by comparison with the Wiley database 275, the Registry of Mass Spectral Data (McLafferty & Stauffer 1989), Stokes et al. (1983), Rocca et al. (1992), and Adams (2007).
[c]Values shown are mean % of the relative abundance (±standard error; *N*=10). Tr – trace amount.

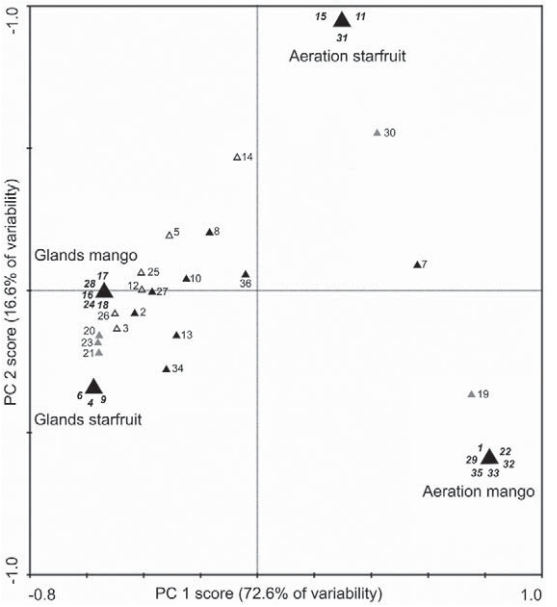

Fig. 1. A graphical result of the canonical correspondence analysis (CCA) of the compounds identified by GC-MS analyzes in the male aeration and salivary-gland extracts of 2 wild populations of *Anastrepha obliqua* (host fruit: mango and starfruit, N = 10 for both types of extracts and populations). The numbers represent the compounds from Table 1. The bold italic numbers stand for unique compounds for the population and extract type. The big black triangles represent groups of different populations and extracts; the small triangles stand for the compounds identified, the black triangles have been used for compounds shared by all four types of extracts [(2) 2-heptanone, (7) 1-heptanol, (8) 3-octanone, (10) ethyl hexanoate, (13) 2-ethylhexan-1-ol, (27) ethyl octanoate, (34) (*E*)-α-bergamotene and (36) (*E,E*)-α-farnesene], white for compounds shared by salivary-gland extracts of both populations and aeration extract of starfruit population [(3) 2,5-dimethylpyrazine, (5) 2-methylheptan-4-one, (12) methyl heptanoate, (14) limonene, (25) 3-decanone, (26) 2-decanone], gray for the exclusive compounds [(19) linalool, (30) α-copaene] found only in aeration extracts, and only in salivary-gland extracts [(20) ethyl heptanoate, (21) methyl octanoate, (23) 1-nonanol]. The first canonical axis explains 72.6% of the variability in the data set and the second 16.6%.

of *A. obliqua* pests on mango has a higher proportion of 2-methylheptan-4-one (5), 1-heptanol (7), methyl heptanoate (12,) 2-ethylhexan-1-ol (13), linalool (19), ethyl octanoate (27) and (*E*)-α-bergamotene (34), whereas the starfruit population is characterized by a higher abundance of 2-heptanone (2), 2,5-dimethylpyrazine (3), 2-methylheptan-4-one (5), 3-octanone (8), ethyl hexanoate (10), methyl heptanoate (12), limonene (14), 3-decanone (25), 2-decanone (26), α-copaene (30), and (*E*)-α-bergamotene (34) (Fig. 2).

