

PHEROMONE ANALYSES OF THE *ANASTREPHA FRATERCULUS*
(DIPTERA: TEPHRITIDAE) CRYPTIC SPECIES COMPLEX

RADKA BŘÍZOVÁ^{1,2}, ADRIANA L. MENDONÇA³, LUCIE VANÍČKOVÁ^{3,*}, ALANA L. MENDONÇA³, CARLOS EDUARDO DA SILVA³,
ALEŠ TOMČALA¹, BEATRIZ AGUIAR JORDÃO PARANHOS⁴, VANESSA SIMÕES DIAS⁵, IARA SORDI JOACHIM-BRAVO⁵,
MICHAL HOSKOVEC¹, BLANKA KALINOVÁ¹ AND RUTH R. DO NASCIMENTO³

¹Institute of Organic Chemistry and Biochemistry AS CR, Flemingovo nám. 2, CZ-166 10 Prague 6, Czech Republic

²Institute of Chemical Technology in Prague, Technická 5, CZ-166 28 Prague 6, Czech Republic

³Instituto de Química e Biotecnologia, Universidade Federal de Alagoas, Avenida Lourival de Melo Mota, s/n,
Tabuleiro, 57072-970, Maceió, AL, Brazil

⁴Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA-Semiárido), BR 428, Zona Rural, 56302-970,
Petrolina, PE, Brazil

⁵Universidade Federal da Bahia, Instituto de Biologia, Departamento de Biologia Geral. R. Barão do Geremoabo
s/n, Campus Universitário de Ondina, 40170-290, Salvador, BA, Brazil

*Corresponding author; E-mail: luci.vanickova@gmail.com

Supplementary material for this article in Florida Entomologist 96(3) (2013) is online
at <http://purl.fcla.edu/fcla/entomologist/browse>

ABSTRACT

The South American fruit fly *Anastrepha fraterculus* (Wiedemann) (Diptera: Tephritidae) cryptic species complex is presently one of the most studied pest models in terms of speciation and population mating compatibility. The improvement of pest-control techniques has strongly relied on successful implementation of laboratory strains into wild populations. Pheromone communication plays an important role in the mating process in the South American fruit fly. Therefore, the main goal of the present study was to investigate the pheromone composition of 7 different populations, originating from geographically distant locations in Brazil and Argentina. Fourteen volatile compounds were identified in calling male emanations by GC×GC/TOF-MS and the data obtained were subsequently analyzed by multivariate statistics. The pheromone composition varied both quantitatively and qualitatively among the studied populations. These results will serve as the basis for further electrophysiological analyses.

Key Words: South American fruit fly, two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GC×GC/TOF-MS), principal component analysis (PCA)

RESUMO

O complexo de espécies crípticas *Anastrepha fraterculus* é, hoje em dia, um dos modelos de insetos-praga mais estudados no que se refere a especiação e compatibilidade populacional. O aperfeiçoamento das técnicas empregadas no controle de insetos-praga é fundamentada no sucesso na introdução de populações de laboratório entre as populações selvagens, em condições de campo. A comunicação mediada por feromônios, desempenha uma importante função no processo de acasalamento da mosca-das-frutas Sul Americana. Deste modo, o principal objetivo do presente estudo foi investigar a composição do feromônio de 7 populações diferentes de *A. fraterculus*, oriundas de regiões brasileiras geograficamente distintas e da Argentina. 14 compostos, provenientes de misturas liberadas por machos em chamamento, foram identificados por cromatografia gasosa bidimensional acoplada a detector time-of-flight e espectrometria de massas e os dados obtidos foram subsequentemente submetidos a análises estatísticas multivariada. A composição do feromônio variou qualitativamente e

quantitativamente entre as populações estudadas. Estes resultados irão servir para futuras análises eletrofisiológicas.

Palavras Chave: mosca das frutas Sul-Americana, Cromatografia Gasosa Bidimensional acoplada a detector Time-of-Flight e Espectrometria de Massas (CG×CG/TOF-EM), Análises de Componente Principal (ACP).

Anastrepha Schiner (1868) is the largest and most economically important genus of Tephritidae in tropical and subtropical Americas, including major pest species such as the South American fruit fly *A. fraterculus* (Wiedemann) (Norrbom & Korytkowski 2011). The *fraterculus* group includes 29 species (Smith-Caldas et al. 2001; Norrbom & Korytkowski 2011) and is currently known to be a complex of cryptic species (AF complex) that exhibit considerable levels of prezygotic and postzygotic reproductive isolation (Selivon et al. 1999; Vera et al. 2006; Cáceres et al. 2009; Segura et al. 2011). Numerous studies of AF complex revealed differences among populations in morphology (Stone 1942), karyotypes (Bush 1962), isozymes (Morgante et al. 1980), and host preference (Malvasi et al. 1983). More recently, differences among populations have been observed in egg morphology, embryonic development, mitochondrial and highly repetitive DNA, morphometric traits and mating incompatibility between some South American populations (Selivon & Perondini 1998; Steck & Shepard 1993; Smith-Caldas et al. 2001; Rocha & Selivon 2004; Selivon et al. 1999, 2005; Hernández-Ortiz et al. 2004, 2012; Ludena et al. 2010; Solferini & Morgante 1987; Vera et al. 2006; Cáceres et al. 2009, Rull et al. 2012). Based on a comprehensive morphological description, seven morphotypes of *A. fraterculus* have been determined (Hernández-Ortiz et al. 2012). In Brazil, 3 of these entities have been referred to as *A. sp. 1* aff. *fraterculus*, *A. sp. 2* aff. *fraterculus* (Yamada & Selivon 2001), and *A. sp. 3* aff. *fraterculus* (Selivon et al. 2005). Studies of 1 population from the southeast of Brazil have shown mating compatibility among some populations of *A. fraterculus* and have indicated that others are not compatible (Vera et al. 2006). Recently, no evidence of reproductive isolation has been found among 2 Brazilian populations from the southern region and one population from Argentina (Rull et al. 2012). Furthermore, there are differences in the time of sexual activity, with some populations mating in the morning, others at midday and still others in the afternoon (Vera et al. 2006; Segura et al. 2011).

