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Identification of host plant volatile stimulants of *Anastrepha fraterculus* male courtship behavior

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In some tephritid fruit flies, exposure to volatile compounds from host plants increases male sexual success. This phenomenon has been used to boost sterile males' sexual competitiveness in the framework of the sterile insect technique (SIT). Previous studies revealed that males of *Anastrepha fraterculus* (Diptera: Tephritidae) exposed to volatiles from guava (*Psidium guajava*) fruit (GF) and guava essential oil (GEO) exhibit intensified courtship behavior and have greater copulatory success relative to unexposed males. Similar results were achieved in these flies through exposure to moradillo (*Schinus polygama*) essential oil or lemon (*Citrus limon*) essential oil. To identify the responsible compounds involved in these effects, we compared the volatile chemical profiles of GF, GEO, moradillo essential oil, and lemon essential oil. We selected five candidate compounds: (E)-β-ocimene, (Z)-β-ocimene, limonene, β-caryophyllene, and α-humulene. Using the electroantennographic detection (EAD) technique, we verified that males are able to detect all the candidate compounds and built dose-response curves between 0.01 and 100 μg/μl for each compound. We confirmed a stimulating effect on the courtship behavior of males for (E/Z)-β-ocimene and (R)-limonene, whereas β-caryophyllene and α-Humulene did not affect male courtship behavior. For those compounds that sexually stimulated males, we found a dose-dependent effect. Males' behavioral response to the semiochemicals was maximum when (R)-limonene was combined with

(E/Z)-β-ocimene, but the response was reduced when β-caryophyllene and α-humulene were included, which suggests some sort of negative interaction between them. Our results may contribute to the ongoing development of the SIT in this species.

KEYWORDS

semiochemicals, β-ocimene, limonene, pheromone calling, phytochemicals, fruit flies, sterile insect technique

Introduction

Many tephritid species exhibit a lek mating system in which males aggregate in specific sites to attract or court females (Arita and Kaneshiro, 1989; Díaz-Fleischer and Aluja, 1999). This calling behavior consists of emitting acoustical, visual, and chemical signals, and involves the display of energetically expensive behaviors to release pheromones (e.g., wing fanning and expansion of salivary glands) (Yuval et al., 1998; Sivinski et al., 1999). Males' attractiveness and mating success positively correlate with the calling effort (Whittier et al., 1994; Segura et al., 2007; Niyazi et al., 2008). Therefore, the male capability to synchronize calling activity with the availability of food, among other suitable environmental conditions, is expected to be critical to improving male fitness. In this sense, volatile organic compounds (VOCs) from hosts could act not only as conductive feeding, oviposition, and rendezvous sites (Landolt and Phillips, 1997; Tumlinson, 2014; Moreau et al., 2017; Xu and Turlings, 2018) but also as environmental signals that trigger the synthesis/release of pheromones or switch on sexual behaviors (Reddy and Guerrero, 2004; Borrero-Echeverry et al., 2018). In fact, host plant VOCs stimulate calling behavior and sexual success in a number of tephritid fruit fly species (reviewed by Segura et al., 2018). For example, *Ceratitis capitata* males increase their mating success when they are exposed to α-copaene, a phytochemical widely distributed among *C. capitata* host plants (Shelly, 2001). This compound also acts as a long-distance cue for male aggregation in lekking sites (Nishida et al., 2000). Similarly, the male mating success of *Bactrocera oleae* increases when males are exposed to α-pinene, a compound emitted by olive fruit, the only host of this species (Gerofotis et al., 2013; Kokkari et al., 2021). The stimulating effects of these plant VOCs may have probably evolved during the adaptation of these flies to the exploitation of their hosts. In addition, in some cases, the same compounds released by host plants are also present in males' pheromonal blends (Segura et al., 2018). For example, α-pinene and α-copaene are found in the sex pheromone blend of *B. oleae* and *C. capitata*, respectively (Mazomenos and Haniotakis, 1981; Vaníèková et al., 2012). This is in line with the sensory exploitation hypothesis, according to which host-like pheromone odors have evolved to exploit females' pre-existing sensory bias toward host fruit odors (Ryan, 1990).

Anastrepha, like *Ceratitis* and *Bactrocera*, is a tephritid genus of fruit flies. However, only a few studies have evaluated male sexual responses to VOCs exposure in *Anastrepha* (Segura et al., 2018). Volatiles from ginger root oil (GRO), which majorly contain α-copaene, were tested on several *Anastrepha* species, namely *Anastrepha fraterculus*, *Anastrepha ludens*, *Anastrepha obliqua*, and *Anastrepha serpentina* (Flores et al., 2011; Ruiz et al., 2021). However, except for *A. serpentina*, male mating success was not enhanced by the exposure to GRO volatiles in these species. On the other hand, fruits of preferred host plants have provided better sources of sexually stimulating VOCs in these flies (Vera et al., 2013; Bachmann et al., 2015; Morató et al., 2015). For instance, *A. ludens* mating success is increased in males exposed to volatiles from grapefruit essential oil (Morató et al., 2015).