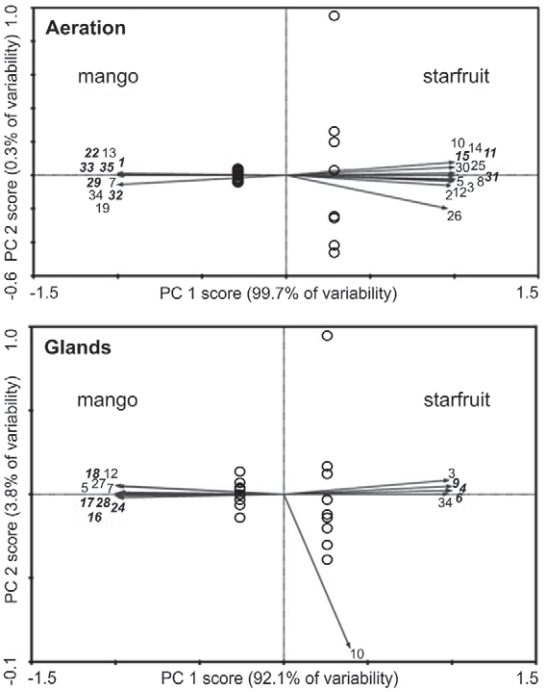

Fig. 2. The results of the multivariate redundant analysis (RDA) of the *Anastrepha obliqua* male aeration extracts (aeration) and salivary-gland extracts (glands) from the 2 wild populations. The empty circles represent a particular sample in the dataset (N = 10) and the numbers stand for the compounds identified. The bold italic numbers have been used for unique compounds for the population and extract type. The numbering corresponds to Table 1. The arrows show the quality (direction) and quantity (length) of the change in the data set with a comparison to the mean value of the compound. The first canonical axis explains 99.7% of the variability in the aeration extract and 92.1% in the salivary-gland extracts.

Behavioral Responses of *A. obliqua* Females to the Aeration and Salivary-Gland Extracts of Conspecific Males.

The virgin females of *A. obliqua* were as much attracted to the salivary-gland extract as they were attracted to the aeration extracts of calling males. These results differ from the control treatment as can be seen in Figs. 3 and 4.

Discussion

Both studied populations of *A. obliqua* produce 11 compounds in common, i.e., 1-heptanol, 3-octanone, ethyl hexanoate, 2-ethylhexan-1-ol, limonene, linalool, (*E,Z*)-3,6-nonadien-1-ol, ethyl octanoate, α-copaene, (*E*)-α-bergamotene and (*E,E*)-α-farnesene. Three of these compounds, i.e. (*Z,Z*)-3,6-nonadien-1-ol, (*E*)-α-bergamotene and (*E,E*)-α-farnesene, have also been detected in the

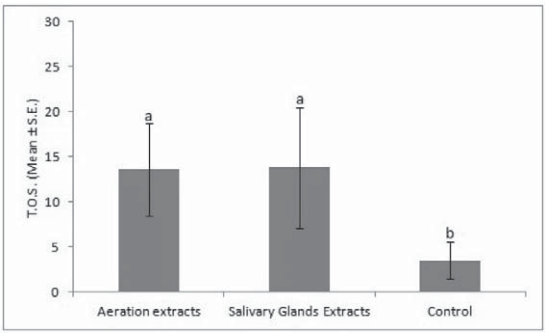

Fig. 3. The mean of the response of *Anastrepha obliqua* virgin females (from the wild population with the host fruit *Averrhoa carambola*) to the volatile conspecific male extracts from the salivary gland, to the volatiles from conspecific male aeration and to control. The response of females was touching the odor source using the proboscides (T.O.S.). The different lowercase letters represent the significant statistical difference by Tukey's HSD test ($P < 0.05$).

volatiles released by the males of *A. suspensa* and *A. ludens* (Nation 1983, 1990; Rocca et al. 1992). Limonene has previously been detected in the aeration extracts of *A. ludens* (Rocca et al. 1992) and of *A. fraterculus* (Lima et al. 2001), but its presence has not been detected in *A. suspensa*. The (*E,E*)- and (*Z,E*)-isomers of α-farnesene have been determined in the extracts of the salivary glands from the calling males of *A. fraterculus* (Lima et al. 1996, 2001) and also in the extracts of *A. obliqua* calling males (López-Guillén et al. 2011).

The lactones, anastrephin, epianastrephin and (3*E*,8*E*)-suspensolide, which have been identified in the aeration extracts of *A. suspensa* and *A. ludens* males (Esponda-Gaxiola 1977; Battiste et al. 1983, 1988; Nation 1983, 1990; Chuman et al. 1988; Rocca et al. 1992; Lu & Teal 2001), were not detected in the present study. This finding was also confirmed by López-Guillén et al. (2011). On the other hand, Lu & Teal (2001) reported the presence of anastrephin, epianastrephin and (3*E*,8*E*)-suspensolide in the oral secretion of *A. suspensa* males, whereas our study focused on the whole-body aeration and salivary-gland extracts of the calling males of *A. obliqua*.