These findings are very interesting and encourage the study of chemical communication, which plays an important role among the auditory and visual stimuli leading to mating in the AF complex. Differences in the composition of sex pheromone in Dipterans, within cryptic

species complexes, have recently been found in *Dasineura oxycoccana* (Johnson) (Diptera: Cecidomyiidae) midges collected from cranberry (*Vaccinium macrocarpon* Aiton; Ericaceae) and from highbush blueberry (*Vaccinium corymbosum* L.; Ericaceae) (Fitzpatrick et al. 2013). Evidence of male pheromone polymorphism comes from studies of *Drosophila* (Jallon 1987; Rouault 2001, 2004; Fedina et al. 2012; Grillet et al. 2012, Bontonou et al. 2012), and from cryptic species complexes in bees (Eltz et al. 2011), wasps (Niehuis et al. 2013) and moths (Droney et al. 2012; Groot et al. 2013).

The male sex pheromone of *A. fraterculus* has recently been studied with laboratory strains, and quantitative and qualitative differences in the composition of volatiles released by sexually mature flies from populations of southern Brazil, Argentina and Peru and their hybrids (Lima et al. 2001; Cáceres et al. 2009) were found, suggesting that pheromone variations would be indicative of incipient speciation. Nevertheless, there is a lack of studies investigating the composition of pheromone that would cover a broader sample of Brazilian populations. Therefore, we have conducted a comparative study of male-produced volatiles, aiming to describe the differences or similarities in the pheromone of 7 geographically distant populations of the AF complex. Moreover, the present work has been performed in order to provide the basic chemical study for future electroantennographic and behavioral analyses.

MATERIALS AND METHODS

Insects

The pupae of laboratory populations of *A. fraterculus* were provided by several places: the Entomology Laboratory of the Food and Agriculture Organization/International Atomic Energy Agency (FAO/IAEA, Seibersdorf, Austria; originally from Tucúman, Argentina); Embrapa Grape and Wine (Bento Gonçalves, Rio Grande do Sul, southern Brazil), Departamento de Biologia Animal, Universidade Federal da Bahia (UFBA), Salvador, Brazil and Laboratório de Ecologia Química, Universidade Federal de Alagoas (UFAL), Maceio, Brazil. The population from Seibersdorf was kept at the Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic, whereas the remaining populations were

kept at the Departamento de Biologia Animal da UFBA and Instituto de Química e Biotecnologia da UFAL. Identical protocols were used to feed and maintain these populations under laboratory conditions. The flies were allowed to emerge and the sexes were kept separate. They were fed an artificial diet composed of a mixture of brewer's yeast and cane sugar (3:1) and mineral water. The insect-rearing rooms were kept at 25 °C, 60% RH and 14:10 h L:D. Sexually mature flies, 20 days old, were used for the experiments.

The Collection of Volatiles

Volatile chemicals released by calling males of all populations, were obtained using the standard dynamic headspace procedures using a push-pull system. Specifically, groups of 20 virgin males were placed in glass chambers (500 mL). The chambers were aerated using clean air (1 mL/min). The volatiles released by the calling males were entrained by the air stream and trapped on filters containing an adsorbent (100 mg of SuperQ, Chrompack), which were placed at the chamber outlet. Volatile chemicals were collected for 24 h, starting from 9.00 a.m. and finishing at 9.00 a.m. on the following day, and this was replicated 5 times. The volatiles were subsequently rinsed from the filters by 500 µL of redistilled trace analysis grade hexane (Sigma-Aldrich). The extracts were stored in the freezer until chemical analyses.

GC×GC/TOF-MS

The two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GC×GC/TOF-MS) was used for the identification of headspace volatiles. The analysis was carried out on a LECO Pegasus 4D instrument (LECO Corp., St. Joseph, Missouri) equipped with a non-moving quad-jet cryomodulator. A DB-5 column (J&W Scientific, Folsom, California; 30 m × 250 µm i.d. × 0.25 µm film) and a BPX-50 column (SGE Inc., Austin, Texas; 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 rate of 1 ml/min. Sample injection was done with the HP 7683 autosampler; 1 µL of the sample was injected in the splitless mode. The temperatures of the GC×GC/TOF-MS 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 °C at 5 °C/min, and finally 190-320 °C 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 modula-

tion 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 with LECO ChromaTOF™ software. A standard of *n*-alkanes (C₈-C₂₂; Sigma-Aldrich) was co-injected with authentic samples to determine their retention indices (*RI*). The compounds were identified by a comparison of their mass-spectra fragmentation patterns, retention times and retention indices with previously published data and authentic standards (Sigma-Aldrich). In the absence of the standards, identifications were carried out by comparison with the NIST spectral reference library, the Wiley/NBS registry of mass spectral data (McLafferty et al. 1989) and published retention indices (Adams 2007; Jang et al. 1989, Vanícková et al. 2012a, 2012b).