Anastrepha fraterculus sp. 1 morphotype constitutes another example of tephritid flies that are sexually stimulated by host plant VOCs. This species belongs to the *Anastrepha fraterculus* cryptic species complex, which has a wide distribution range in the American continent (Hernández-Ortiz et al., 2019) and generates a high economic impact on the agribusiness of this region (Cladera et al., 2014; De León et al., 2021). A recent review of their natural hosts showed that, despite the differences in the host range used by different populations at the regional level, the native *Psidium guajava* is the only host shared by all populations (Hernández-Ortiz et al., 2019). Therefore, the chemosensory system of *A. fraterculus* is likely adapted to perceive VOCs from guava fruit as an environmental cue for host availability or the localization of sexual mates. In fact, previous studies revealed that males exposed to volatiles from guava (*P. guajava*) fruit (GF) perform calling behavior more frequently, release a higher amount of sex pheromone, and achieve a higher copulatory success relative to unexposed males (Vera et al., 2013; Bachmann et al., 2015). Similar results were obtained when males were exposed to a commercial guava essential oil (GEO) (Belliard et al., 2022) or a blend of seven representative compounds of GF [(R)-limonene, β-myrcene, (E)-β-ocimene, α-humulene, E-2-hexenal, ethyl butanoate, and ethyl hexanoate], although the effect of the blend was lower compared to the GF effect (Bachmann et al., 2015). Knowing which of these compounds are responsible for stimulating *A. fraterculus* male sexual behavior could help to understand this phenomenon and further evaluate its potential for pest management.

Recently, Ruiz et al. (2021) assessed the effect of odors from seven essential oils, from exotic hosts and non-host plants, as well as two standard compounds [(R)-limonene 98% and citral (mixture of neral and geranial) 95%] on *A. fraterculus* male mating success. They found an increased male mating success when males were exposed to lemon (*Citrus limon*) essential oil or moradillo (*Schinus polygama*) essential oil. In addition, when males were exposed to R-limonene, the major compound of lemon essential oil (LO), they achieved a higher number of mates. However, when males were exposed to grapefruit (*Citrus paradisi*) essential oil (GFO) and orange (*Citrus sinensis*) essential oil (OO), which also contain R-limonene as the major compound, their sexual success was not affected (Ruiz et al., 2021). The limonene relative abundance varies between citrus species (González-Mas et al., 2019), and detrimental effects have been shown for tephritid flies exposed to sources with high concentrations of this compound (Salvatore et al., 2004; Papachristos et al., 2009; Oviedo et al., 2020; Zeni et al., 2021). Thus, limonene's effect on male sexual behavior appears to be dose-dependent, but it may also depend on the presence of other compounds. Guava and moradillo are native plants with marked differences in their chemical composition (Soares et al., 2007; Jofré Barud, 2018). The fact that volatiles from these fruits, for which limonene is not a major compound, act as male sexual enhancers in *A. fraterculus* (Vera et al., 2013; Bachmann et al., 2015; Ruiz et al., 2021; Belliard et al., 2022) suggests that other compounds should be involved.

Due to the economic impact of *A. fraterculus*, diverse control methods are under development, including the sterile insect technique (SIT) (Cladera et al., 2014; Mastrangelo et al., 2018, 2019, 2021; De León et al., 2021). This technique involves the field release of mass-reared sterile males, which must achieve mating with wild females to compromise the reproductive output of the wild population (Knipling, 1955). Therefore, the success of the SIT strongly depends on the sterile males' sexual competitiveness. In this context, phytochemicals have been used in the framework of the SIT to boost male sexual competitiveness in other fruit fly pests (Hendrichs, 2000; Enkerlin, 2005; Pereira et al., 2013; Enkerlin et al., 2017). For example, the exposure of *C. capitata* sterile males to volatile compounds of ginger root oil (GRO) is currently being used to increase their sexual competitiveness (Pereira et al., 2021). The identification of α-copaene as a stimulator of male sexual behavior in this species (Nishida et al., 2000; Shelly, 2001) was key to find GRO (which is rich in α-copaene) as an efficient and easy-to-use source of stimulating phytochemicals (Shelly T. E. et al., 2004). Transferring this methodology to *A. fraterculus* or other pests implies finding compounds capable of stimulating male sexual behavior and mating success. Therefore, studying the volatile compounds released from host plants and present in the sex pheromone blend can be a good approximation to identify candidate stimulating compounds.

The goal of this study was to identify candidate compounds involved in the exacerbated male sexual behavior that follows exposure to guava volatiles in *A. fraterculus*, providing insights from applied and evolutionary perspectives. We characterized the volatiles released by GF and GEO through chromatography coupled to mass spectrometry (GC-MS) and searched for common compounds. We selected candidate compounds after comparing our findings with the previously published chemical profiles of the essential oils tested by Ruiz et al. (2021). In addition, we used electroantennographic detection (EAD) to confirm that males are able to detect these candidate compounds and GC-MS to examine if these compounds are present among the volatiles released by *A. fraterculus* males. Furthermore, we performed bioassays to test if exposure to each candidate compound stimulates male sexual behavior, taking into account the possibility of dose-dependent and between-compounds interaction effects.

