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Authors: Břízová, Radka, Mendonça, Adriana L., Vanícková, Lucie, Mendonça, Alana L., Eduardo Da Silva, Carlos, et. al.

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

RADKA BŘÍZOVÁ1,2, ADRIANA L. MENDONÇA3, LUCIE VANÍČKOVÁ1,2, ALANA L. MENDONÇA1, CARLOS EDUARDO DA SILVA1, ALEŠ TOMÁLÁ1, BEATRIZ AGUIAR JORDÃO PARANHOS1, VANESSA SIMÕES DIAS1, IRA SORDI JOACHIM-BRAVO1, MICHAL HOSKOVEC1, BLANKA KALINOVÁ1 AND RUTH R. DO NASCIMENTO3

1 Institute of Organic Chemistry and Biochemistry AS CR, Flemingovo nám. 2, CZ-166 10 Prague 6, Czech Republic
2 Institute of Chemical Technology in Prague, Technická 5, CZ-166 28 Prague 6, Czech Republic
3 Instituto de Química e Biotecnologia, Universidade Federal de Alagoas, Avenida Lourival de Melo Mota, s/n, Tabuleiro, 57072-970, Maceió, AL, Brazil
4 Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA-Semiárido), BR 428, Zona Rural, 56302-970, Petrolina, PE, Brazil
5 Universidade 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

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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 cripticas 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 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 (CGxCG/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 (Malavasi 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 oxycocca (Johnson) (Diptera: Cecidomyiidae) through studies of 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 Tucumán, 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 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 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 with LECO ChromaTOF™ software. A standard of n-alkanes (Cn-C23, 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, Vaníčková 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 (Biometrics, 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

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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 emissions from each of the remaining populations. The volatile productions of the latter populations were comparably about equal to each other. The mean of the GCxGC/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 Tucumán, 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 \((E,E)\)-\(\alpha\)-farnesene (12) were the major components (> 15 %) in male emanations from Piracicaba, Bento Gonçalves, São Joaquim, Tucumán and Alagoas. Minor components (1-12 %) in all the populations included \((Z)\)-3-nonanol-1-ol (6), decenal (8), \((Z,E)\)-\(\alpha\)-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 Tucumán (traces). \((Z)\)-\(\beta\)-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 Tucumán 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 (Brízová et al. 2010, 2011; Vaníčková et al. 2010) of the volatiles released by males from the Tucumán population revealed that 5 compounds elicit
| No. | Compound | RF | PIR | BEN | VAC | PEL | SJ | TUC | AL |
|-----|----------|----|-----|-----|-----|-----|----|-----|----|
| 1   | p-Cymene | 1022 | 2.61 ± 0.62 | 1.50 ± 1.02 | 6.94 ± 1.42 | 35.75 ± 8.62 | 3.71 ± 1.00 | 3.29 ± 1.39 | 12.19 ± 4.00 |
| 2   | 2-Ethylhexan-1-ol b | 1029 | 2.10 ± 0.69 | 16.39 ± 6.58 | 35.89 ± 5.59 | 0.34 ± 0.12 | 0.75 ± 0.23 | 7.18 ± 2.74 | Tr |
| 3   | Limonene a | 1041 | 0.50 ± 0.07 | 8.12 ± 0.70 | 17.68 ± 2.67 | 28.68 ± 7.38 | 20.30 ± 4.31 | 10.36 ± 3.18 | 37.79 ± 1.16 |
| 4   | (Z)-β-Ocimene a | 1050 | 13.18 ± 0.63 | 1.18 ± 0.81 | Tr | 4.71 ± 2.06 | 5.16 ± 3.67 | 11.07 ± 3.82 | — |
| 5   | Nonanal a | 1107 | 2.47 ± 0.56 | 9.74 ± 1.09 | 24.65 ± 6.95 | 1.90 ± 0.35 | 4.80 ± 2.54 | Tr | 1.69 ± 0.68 |
| 6   | (Z)-3-Nonen-1-ol a | 1159 | 0.18 ± 0.25 | 8.91 ± 0.09 | 0.23 ± 0.02 | Tr | 0.84 ± 0.40 | 4.23 ± 1.64 | 0.45 ± 0.07 |
| 7   | (E,Z)-3,6-Nonadien-1-ol a | 1161 | 33.73 ± 5.60 | 28.48 ± 1.74 | 0.58 ± 0.06 | Tr | 13.34 ± 3.96 | 15.62 ± 1.99 | 31.20 ± 2.78 |
| 8   | Decenal a | 1210 | 1.18 ± 0.08 | 2.98 ± 0.62 | 6.65 ± 1.27 | 5.55 ± 0.93 | 2.56 ± 1.11 | Tr | 0.65 ± 0.38 |
| 9   | (Z,E)-α-Farnesene a | 1495 | 1.74 ± 0.28 | 0.73 ± 0.38 | 0.26 ± 0.05 | 1.39 ± 0.24 | 3.21 ± 1.74 | 2.92 ± 1.32 | 0.17 ± 0.06 |
| 10  | Germacrene D a | 1498 | 0.44 ± 0.11 | 0.37 ± 0.08 | 2.24 ± 0.28 | 1.04 ± 0.49 | 0.35 ± 0.06 | Tr | 1.23 ± 0.42 |
| 11  | (E,E)-Suspensolide a | 1506 | 8.93 ± 3.25 | 9.52 ± 4.11 | 0.53 ± 0.05 | 5.18 ± 1.41 | 6.48 ± 0.77 | 6.46 ± 1.33 | 7.62 ± 1.26 |
| 12  | (E,Z)-α-Farnesene a | 1512 | 27.14 ± 3.88 | 7.39 ± 0.39 | 0.89 ± 0.05 | 9.78 ± 2.48 | 23.97 ± 6.79 | 37.75 ± 1.63 | 2.81 ± 0.20 |
| 13  | Anastrephin a | 1617 | 1.98 ± 0.49 | 0.82 ± 0.08 | 0.59 ± 0.06 | 1.65 ± 0.21 | 2.93 ± 1.12 | 0.42 ± 0.11 | 1.85 ± 0.46 |
| 14  | Epianastrephin a | 1621 | 3.80 ± 0.28 | 3.87 ± 1.12 | 2.86 ± 0.23 | 4.03 ± 0.48 | 11.59 ± 4.48 | 0.71 ± 0.36 | 2.35 ± 0.69 |

*Compounds 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 (Brízová et al., 2010, 2011, Vaníčková et al., 2010). Tr ≤ 0.1 %.
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 \((\text{Z})-3\text{-nonen-1-ol} (6), (\text{E,Z})-3,6\text{-nonadien}-1\text{-ol} (7), (\text{Z,E})-\alpha\text{-farnesene} (9), (\text{E,E})-\alpha\text{-farnesene} (12), \) 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 (Tucumán) 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-
might induce repellency on conspecific and heterospecific 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 Vaníčková et al. (2010) demonstrated that 5 compounds found in extracts from laboratory males of *A. fraterculus* from Argentina (Tucumán), 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 Tucumán population.

Vera et al. (2006) reported partial mating incompatibility between *A. fraterculus* populations from Tucumán (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.

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