Statistical Analysis

The data obtained from the chemical analysis (*N* = 5) of male emanations for each population were statistically evaluated. For the statistical analyses, the peak areas of the 14 compounds identified in the volatile mixtures released by 20-day-old males of all studied populations of *A. fraterculus* were used. For the further analysis only the 5 antennally active compounds were used. The differences in the chemical composition of the samples from all of the populations were analyzed by principal component analysis (PCA). Prior to the PCA analysis, the peak areas were subjected to logarithmic transformation, scaling was focused on inter-species correlation, each species score was divided by its standard deviation and the data were centered by species. In the PCA analyses, samples with similar chemical profiles cluster together and segregate from those that are different. 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 7 populations each stood as a categorical predictor. RDA analysis is also able to reveal compounds responsible for sample segregation. 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. Some of the data from the latter analyses can be found at the supplementary material for this article in Florida Entomologist 96(3) (2013) is online at <http://purl.fcla.edu/fcla/entomologist/browse>

RESULTS

Significant qualitative and quantitative differences in pheromone composition were discovered in the hexane extracts obtained from the headspace samples of the male volatiles of the 6 Brazilian populations and 1 Argentinean population of *A. fraterculus* (Fig. 1). The total amounts of the male produced volatiles varied among the 7 populations (Fig. 2). The volatile production originating from each of 2 populations from southern Brazil (Bento Gonçalves and São Joaquim) was on average 2.4 fold greater than the male emanations from each of the remaining populations. The volatile productions of the latter populations were comparably about equal to each other. The mean of the GC×GC/TOF-MS total peak areas of the identified male volatile components of the São Joaquim and Bento Gonçalves populations reached $5.41 \times 10^7 \pm 0.40 \times 10^7$ counts and $4.34 \times 10^7 \pm 0.33 \times 10^7$ counts, while the total peak areas of volatiles of the remaining populations from Tucúman, Pelotas, Alagoas and Piracicaba reached $2.98 \times 10^7 \pm 0.30 \times 10^7$ counts, $2.23 \times 10^7 \pm 0.25 \times 10^7$ counts, $2.15 \times 10^7 \pm 0.26 \times 10^7$ counts and $1.87 \times 10^7 \pm 0.19 \times 10^7$ counts, respectively. The lowest volatile production was observed in Vacaria population reaching $1.39 \times 10^7 \pm 0.15 \times 10^7$ counts of the total peak areas.

In total, 14 volatile compounds were detected in the male emanations consisting of terpenoids, alcohols and aldehydes (Table 1). Limonene (3) was one of the major components (> 17 %) in Vacaria, Pelotas, São Joaquim and Alagoas. But 2-ethylhexan-1-ol (2) was the major compound (> 16 %) present in Vacaria and Bento Gonçalves populations. (*E,Z*)-3,6-nonadien-1-ol (7) and/or (*E,E*)-

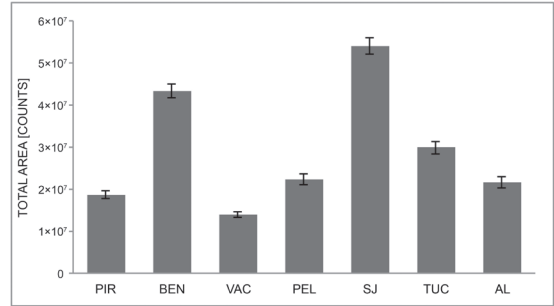


Fig. 2. Comparison of the total area of the volatile mixture released by each group of 25 calling males of *Anastrepha fraterculus* from 6 Brazilian populations (AL-Alagoas, SJ-São Joaquim, PEL-Pelotas, VAC-Vacaria, BEN-Bento Gonçalves and PIR-Piracicaba) and 1 population from Argentina (TUC-Tucúman).

α -farnesene (12) were the major components (> 15 %) in male emanations from Piraciaba, Bento Gonçalves, São Joaquim, Tucúman and Alagoas. Minor components (1-12 %) in all the populations included (*Z*)-3-nonen-1-ol (6), decenal (8), (*Z,E*)- α -farnesene (9), germacrene D (10), suspensolide (11), anastrephin (13) and epianastrephin (14). Nonanol (5) was a minor compound in 5 populations with exception of Vacaria (24%) and Tucúman (traces). (*Z*)- β -ocimene (4%) was not present in aerations of the Alagoas population, while in remaining populations it was present as a minor or trace compound.

The PCA and RDA analyses of the pheromone-compound peak-area dataset show groupings of particular populations. The compounds obtained from the Vacaria population differed the most (Fig. 3), and the compounds responsible for this divergence are germacrene D (10), decenal (8) and nonanal (5) (Fig. 4). It has to be noted that the arrows do not represent the concentrations of particular compounds but describe the significance of some compounds in the species-specific mixture of 14 male volatile compounds of *A. fraterculus*. Due to the occurrence of *p*-cymene (1) (Fig. 4), the second largest difference was observed in the Pelotas population (Fig. 3). The populations from Piracicaba and São Joaquim appear to be similar (Fig. 3), but the RDA analyses revealed a significant differentiation ($P < 0.01$) in their pheromone-composition datasets (Fig. 4). The results show that the populations from Pelotas, Vacaria, Alagoas and Tucúman are clearly segregated, whereas there is a degree of similarity among the populations from Bento Gonçalves, São Joaquim and Piracicaba. Among all the populations compared, the populations from Alagoas and Pelotas differ the most from the others.

Considering that the earlier GC-EAD analyses (Břízová et al. 2010, 2011; Vaníčková et al. 2010) of the volatiles released by males from the Tucúman population revealed that 5 compounds elicit

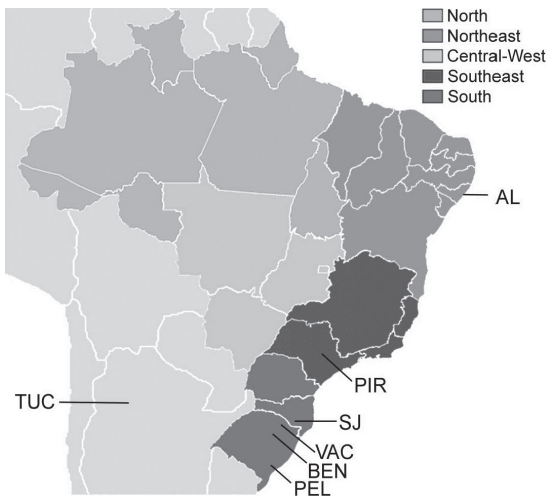


Fig. 1. The locations of the 7 populations of the *Anastrepha fraterculus* cryptic species complex. The population designations corresponds to those in Table 1.