Materials and methods

Insects

All insects employed in this study correspond to the Brazilian 1 morphotype of *A. fraterculus* sp. 1 and belong to a laboratory colony that was kept for ca. 200 generations (approximately 10 generations per year) at Instituto Nacional de Tecnología Agropecuaria (INTA, Buenos Aires, Argentina). The colony derived from pupae obtained from guavas (*P. guajava*) collected in Horco Molle (Tucumán, Argentina) in 1997. Assayed flies emerged from pupae kept in glass containers inside an incubator (Sanyo MLR350, Japan) at 25 ± 1°C and under a photoperiod of 12L:12D. Emerged females were discarded since we only address male calling behavior in this study.

Fruits, essential oil, and synthetic compounds

Guava fruits were collected in Horco Molle (Tucuman, Argentina) and shipped within 48 h to INTA (Buenos Aires, Argentina), while undiluted guava essential oil obtained from fruits by cold-pressed was purchased from Salvia Cosmeceuticals (Delhi, India). The synthetic compounds

R-limonene (CAS 5989-27-5), β-caryophyllene (CAS 87-44-5), and β-ocimene E-Z isomers mix (CAS 13877-91-3) were purchased from Sigma-Aldrich (Saint Louis, MO, United States), while α-humulene (CAS 6753-98-6) was obtained from PhytoLab (Vestenbergsgreuth, Germany). All of them presented purities between 80 and 97%.

Volatile's collection

In order to obtain volatile compounds from GF and GEO, a piece of 3 g of GF and 200 μl of GEO (dispensed on a filter paper) were placed separately in glass aeration chambers (46 cm long × 4 cm i.d.). An airstream (500 ml/min), filtered using activated charcoal, was pushed through the chambers for 4 h (9:30–13:30) at 25°C. In addition, empty glass aeration chambers and glass aeration chambers containing clean filter paper were used as negative controls for GF and GEO collections, respectively. Volatiles from each chamber were collected onto traps with 20 mg of Hayesep Q adsorbent (Grace, Deerfield, IL, United States). The same procedure was applied to collect volatile compounds released by *A. fraterculus* calling-males. In this case, 15 mature males were placed in each glass aeration chamber during the calling activity peak (8:30–12:30). Empty glass aeration chambers were used as blanks. Ten replicates were performed for each guava volatile source and seven replicates for calling males. Collected compounds were eluted from the traps using 200 μl of dichloromethane (HPCL grade, Sintorgan, Argentina) and stored at -20°C until chemical analysis.

Chemical characterization of volatile samples and selection of candidate compounds

Chemical characterization of guava and flies' volatiles was performed by gas chromatography coupled to mass spectrometry detector (GC-MS, Agilent Technologies, CA, United States). The GC was equipped with an HP-5 capillary column (30 m length, 0.32 mm inner diameter and 0.25 μm film thickness; Agilent Technologies, CA, United States) and the MS was set in electron impact (EI) ionization at 70 eV mode. One microliter of each sample was injected at 240°C in splitless mode and Helium was used as the carrier gas at 0.7 ml/min (inlet pressure: 20.48 kPa). The oven temperature was programmed to increase at 10°C/min from 35°C (isothermal for 1 min) to 230°C and hold at 230°C for 15 min. GF and GEO compounds were identified by comparing their relative retention index (RI) with those reported in bibliography (Adams, 2007) and their mass spectra with those provided by NIST/EPA/NIH mass spectral library (NIST 08, National Institute of Standards and Technology, MD, United States). For each identified compound, its peak area was calculated and relativized to the total area of all identified compounds in the sample.

We considered as candidate compounds those present in both guava volatile profiles (GF and GEO) and also in volatile profiles of other odor sources that were shown to improve the mating success of *A. fraterculus* males through volatiles exposure (Ruiz et al., 2021). We confirmed the identity of candidate compounds by chromatographic analysis of synthetic standards. The chromatographic column that we used to identify guava's volatiles does not separate limonene optical isomers (R and S). However, the detection of limonene was considered as indicative of the presence of R-limonene since this isomer was previously reported in EOs from guava and other Myrtaceae species (Siddique et al., 2015; Hassan et al., 2020).

Electroantennography

The olfactory response of *A. fraterculus* males was tested by electroantennography (EAG) for five dilutions (0.01, 0.1, 1, 10, 100 μg/μl) of each candidate compound using n-hexane (HPLC grade, Sintorgan, Argentina) as solvent.

For each replicate, a fresh male head was carefully excised and mounted through its base on the tip of a glass capillary with phosphate buffer saline solution (PBS) (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 2 mM KH_2PO_4) containing the reference electrode. Using another PBS-filled capillary, the distal end of an antenna was contacted with the recording electrode. Electrodes were connected from their base to a signal amplifier (iDAC2; Syntech, Hilversum, Netherlands). The mounted antenna was placed under a stream of charcoal-filtered and humidified air (5 ml/min) which was delivered through a glass tube (1 cm i.d.). Five microliters of each sample were adsorbed onto a filter paper (2 cm × 1 cm; Whatman N1, Whatman, United Kingdom). After solvent evaporation, the filter paper was placed inside a Pasteur pipette. The tip of the Pasteur pipette was supported on a glass tube hole and the sample was injected into the airstream by an air puff from the base of the pipette. Using 1-hexanol (1μg/μl) as a positive standard and n-Hexane as the solvent control, samples were delivered to each antenna in the following order: positive standard, solvent control, different doses of the tested compound in increasing order, solvent control, and positive standard. Each stimulus was applied for 1 s and consecutive stimulus were separated in time by 60 s to prevent or minimize antennal adaptation. Eight to ten replicates were performed for each tested compound so each mounted antenna was assayed for only one candidate compound.