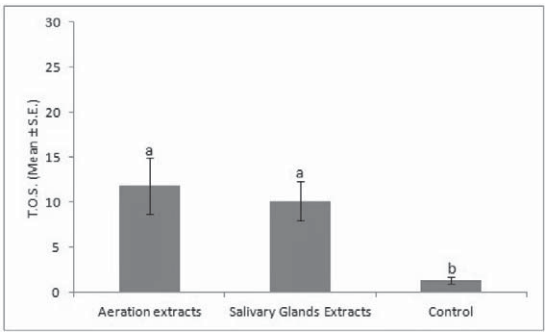

Fig. 4. The mean of the response of *Anastrepha obliqua* virgin females (from the wild population with the host fruit *Mangifera indica*) to the volatile male extracts from the salivary gland, to the volatiles from male aeration and to control. The response of females was touching the odor source using the proboscides (T.O.S.). The Tukey's HSD test, used for the data evaluation, did not show any significant difference ($P \leq 0.05$). The different lowercase letters represent a significant statistical difference.

(*Z*)-3-Nonenol, identified in *A. suspensa*, *A. ludens*, *A. fraterculus* and the Mexican population of *A. obliqua* (Battiste et al. 1983; Rocca et al. 1992; López-Guillén et al. 2011; Vaníčková 2012a), was not found either in the aeration extracts nor in the salivary-gland extracts of any of the 2 wild populations of *A. obliqua* described in the present study. The presence of this compound cannot be excluded, but its amount has to be very low.

Unlike the recent study by López-Guillén et al. (2011), we identified a very complex volatile mixture containing 22 compounds in the aeration extracts obtained from wild *A. obliqua* males. For the first time, we reported here the presence of δ-elemene, α-copaene, β-elemene, α-humulene as pheromone volatile components of *A. obliqua*. These compounds are common plant volatiles and may hence be the residues of the larval food of wild fruit flies. The difference between our results and those published previously may be caused by the different diet given to the insect during the larval stage, which may have a direct influence on the volatile produced and released by the adult males (Etges et al. 2006; Vaníčková 2012a; Vaníčková et al. 2012b).

With respect to previous reports, it is clear that (*Z,Z*)-3,6-nonadien-1-ol, (*E*)-β-caryophyllene, (*E*)-α-bergamotene, (*E,E*)-α-farnesene and limonene can be found in the volatile emissions of *Ceratitis capitata*, *A. suspensa* and *A. ludens* (Rocca et al. 1992), and limonene and 2,5-dimethylpyrazine are shared by *A. obliqua*, *C. capitata* and *A. fraterculus* (Gonçalves et al. 2006; Lima et al. 2001). Analogically to the situation in *C. capitata* (Gonçalves et al. 2006), (*Z,Z*)-3,6-nonadien-1-ol was not found in the solvent extracts of *A. obliqua* males. This result is in agreement with the hypothesis by Nation (1989) that the nonenols are stored in the intestine of *A. suspensa*. Nation (1989) also postulated that (*E*)-α-bergamotene and (*E,E*)-α-farnesene are produced in the salivary glands and released from the proboscis, whereas the nonenols are released from the anal gland.

Considering that approximately 50% of the compounds identified in the aeration extracts from wild *A. obliqua* calling males were also present in the extracts of the salivary glands, it is

reasonable to suggest that these glands are the site of the storage and probably also synthesis of these compounds in *A. obliqua*. This hypothesis is corroborated by the evidence that *A. obliqua* females are attracted to salivary-gland extracts in the same extent as they are attracted to the extracts of calling males in the laboratory assays.

We have demonstrated that the volatile mixtures of the compounds released by the males from the 2 wild populations of *A. obliqua* share approximately 60% of their composition. The same has also been observed in the salivary-gland extracts of these 2 populations, which have more than 70% of chemicals in common. Therefore, we can speculate that these 2 populations are in fact 1 single entity and the small differences observed arise from the diet. The pilot studies currently running in our laboratory are focused on the identification of the volatiles released by ripe mangoes and starfruits and their comparison with the pheromone composition of *A. obliqua* males. In the case of the starfruit, we have already identified a set of esters (Do Nascimento et al., unpublished) which may be a possible candidate for antennally active compounds of this particular fruit-fly species.

Nevertheless, with respect to the compounds identified in the volatiles released by the calling males of the 2 populations, electrophysiological and behavioral assays should be conducted in order to determine which of the identified components released by the calling males of *A. obliqua* possess female attractant activities, the possible candidates for bait control in the field.

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