TABLE 1. THE CHEMICAL COMPOUNDS (AVERAGE RELATIVE PERCENT \pm % RSD) IDENTIFIED BY GC \times GC/TOF-MS IN THE AERATION EXTRACTS OF CALLING MALES OF *ANASTREPHA FRATERCULUS* FROM THE FOLLOWING 7 POPULATIONS: PIR (PIRACICABA), BEN (BENTO GONÇALVES), VAC (VACARIA), PEL (PELOTAS), SJ (SÃO JOAQUIM), TUC (TUCÚMAN), AL (LAGOAS).

No.	Compound	RF	PIR	BEN	VAC	PEL	SJ	TUC	AL
1	<i>p</i> -Cymene ^b	1022	2.61 \pm 0.62	1.50 \pm 1.02	6.94 \pm 1.42	35.75 \pm 8.62	3.71 \pm 1.00	3.29 \pm 1.39	12.19 \pm 4.00
2	2-Ethylhexan-1-ol ^b	1029	2.10 \pm 0.69	16.39 \pm 6.58	35.89 \pm 5.59	0.34 \pm 0.12	0.75 \pm 0.23	7.18 \pm 2.74	Tr
3	Limonene ^a	1041	0.50 \pm 0.07	8.12 \pm 0.70	17.68 \pm 2.67	28.68 \pm 7.38	20.30 \pm 4.31	10.36 \pm 3.18	37.79 \pm 1.16
4	(<i>Z</i>)- β -Ocimene ^a	1050	13.18 \pm 0.63	1.18 \pm 0.81	Tr	4.71 \pm 2.06	5.16 \pm 3.67	11.07 \pm 3.82	—
5	Nonanal ^b	1107	2.47 \pm 0.56	9.74 \pm 1.09	24.65 \pm 6.95	1.90 \pm 0.35	4.80 \pm 2.54	Tr	1.69 \pm 0.68
6	(<i>Z</i>)-3-Nonen-1-ol ^{b,*}	1159	0.18 \pm 0.25	8.91 \pm 0.09	0.23 \pm 0.02	Tr	0.84 \pm 0.40	4.23 \pm 1.64	0.45 \pm 0.07
7	(<i>E,Z</i>)-3,6-Nonadien-1-ol ^{a,*}	1161	33.73 \pm 5.60	28.48 \pm 1.74	0.58 \pm 0.06	Tr	13.34 \pm 3.96	15.62 \pm 1.99	31.20 \pm 2.78
8	Decenal ^b	1210	1.18 \pm 0.08	2.98 \pm 0.62	6.65 \pm 1.27	5.55 \pm 0.93	2.56 \pm 1.11	Tr	0.65 \pm 0.38
9	(<i>Z,E</i>)- α -Farnesene ^{a,*}	1495	1.74 \pm 0.28	0.73 \pm 0.38	0.26 \pm 0.05	1.39 \pm 0.24	3.21 \pm 1.74	2.92 \pm 1.32	0.17 \pm 0.06
10	Germacrene D ^b	1498	0.44 \pm 0.11	0.37 \pm 0.08	2.24 \pm 0.28	1.04 \pm 0.49	0.35 \pm 0.06	Tr	1.23 \pm 0.42
11	(<i>E,E</i>)-Suspensolide ^a	1506	8.93 \pm 3.25	9.52 \pm 4.11	0.53 \pm 0.05	5.18 \pm 1.41	6.48 \pm 0.77	6.46 \pm 1.33	7.62 \pm 1.26
12	(<i>E,E</i>)- α -Farnesene ^{a,*}	1512	27.14 \pm 3.88	7.39 \pm 0.39	0.89 \pm 0.05	9.78 \pm 2.48	23.97 \pm 6.79	37.75 \pm 1.63	2.81 \pm 0.20
13	Anastrephin ^a	1617	1.98 \pm 0.49	0.82 \pm 0.08	0.59 \pm 0.06	1.65 \pm 0.21	2.93 \pm 1.12	0.42 \pm 0.11	1.85 \pm 0.46
14	Epianastrephin ^{a,*}	1621	3.80 \pm 0.28	3.87 \pm 1.12	2.86 \pm 0.23	4.03 \pm 0.48	11.59 \pm 4.48	0.71 \pm 0.36	2.35 \pm 0.69

^aCompounds identified by comparison with synthetic standards.

^bCompounds identified by comparison with the NIST library and the Wiley/NBS registry.

^cThe retention index calculated from retention times on a DB-5 capillary column.

^{*}Antennally active compounds identified by GC-EAD from the Tucuman population (Břizová et al., 2010, 2011, Vaníčková et al., 2010). Tr \leq 0.1 %.