All males used in this assay were 8 to 12-day-old adults maintained in containers with water and food [3:1, sugar: brewer's yeast (CALSA, Tucumaìn, Argentina)] *ad libitum* since their emergence.

Bioassay I: Male calling behavior after exposure to candidate compounds

In *A. fraterculus*, the time males spend calling within a lek positively correlates with mating success (Segura et al., 2007; Bachmann et al., 2015). Therefore, to survey the potential effect of exposure on male mating success, we observed calling behavior on males exposed to four different concentrations of each candidate compound. Based on the calling behavior described for *A. fraterculus* males (Gomez Cendra et al., 2011), we considered that males were calling when they were observed performing wing fanning and/or exposing its salivary glands and anal tissue. These behaviors have been used in our previous studied (Bachmann et al., 2015, 2019; Belliard et al., 2022) as indicative of male calling. We tested four doses as follows: 0 (solvent control), 1, 10, and 100 µg/µl. n-Hexane (HPLC grade, Sintorgan, Argentina) was used as solvent for the dilutions. The selection of these concentrations followed a pilot test in which we found (for limonene) that higher concentrations were detrimental for male calling activity.

Newly emerged males were transferred randomly in groups of five individuals to 3 L glass containers with water, where they were fed *ad libitum* [3:1, sugar: brewer's yeast (CALSA, Tucumàn, Argentina)]. Each group (i.e., replicate) was randomly assigned to one of the four possible doses. Exposure of males to different compound concentrations or solvent (control) was carried out for 4 h (12:00–16:00) for two days starting 10 days after males' emergence. For this procedure, 20 µl of the assigned dilution were dispensed on a piece of filter paper (2 cm^2), which was placed inside a plastic container covered with a mesh to prevent contact between the filter paper and the insects. The plastic container with the filter paper was then placed inside the glass container that housed the experimental males. When males were 12 days old, the number of calling males per group were recorded four times for 1 hour (8:30, 8:50, 9:10, and 9:30). We used three batches of flies (groups of flies obtained from eggs collected the same day from the laboratory colony), totalizing 24–25 replicates (7–9 per batch) per dose, for each candidate compound. The containers with males were kept inside an environmental chamber (Sanyo MLR350, Japan) at 25 ± 1°C and a photoperiod of 12L:12D. Four chambers were employed, one for each dose treatment. From batch to batch, treatment levels were rotated among chambers.

Bioassay II: Male calling behavior after exposure to selected volatile blends

In order to study possible interactions among compounds, we assayed exposure to two mixtures of candidate compounds. One of them contained all candidate compounds and the other contained only those that proved to increase male calling activity in bioassay I, which were limonene and β-ocimene. In both mixtures, each compound was used at the concentration at which it elicited the highest response in bioassay I. n-hexane and limonene (10 g/µl) were used as solvent control and a positive control, respectively. Exposure was performed as in bioassay I. Three batches of flies were used to assess 24 replicates (7–9 per batch) per treatment.

Data analysis

All statistical analyses were carried out in R (v 4.1.1; R Core Team, 2021). We used Agilent MassHunter Qualitative Analysis Software (B.06.00) to calculate relative areas of identified compounds from GC-MS assays. For the chemical characterization of volatile samples, we calculated descriptive statistics with *stat.desc* function from *pastecs* package (Grosjean and Ibanez, 2018).

For electroantennography analysis, the amplified signals were recorded, calculated, and normalized to the positive standard by using the Syntech software (v1.2.5, Syntech, Hilversum, Netherlands). For each candidate compound separately, the normalized EAG response was compared between n-hexane (solvent control) and each dilution. We applied a Restricted Maximum Likelihood (REML) approach for fitting a linear mixed-effects model with a Gaussian error structure using the *lmer* function from the *lme4* and *lmerTest* packages (Bates et al., 2015; Kuznetsova et al., 2017). The model included the antenna as a random factor and the concentration as a within-antenna, categorical fixed factor. Model assumptions of normal distribution and homoscedasticity of residuals were evaluated with the *shapiro.test* function from the *stats* package (R Core Team, 2021) and *leveneTest* function from *car* package (Fox and Weisberg, 2019), respectively, and also graphically. In order to compare the male's olfactory response between candidate compounds, the net normalized EAG response (i.e., absolute response minus solvent control response) was modeled with the *nls* function from the *stats* package (R Core Team, 2021). The response of each antenna was described as a function of concentration using the following two-parameter, first-order kinetic function: $a \times (1 - \exp(-b \times \text{concentration}))$, where a represents the expected maximum response (at saturating dosages) and b represents the sensitivity of the response to pre-saturating concentration (Byers, 2013). As this function is strictly increasing, for those antennae that exhibited a response drop at the highest concentrations, the last response values needed to be adjusted prior to fitting the model; that is, each of the dropping records was replace with the drop values average so the drop was removed without affecting the antenna average response. Following parameter estimations, we compared both parameters separately across candidate compounds by performing Kruskal—Wallis tests using the *kruskal.test* function from the *stats* package (R Core Team, 2021).

To estimate the effects of each candidate compound at different doses (bioassay I) or mixture treatments (bioassay II) on calling activity, we first calculated a calling index defined as the number of calling males divided by the total number of males of the group (Belliard et al., 2022). Then, we performed a generalized linear model with binomial distribution [logit link function, *glmer* function from *lme4* package (Bates et al., 2015)]. Repeated measurements of the calling index were modeled as a function of dose or mixture treatment, for bioassay I or II, respectively. In each case, dose or treatment was included as a fixed factor, while the batch was treated as a random factor. For both bioassays, all pairwise comparisons were performed using *emmeans* function from *emmeans* package (Lenth et al., 2018).

Results

Chemical characterization of volatile samples and selection of candidate compounds

A total of 24 and 22 putative compounds were identified from GF and GEO samples, respectively. The identity of these compounds and their peak areas relative to the total area are shown in **Table 1**. Seven compounds were shared by both volatile profiles. However, only five of them—limonene, E-β-ocimene, Z-β-ocimene β-caryophyllene, and α-humulene—have also been reported during chemical characterization of other sources of volatile phytochemicals that increased *A. fraterculus* mating success following exposure (**Table 1** and **Figure 1**).

E-β-ocimene was found among the five major volatile compounds released by calling-males (% Relative area ± SD = 10.11 ± 0.45). The other candidate compounds were absent (β-caryophyllene and α-humulene) or only in trace (Relative area <0.1) amounts (limonene) (**Table 2** and **Figure 1**).

Electroantennography

All the candidate compounds evaluated (R-limonene, (E/Z)-β-ocimene, β-caryophyllene, and α-humulene) were detected by the insect antenna (**Figure 2**) The male's olfactory response to these compounds was stronger than for the solvent controls for at least 3 out of the 5 tested doses (all p-values < 0.01). However, neither the response triggered at saturating dosages (parameter a) nor the sensitivity of the response to pre-saturating concentration (parameter b) differed between compounds (p-values = 0.713 and 0.142, respectively).

Bioassay I: Male calling behavior after candidate compounds exposure

Males exposed to β-ocimene and R-limonene showed a higher calling index than control males when they were exposed to 1 and 10 μg/μl, respectively. For limonene, the male calling index odds (which stands for the probability that a male is calling divided by its complement) in males exposed to 10 μg/μl were on average 2.65 times ($Z_{ratio} = 5.16$, $p < 0.0001$) higher than in control males and 4.06 times ($Z_{ratio} = 6.40$, $p < 0.0001$) higher than in males exposed to 100 μg/μl (**Figure 3A**). In addition, limonene showed a detrimental effect on the calling index when males were exposed to pure limonene (∼800 μg/μl). In this case, the calling index odds were on average 2.22 times ($Z_{ratio} = 3.36$, $p = 0.001$) lower in exposed males than in control males (**Figure 3A**). For β-ocimene treatment, the male calling index odds were on average 2 times ($Z_{ratio} = 4.63$, $p < 0.0001$) higher in males exposed to 1 μg/μl than in control males and 2.60 times ($Z_{ratio} = 5.39$, $p < 0.0001$) higher than in males exposed to 100 μg/μl (**Figure 3B**). No effects on male calling index for any of the tested doses were found after exposure to β-caryophyllene (Chisq = 5.42; $p = 0.143$) (**Figure 3C**) or α-humulene (Chisq = 1.07, $p = 0.785$) (**Figure 3D**).

Bioassay II: Male calling behavior after candidate compounds blends exposure

Males exposed to the mixture of all the four candidate compounds showed a calling index odds 1.6 times higher than non-exposed males ($Z_{ratio} = 4.49$, $p < 0.0001$). However, the effect of this mixture on the calling index did not differ from the effect of 10 μg/μl of limonene ($Z_{ratio} = 1.33$, $p = 0.55$), which was on average 1.06 times higher than control ($Z_{ratio} = 4.49$, $p < 0.0001$). The maximum calling index was found for males exposed to the mixture of limonene and β-ocimene. Males exposed to this mixture showed calling index odds on average 4.24 times ($Z_{ratio} = 8.35$, $p < 0.0001$) higher than that for non-exposed males and 1.5 times ($Z_{ratio} = 4.51$, $p < 0.0001$) higher than males exposed to 10 μg/μl of limonene (**Figure 4**).

Discussion

In this study, we explored the chemical profiles of guava volatiles sources (GF and GEO) that can accentuate *A. fraterculus* male courtship behavior so as to find specific compounds that trigger this phenomenon. We first selected five candidate compounds (α-humulene, β-caryophyllene, E-β-ocimene, Z-β-ocimene, and R-limonene) which were not only present in GF and GEO but also in other volatile sources that stimulate male sexual behavior (LO and SpO). Our analyses

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TABLE 1 Volatile compounds from guava (*Psidium guava*) fruit and guava essential oil identified by GC-MS.