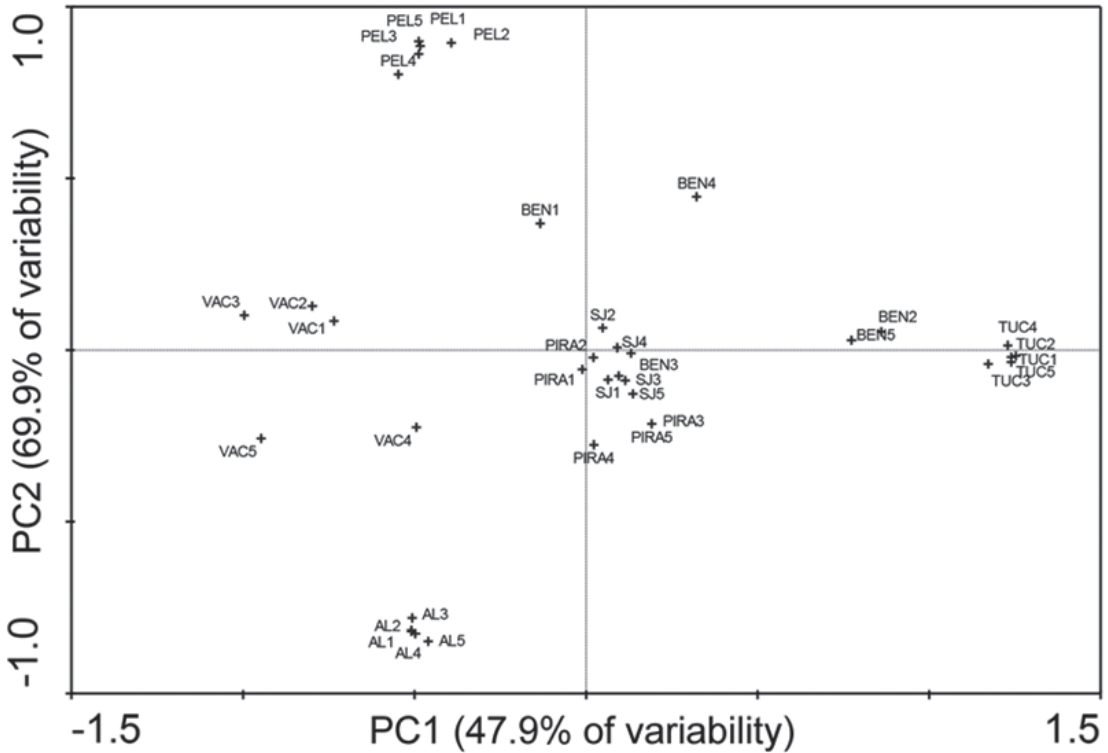


Fig. 3. The results of the multivariate principal component analysis (PCA) of the sex pheromone of the males of *A. fraterculus* originating from the 7 different populations of *A. fraterculus* (Bento Gonçalves-BEN 1-5, Pelotas-PEL 1-5, Piracicaba-PIRA 1-5, São Joaquim-SJ 1-5, Vacaria-VAC 1-5, Alagoas-AL 1-5 and Tucúman-TUC 1-5). The populations of Alagoas, Vacaria, Pelotas and Tucúman comprise 4 clearly segregated groups. The populations of Piracicaba, São Joaquim and Bento Gonçalves result in 1 mixed group. The number of the samples (N) analyzed for each population was 5.

female antennal activity, we further analyzed the data from all studied populations and found that those antennally active compounds were also present in all of them. Therefore the RDA was used to compare the importance of these 5 compounds [(*Z*)-3-nonen-1-ol (6), (*E,Z*)-3,6-nonadien-1-ol (7), (*Z,E*)- α -farnesene (9), (*E,E*)- α -farnesene (12), and epianastrephine (14)], and this revealed a significant differentiation ($P < 0.01$) among the 7 populations (Suppl. Fig. 1).

DISCUSSION

The present work demonstrates that there exist differences in the pheromone composition of geographically distinct populations of the South American fruit fly. The 4 studied populations of *A. fraterculus* from southern Brazil (Pelotas, Bento Gonçalves, Vacaria, São Joaquim), one from southeastern Brazil (Piracicaba), one from northeastern Brazil (Alagoas) and one from Argentina (Tucúman) differ both quantitatively and qualitatively in the mixture of volatiles released by sexually mature calling males. Differences

in the composition of the male sex pheromone within the *A. fraterculus* complex have been published by Lima et al. (2001) based on a laboratory population from southern Brazil (Pelotas) and by Cáceres et al. (2009) with laboratory populations from Peru and Argentina.

According to the literature, the chemical composition of the pheromone mixtures of some insects can be affected by at least 3 factors, which include gene control, environmental conditions and diet. The role of genetic variation on sex pheromone composition of closely related species has been reported for moths (Löfstedt 1990; Roelofs et al. 2002; Roelofs & Rooney 2003). The effect of the environment on the composition of sex pheromone of *Drosophila melanogaster* (Meigen) was found in studies of populations encountered at high and low latitudes (Rouault et al. 2001, 2004). This male pheromone polymorphism in *Drosophila* is also responsible for reproductive isolation between pheromonal races (Grillet et al. 2012; Bontonou et al. 2012). Differences have been reported concerning the chemical composition of cuticular hydrocarbons of *Drosophila ser-*

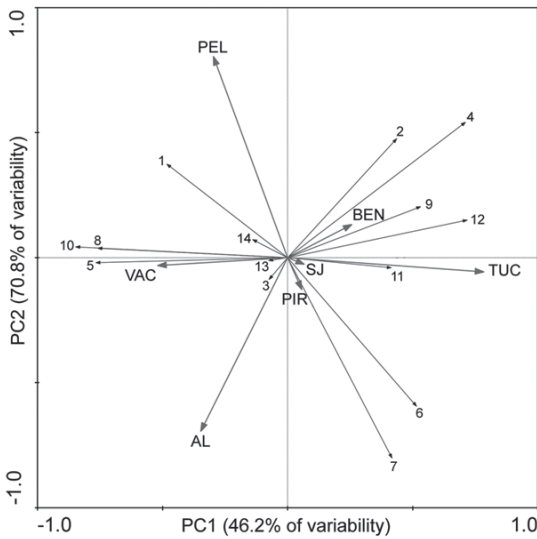


Fig. 4. The results of the multivariate redundant analysis (RDA) of the sex pheromone of the males of *A. fraterculus* from the 7 different populations (AL-Alagoas, SJ-São Joaquim, PEL-Pelotas, VAC-Vacaria, BEN-Bento Gonçalves and PIR-Piracicaba) and 1 population from Argentina (TUC-Tucúman). The vertical line separates the Bento Gonçalves, São Joaquim, Piracicaba and Tucúman populations from the Pelotas, Vacaria and Alagoas populations. The horizontal line separates the Vacaria, Alagoas, Piracicaba, São Joaquim and Tucúman populations from the Bento Gonçalves and Pelotas populations. The arrows represent the 14 compounds that are characteristic of the respective populations. The compounds are numbered according to Table 1.