R.I.[a]	Name	IUPAC name	CAS No	Formula	% Relative area[b] ± SD	
					Fruit	Essential oil
802	Z-3-hexenal	(Z)-hex-3-enal	6789-80-6	$C_6H_{10}O$	25.99 ± 12.05	–
803	Ethyl butyrate	ethyl butanoate	105-54-4	$C_6H_{12}O_2$	22.13 ± 8.48	–
	Unknown				2.15 ± 1.64	–
851	(Z)-2-hexenal	(Z)-hex-2-enal	16635-54-4	$C_6H_{10}O$	1.41 ± 0.38	–
856	2-Hexenal	(E)-hex-2-enal	6728-26-3	$C_6H_{10}O$	4.37 ± 1.99	–
881	Isoamyl acetate	3-methylbutyl acetate	123-92-2	$C_7H_{14}O_2$	0.59 ± 0.44	–
883	2-Methylbutyl acetate	2-methylbutyl acetate	624-41-9	$C_7H_{14}O_2$	0.47 ± 0.21	–
	Unknown				0.62 ± 0.33	–
912	2,4-Hexadienal	(2E,4E)-hexa-2,4-dienal	142-83-6	C_6H_8O	0.80 ± 0.24	–
929	α-Phellandrene	2-methyl-5-propan-2-ylcyclohexa-1,3-diene	99-83-2	$C_{10}H_{16}$	–	0.5 ± 0.09
	Unknown				0.19 ± 0.06	–
935	α-Pinene	(1R,5R)-2,6,6-trimethylbicyclo[3.1.1]hept-2-ene	7785-70-8	$C_{10}H_{16}$	–	4.34 ± 1.05
950	Camphene	2,2-dimethyl-3-methylidenebicyclo[2.2.1]heptane	79-92-5	$C_{10}H_{16}$	–	0.63 ± 0.12
977	β-Pinene	6,6-dimethyl-2-methylidenebicyclo[3.1.1]heptane	127-91-3	$C_{10}H_{16}$	–	3.22 ± 0.44
992	Myrcene	7-methyl-3-methylideneocta-1,6-diene	123-35-3	$C_{10}H_{16}$	–	1.99 ± 0.22
	Unknown				–	0.33 ± 0.07
1001	Ethyl hexanoate	ethyl hexanoate	123-66-0	$C_8H_{16}O_2$	2.01 ± 0.62	–
	Unknown				–	1.29 ± 0.07
	Uknown				1.34 ± 0.94	–
1009	(E)-3-hexenyl acetate	[(E)-hex-3-enyl] acetate	3681-82-1	$C_8H_{14}O_2$	5.43 ± 3.06	–
1010	3-Carene	3,7,7-trimethylbicyclo[4.1.0]hept-3-ene	13466-78-9	$C_{10}H_{16}$	–	1.82 ± 0.09
1017	Hexyl acetate	hexyl acetate	142-92-7	$C_8H_{16}O_2$	1.20 ± 0.52	–
1017	α-Terpinene	1-methyl-4-propan-2-ylcyclohexa-1,3-diene	99-86-5	$C_{10}H_{16}$	–	5.87 ± 0.38
1022	Menthene	1-methyl-4-propan-2-ylcyclohexene	5502-88-5	$C_{10}H_{18}$	–	0.23 ± 0.03
1026	p-Cymene	1-methyl-4-propan-2-ylbenzene	99-87-6	$C_{10}H_{14}$	–	6.53 ± 0.52
1030	Limonene*	(4R)-1-methyl-4-prop-1-en-2-ylcyclohexene	5989-27-5	$C_{10}H_{16}$	5.58 ± 5.54	67.48 ± 0.92
1041	Z-β-ocimene*	(3Z)-3,7-dimethylocta-1,3,6-triene	3338-55-4	$C_{10}H_{16}$	0.71 ± 0.62	0.27 ± 0.05
1052	E-β-ocimene*	(3E)-3,7-dimethylocta-1,3,6-triene	3779-61-1	$C_{10}H_{16}$	0.35 ± 0.27	0.08 ± 0.02
1061	γ-Terpinene	1-methyl-4-propan-2-ylcyclohexa-1,4-diene	99-85-4	$C_{10}H_{16}$	–	1.94 ± 0.29
	Uknown				–	0.06 ± 0.02
1089	Terpinolene	1-methyl-4-propan-2-ylidenecyclohexene	586-62-9	$C_{10}H_{16}$	–	1.85 ± 0.29
1133	Allocimene	(4E,6Z)-2,6-dimethylocta-2,4,6-triene	7216-56-0	$C_{10}H_{16}$	0.44 ± 0.38	0.12 ± 0.03

(Continued)

TABLE 1 (Continued)

R.I.[a]	Name	IUPAC name	CAS No	Formula	% Relative area[b] ± SD	
					Fruit	Essential oil
1159	Menthone	(2S,5R)-5-methyl-2-propan-2-ylcyclohexan-1-one	14073-97-3	$C_{10}H_{18}O$	2.80 ± 3.71	0.12 ± 0.03
	Ethyl benzoate	ethyl benzoate	93-89-0	$C_9H_{10}O_2$	0.26 ± 0.16	–
1190	(Z)-3-hexenyl butyrate	[(Z)-hex-3-enyl] butanoate	16491-36-4	$C_{10}H_{18}O_2$	0.42 ± 0.34	–
	Unknown				–	0.38 ± 0.14
	Unknown				–	0.05 ± 0.02
1249	I-Carvone	(5R)-2-methyl-5-prop-1-en-2-ylcyclohex-2-en-1-one	6485-40-1	$C_{10}H_{14}O$	–	0.13 ± 0.06
	Unknown				–	0.02 ± 0.01
1262	Phenethyl acetate	2-phenylethyl acetate	103-45-7	$C_{10}H_{12}O_2$	0.38 ± 0.28	–
	Unknown				–	0.04 ± 0.02
	Unknown				–	0.08 ± 0.04
1289	Stragole	1-methoxy-4-prop-2-enylbenzene	140-67-0	$C_{10}H_{12}O$	–	0.09 ± 0.03
	Unknown				–	0.03 ± 0.01
1367	Neryl acetate	[(2Z)-3,7-dimethylocta-2,6-dienyl] acetate	141-12-8	$C_{12}H_{20}O_2$	–	0.16 ± 0.08
1375	3-Phenylpropyl acetate	3-phenylpropyl acetate	122-72-5	$C_{11}H_{14}O_2$	9.98 ± 4.46	
1381	α-Copaene	(1R,2S,6S,7S,8S)-1,3-dimethyl-8-propan-2-yltricyclo[4.4.0.02,7]dec-3-ene	3856-25-5	$C_{15}H_{24}$	–	0.02 ± 0.01
	Unknown				–	0.02 ± 0.01
1428	β-Caryophyllene*	(1R,4E,9S)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene	87-44-5	$C_{15}H_{24}$	2.96 ± 3.14	0.3 ± 0.12
1463	α-Humulene*	(1E,4E,8E)-2,6,6,9-tetramethylcycloundeca-1,4,8-triene	6753-98-6	$C_{15}H_{24}$	6.04 ± 6.27	0.08 ± 0.04
1484	γ-Selinene	(4aS,8aR)-8a-methyl-4-methylidene-6-propan-2-ylidene-2,3,4a,5,7,8-hexahydro-1H-naphthalene	515-17-3	$C_{15}H_{24}$	0.18 ± 0.14	–
1496	β-Selinene	(3S,4aR,8aS)-8a-methyl-5-methylidene-3-prop-1-en-2-yl-1,2,3,4,4a,6,7,8-octahydronaphthalene	17066-67-0	$C_{15}H_{24}$	1.35 ± 1.22	–
1504	α-Selinene	(3R,4aR,8aR)-5,8a-dimethyl-3-prop-1-en-2-yl-2,3,4,4a,7,8-hexahydro-1H-naphthalene	473-13-2	$C_{15}H_{24}$	1.36 ± 1.54	–

[a]The retention index calculated from retention times on an HP5-MS capillary column. [b]Relative area to the total area of all identified compounds in the sample. *Compounds compared with synthetic standards [R-limonene, (E/Z)-β-ocimene, β-caryophyllene, α -humulene].

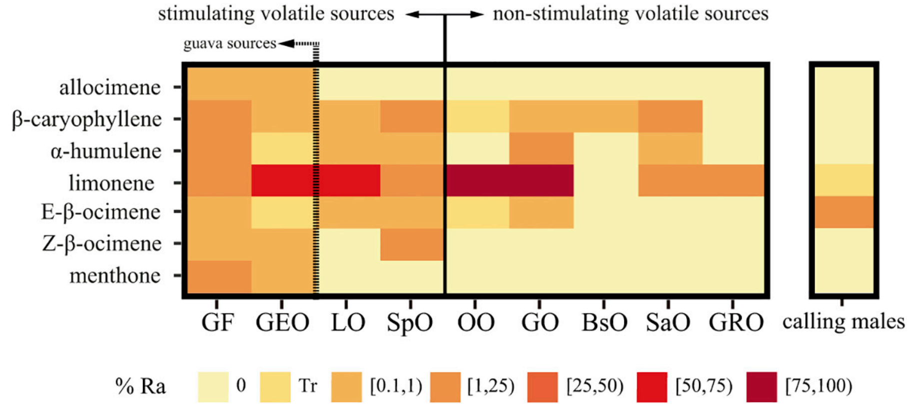

FIGURE 1
Heatmap of the relative abundance of the seven volatile compounds shared by guava fruit and guava essential oil for different volatile plant sources and calling males. Some of these volatile sources [*Psidium guajava* fruit (GF), *Psidium guajava* essential oil (GEO), *Citrus limon* essential oil (LO), and *Schinus polygama* essential oil (SpO)] are known to stimulate *A. fraterculus* male sexual behavior (Vera et al., 2013; Bachmann et al., 2015; Ruiz et al., 2021; Belliard et al., 2022). The rest of them [*Citrus sinensis* essential oil (OO), *Citrus paradisi* essential oil (GO), *Baccharis spartioides* essential oil (BsO), *Schinus areira* essential oil (SaO), and *Zingiber officinale* root oil (GRO)] were found as non-stimulating sources (Ruiz et al., 2021). Calling males volatiles were collected from 11-day-old mature males. Data correspond to peak areas relative to the total area from GC-MS analysis of each volatiles source. Data for GF and GEO were generated in the present study while data for non-guava resources were obtained from Ruiz et al. (2015) and Jofré Barud (2018). Traces (Tr) stands for relative area < 0.1%.