rata (Malloch) (Diptera: Drosophilidae) males fed on 3 different diets (rice, yeast and corn) (Rundle 2005). More recently, Fedina et al. (2012) reported changes in sexual attractiveness and pheromone composition influenced by diet in *D. melanogaster*. Vaničková et al. (2012a, 2012b) described quantitative and qualitative differences in the composition of medfly (*Ceratitidis capitata*) male pheromone originating from 3 different populations. In the case of *A. fraterculus* populations, the factors, which may be involved in the chemical differences among various sex pheromone mixtures, are unknown. Perhaps a combination of genetics and environmental factors may be responsible for the results described here.

For instance, despite the existence of sexual compatibility among the adults of *A. fraterculus* populations from different localities in southern Brazil, namely Bento Gonçalves, São Joaquim, Pelotas and Vacaria reported by Dias (2012), no behavioral study has been carried out to test the biological activity of the compound mixtures released by the calling males from these populations to find out which ones are attractive or might induce repellency on conspecific and het-

erospecific females. In addition, no electrophysiological study has been conducted to determine which components within the mixtures elicit electrophysiological responses in conspecific and heterospecific females from different Brazilian populations. However, research conducted by Břízová et al. (2010, 2011) and Vaničková et al. (2010) demonstrated that 5 compounds found in extracts from laboratory males of *A. fraterculus* from Argentine (Tucúman), used in the present study, are antennally active for conspecific females. The chemical identities of these compounds have been determined as: (*Z*)-3-nonen-1-ol, (*E,Z*)-3,6-nonadien-1-ol, (*Z,E*)- α -farnesene, (*E,E*)- α -farnesene and epianastrephin. The multivariate analyses performed in the present study proved that 4 out of these 5 compounds are specific for the Tucúman population.

Vera et al. (2006) reported partial mating incompatibility between *A. fraterculus* populations from Tucúman (Argentina) and Piracicaba (southern Brazil). More recently, Dias (2012) has reported partial incompatibility between populations from southeastern (Piracicaba) and southern regions (Pelotas, Vacaria, Bento Gonçalves and São Joaquim) of Brazil, whereas the southern Brazilian populations were compatible with each other. Rull et al. (2012) confirmed the compatibility among the southern Brazilian populations from Vacaria and Pelotas. From these results, it is possible to speculate that the partial incompatibility may be, among other factors, caused by different amounts of the 5 antennally active compounds, as reported here. Moreover, we hypothesize that there may also be other compounds of the male pheromone, which are responsible for the attraction, and/or repulsion of conspecific and/or heterospecific females. The differences among the pheromone mixtures released by the males of different Brazilian and Argentinean populations of *A. fraterculus* found here might also be regulated by various genes, which may influence the responses of the females from these populations to change the manner in which they respond to the pheromone mixtures released by homospecific and heterospecific males. However, behavioral, electrophysiological and genetics studies should be conducted to corroborate or reject this hypothesis.

ACKNOWLEDGMENTS

We acknowledge the following sponsors for financial support and scholarships: FAO/IAEA, Vienna, Austria (the funding was provided to Research Contracts No. 16051 and 16106 as part of the Coordinated Research Project on the Resolution of the Cryptic Species Complexes of Tephritid Pests to Overcome Constraints to SIT Application and International Trade), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Proc. Nos. 555094/2010-3 and 308695/2011-9).

REFERENCES CITED

- ADAMS, R. P. 2007. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry. Allured Publishing Corporation, Illinois, USA.
- BONTONOU, G., DENIS, B., AND WICKER-THOMAS, C. 2012. Male pheromone polymorphism and reproductive isolation in populations of *Drosophila simulans*. *Ecol. Evol.* 2: 2527-2536.
- BRÍZOVÁ, R., VANÍČKOVÁ, L., KALINOVÁ, B., DO NASCIMENTO, R. R., AND HOSKOVEC, M. 2010. Sex specific differences in preception of the pheromone in *Anastrepha fraterculus*. Proc. 26th ISCE Annual Meeting, Tours, France, pp. 210.
- BRÍZOVÁ, R. 2011. Analyses of male sex pheromone of *Anastrepha fraterculus* (Diptera: Tephritidae). MSc thesis. Institute of Chemical Technology Prague, Czech Republic.
- BUSH, G. L. 1962. The cytotaxonomy of the larvae of some Mexican fruit flies in the genus *Anastrepha*. *Psyche* 68: 87-101.
- CÁCERES, C., SEGURA, D. F., VERA, M. T., WORNOAY-PORN, V., CLADERA, J. L., TEAL, P., SAPOUNTZIS, P., BOURTZIS, P., ZACHAROPOULOU, A., AND ROBINSON, A. S. 2009. Incipient speciation revealed in *Anastrepha fraterculus* (Diptera; Tephritidae) by studies on mating compatibility, sex pheromones, hybridization, and cytology. *Biol. J. Linn. Soc.* 97: 152-165.
- DIAS, V. S. 2012. Compatibilidade de acasalamento de populações do complexo *Anastrepha fraterculus* (Diptera: Tephritidae) do Brasil. Dissertation thesis. Federal University of Bahia, Brazil.
- DRONEY, D. C., MUSTO, C. J., MANCUSO, K., ROELOFS, W. L., AND LINN, C. E. 2012. The response to selection for broad male response to female sex pheromone and its implications for divergence in close-range mating behavior in the European corn borer moth, *Ostrinia nubilalis*. *J. Chem. Ecol.* 38: 1504-1512.
- ELTZ, T., FRITZSCH, F., PECH, J. R., ZIMMERMANN, Y., RAMIREZ, S. R., QUEZADA-EUAN, J. J. G., AND BEMBE, B. 2011. Characterization of the orchid bee *Euglossa viridissima* (Apidae: Euglossini) and a novel cryptic sibling species, by morphological, chemical, and genetic characters. *Zool. J. Linn. Soc. Lond.* 163: 1064-1076.
- FEDINA, T. Y., KUO, T.-H., DREISEWERD, K., DIERICK, H. A., YEW, J. Y., AND PLETCHER, S. D. 2012. Dietary effects on cuticular hydrocarbons and sexual attractiveness in *Drosophila*. *PLoS ONE*. 7: e49799.
- FITZPATRICK, S. M., GRIES, R., KHASKIN, G., PEACH, D. A. H., IWANSKI, J., AND GRIES, G. 2013. Populations of the gall midge *Dasineura oxycoccana* on cranberry and blueberry produce and respond to different sex pheromones. *J. Chem. Ecol.* 39: 37-49.
- GRILLET, M., EVERAERTS, C., HOUDOT, B., RITCHIE, M. G., COBB, M., AND FERVEUR, J.-F. 2012. Incipient speciation in *Drosophila melanogaster* involves chemical signals. *Sci. Rep.* 2: 1-11.
- GROOT, A. T., STAUDACHER, H., BARTHEL, A., INGLIS, O., SCHÖFL, G., SANTANGELO, R. G., GEBAUER-JUNG, S., VOGEL, H., EMERSON, J., SCHAL, C., HECKEL, D. G., AND GOULD, F. 2013. One quantitative trait locus for intra- and interspecific variation in a sex pheromone. *Mol. Ecol.* 22: 1065-1080.
- HERNÁNDEZ-ORTIZ, V., GÓMEZ-ANAYA, J. A., SÁNCHEZ, A., MCPHERON, B. A., AND ALUJA, M. 2004. Morphometric analysis of Mexican and South American populations of the *Anastrepha fraterculus* complex (Diptera: Tephritidae) and recognition of a distinct Mexican morphotype. *Bull. Entomol. Res.* 94: 487-499.
- HERNÁNDEZ-ORTIZ, V., BARTOLUCCI, A. F., MORALES-VALLES, P., FRIAS, D., AND SELIVON, D. 2012. Cryptic species of the *Anastrepha fraterculus* complex (Diptera: Tephritidae): a multivariate approach for the recognition of South American morphotypes. *Ann. Entomol. Soc. America* 105: 305-318.
- JALLON, J. M., AND DAVID, J. R. 1987. Variations in cuticular hydrocarbons among the 8 species of the *Drosophila melanogaster* subgroup. *Evolution* 41: 294-302.
- JANG, E. B., LIGHT, D. M., FLATH, R. A., NAGATA, J. T., AND MON, T. R. 1989. Electroantennogram responses of the Mediterranean fruit fly, *Ceratitis capitata* to identified volatile constituents from calling males. *Entomol. Exp. Appl.* 50: 7-19.
- LIMA, I. S., HOUSEB, P. E., AND DO NASCIMENTO, R. R. 2001. Volatile substances from male *Anastrepha fraterculus* Wied. (Diptera: Tephritidae): Identification and behavioural activity. *J. Brazilian Chem. Soc.* 12: 196-201.
- LÖFSTEDT, C. 1990. Population variation and genetic control of pheromone communication-systems in moths. *Entomol. Exp. Appl.* 54: 199-218.
- LUDENA, B., BAYAS, R., AND PINTAUD, J.-C. 2010. Phylogenetic relationships of Andean-Ecuadorian populations of *Anastrepha fraterculus* (Wiedemann 1830) (Diptera: Tephritidae) inferred from COI and COII gene sequences. *Ann. Soc. Entomol. Fr.* 46: 344-350.
- MALAVASI, A., MORGANTE, J. S., AND PROKOPY, R. J. 1983. Distribution and activities of *Anastrepha fraterculus* (Diptera: Tephritidae) flies on host and non host trees. *Ann. Entomol. Soc. America* 76: 286-292.
- MCLAFFERTY, F. W., STAUFFER, D. B., STENHAGEN, E., AND HELLER, S. R. 1989. In McLafferty, F. W., Stauffer, D. B. [eds.], *The Wiley/NBS Registry of Mass Spectral Data*, 1st edition. Wiley, New York, USA.
- MORGANTE, J. S., MALAVASI, A., AND BUSH, G. L. 1980. Biochemical systematics and evolutionary relationships of neotropical *Anastrepha*. *Ann. Entomol. Soc. America* 73: 622-630.
- NIEHUIS, O., BUELLESBACH, J., GIBSON, J. D., POTHMANN, D., HANNER, C., MUTTI, N. S., JUDSON, A. K., GADAU, J., RUTHER, J., AND SCHMITT, T. 2013. Behavioural and genetic analyses of *Nasonia* shed light on the evolution of sex pheromones. *Nature* 494: 345-348.
- NORRBOM, A. L., AND KORYTKOWSKI, C. A. 2011. New species of and taxonomic notes on *Anastrepha* (Diptera: Tephritidae). *Zootaxa* 2740: 1-23.
- ROELOFS, W. L., LIU, W., HAO, G., JIAO, H., ROONEY, A. P., AND LINN, C. E. J. 2002. Evolution of moth sex pheromones via ancestral genes. *Proc. Natl. Acad. Sci. USA* 99: 13621-13626.
- ROELOFS, W. L., AND ROONEY, A. P. 2003. Molecular genetics and evolution of pheromone biosynthesis in Lepidoptera. *Proc. Natl. Acad. Sci. USA* 100: 9179-9184.
- ROCHA, L. S., AND SELIVON, D. 2004. Studies on highly repetitive DNA in cryptic species of the *Anastrepha fraterculus* complex, pp. 415-418, In Barnes B.N. [ed.], *Proc. 6th Intl. Symp. on Fruit Flies of Economic Importance*. Irene: Isteg Scientific Publ.
- ROUAULT, J., CAPY, P., AND JALLON, J.-M. 2001. Variations of male cuticular hydrocarbons with geoclimat-

- ic variables: an adaptative mechanism in *Drosophila melanogaster*? *Genetica* 110: 117-130.
- ROUAULT, J.-D., MARICAN, C., WICKER-THOMAS, C., AND JALLON, J.-M. 2004. Relations between cuticular hydrocarbon (HC) polymorphism, resistance against desiccation and breeding temperature; a model for HC evolution in *D. melanogaster* and *D. simulans*. *Genetica* 120: 195-212.
- RULL, J., ABRAHAM, S., KOVALESKI, A., SEGURA, D. F., ISLAM, A., WORNOPYORN, V., DAMMALAGE, T., TOMAS, U. S., AND VERA, M. T. 2012. Random mating and reproductive compatibility among Argentinean and southern Brazilian populations of *Anastrepha fraterculus* (Diptera: Tephritidae). *Bull. Entomol. Res.* 102: 435-443.
- RUNDLE, H. D., CHENOWETH, S. F., DOUGHTY, P., AND BLOWS, M. W. 2005. Divergent Selection and the Evolution of Signal Traits and Mating Preferences. *PLoS Biology* 3: e368.
- SEGURA, D. F., VERA, M. T., RULL, J., WORNOPYORN, V., ISLAM, A., AND ROBINSON, A. S. 2011. Assortative mating among *Anastrepha fraterculus* (Diptera: Tephritidae) hybrids as a possible route to radiation of the *fraterculus* cryptic species complex. *Biol. J. Linnean Soc.* 102: 346-354.
- SELIVON, D., AND PERONDINI, A. L. P. 1998. Eggshell morphology in two cryptic species of the *Anastrepha fraterculus* complex (Diptera : Tephritidae). *Ann. Entomol. Soc. America* 91: 473-478.
- SELIVON, D., PERONDINI, A. L. P., AND MORGANTE, J. S. 1999. Haldane's rule and other aspects of reproductive isolation observed in the *Anastrepha fraterculus* complex (Diptera : Tephritidae). *Gen. Mol. Biol.* 22: 507-510.
- SELIVON, D., PERONDINI, A. L. P., AND ROCHA, L. S. 2005. Karyotype characterization of *Anastrepha* fruit flies (Diptera: Tephritidae). *Neotrop. Entomol.* 34: 273-279.
- SMITH-CALDAS, M. R. B., MCPHERON, B. A., SILVA, J. G., AND ZUCCHI R. A. 2001. Phylogenetic relationships among species of the *fraterculus* group (*Anastrepha*: Diptera: Tephritidae) inferred from DNA sequences of mitochondrial cytochrome oxidase I. *Neotrop. Entomol.* 30: 565-573.
- SOLFERINI, V. N., AND MORGANTE, J. S. 1987. Karyotype study of eight species of *Anastrepha* (Diptera: Tephritidae). *Carylogia* 40: 229-241.
- STECK, G. J., AND SHEPPARD, W. S. 1993. Mitochondrial DNA variation in *Anastrepha fraterculus*, pp. 9-14 *In* M. Aluja and P. Liedo [eds.], *Fruit flies: Biology and Management*. Springer, New York.
- STONE, A. 1942. The fruit flies of the genus *Anastrepha*. U. S. Dep. Agric. Misc. Publ. 439: 1-112.
- VANIČKOVÁ, L., KALINOVÁ, B., DO NASCIMENTO, R. R., BŘÍZOVÁ, R., AND HOSKOVEC, M. 2010. Pheromone production and preception in *Anastrepha fraterculus*. SIGMA conference, Czech Republic. *Chem. Listy* 104: 389-339.
- VANIČKOVÁ, L., DO NASCIMENTO, R.R., HOSKOVEC, M., JEŽKOVÁ, Z., BŘÍZOVÁ, R., TOMČALA, A., KALINOVÁ, B. 2012a. Are the wild and laboratory insect populations different in semiochemical emission? The case of medfly sex pheromone. *J. Agric. Food Chem.* 60: 7168-7176.
- VANIČKOVÁ, L. 2012b. Chemical ecology of fruit flies: genera *Ceratitidis* and *Anastrepha*. Dissertation thesis. Institute of Chemical Technology, Prague, Czech Republic.
- VERA, M. T., CÁCERES, C., WORNOPYORN, V., ISLAM, A., ROBINSON, A. S., DE LA VEGA, M. H., HENDRICH, J., AND CAYOL, J. P. 2006. Mating incompatibility among populations of the South American fruit fly *Anastrepha fraterculus* (Diptera: Tephritidae). *Ann. Entomol. Soc. America* 99: 387-397.
- YAMADA, S. M., AND SELIVON, D. 2001. Rose, an eye color mutation in a species of the *Anastrepha fraterculus* complex (Diptera: Tephritidae). *Ann. Entomol. Soc. America* 94: 592-595.