TABLE 2 Volatile compounds from *Anastrepha fraterculus* sp1 calling males identified by GC-MS.

R.I.[a]	Name	IUPAC name	CAS No	Formula	% Relative area[b] ± SD
1030	limonene*	(4R)-1-methyl-4-prop-1-en-2-ylcyclohexene	5989-27-5	$C_{10}H_{16}$	0.07 ± 0.03
1041	Z-β-ocimene*	(3Z)-3,7-dimethylocta-1,3,6-triene	3779-61-1	$C_{10}H_{16}$	0.22 ± 0.03
1052	E-β-ocimene*	(3E)-3,7-dimethylocta-1,3,6-triene	3338-55-4	$C_{10}H_{16}$	10.11 ± 1.19
1105	Nonanal		124-19-6	$C_9H_{18}O$	0.07 ± 0.06
1136	Allocimene	(4E,6Z)-2,6-dimethylocta-2,4,6-triene	7216-56-0	$C_{10}H_{16}$	0.12 ± 0.02
-	Unknown		-	-	0.30 ± 0.09
1498	(Z,E)- α-farnesene	(3Z,6E)-3,7,11-trimethyldodeca-1,3,6,10-tetraene	26560-14-5	$C_{15}H_{24}$	4.62 ± 1.24
1513	(E,E)- α-farnesene	(3E,6E)-3,7,11-trimethyldodeca-1,3,6,10-tetraene	502-61-4	$C_{15}H_{24}$	67.66 ± 3.89
1601	Anastrephin	(3aα,4β,7aβ)-4-Ethenylhexahydro-4,7a-dimethyl-2(3H)-benzofuranone	86003-24-9	$C_{12}H_{18}O_2$	5.64 ± 1.51
1614	Epianastrephin	4-ethenyl-4,7a-dimethyl-3a,5,6,7-tetrahydro-3H-1-benzofuran-2-one	87248-73-5	$C_{12}H_{18}O_2$	9.09 ± 3.96

[a]The retention index calculated from retention times on an HP5-MS capillary column. [b]Relative area to the total area of all identified compounds in the sample. *Compounds identified by comparison with synthetic standards.

revealed that all candidate compounds elicit a response on male antennae, but only R-limonene and (E/Z)-β-ocimene enhanced calling behavior of exposed males. The male response to these compounds may have evolved as an adaptative response to synchronize sexual behavior with the availability of guava fruit, an important native host of *A. fraterculus*. Our results support the future evaluation of R-limonene, (E/Z)-β-ocimene, and combinations of these compounds in a SIT context (i.e., mass-reared and sterilized males) with the goal of improving the sexual competitiveness of males to be released as part of this pest control strategy.

The repertoires of volatile compounds we identified from GF and GEO were remarkably different: only seven out of 39 compounds were shared between these sources. Also, these common compounds showed very different relative abundances between GF and GEO. These results are not surprising if we take into account that essential oils are the outcome of an extraction process that gives a partial perspective of the volatiles mixture released by the plant (Figueiredo, 2017). In addition, guava fruit used to obtain the commercial essential oil (Nueva Delhi, India) and the fresh guava fruit (collected in Tucuman, Argentina) used in this study likely belong to different *P. guajava* chemotypes with different types and proportions of the chemical constituents (Figueiredo, 2017; de Souza et al., 2018). In this study, however, we ensured to use the same GF chemotype and GEO batch that we had previously showed to enhance *A. fraterculus* male sexual behavior (Vera et al., 2013; Bachmann et al., 2015; Belliard et al., 2022). The fact that different headspace sampling techniques provide non-overlapping lists of compounds (Rizzolo et al., 1992) may indicate that other

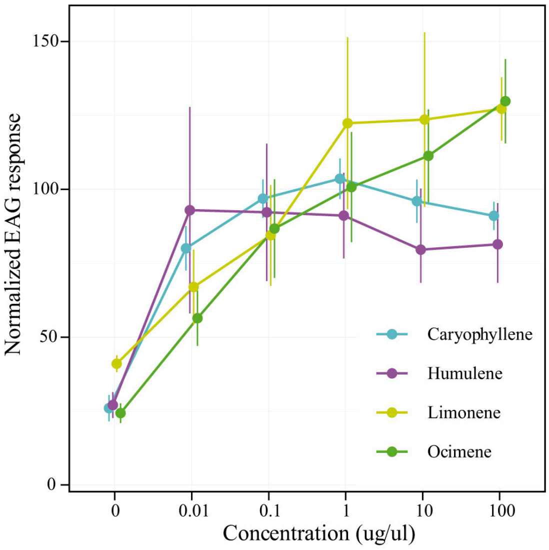

FIGURE 2
Antennal responses of male *A. fraterculus* to candidate compounds. Normalized EAG responses (mean ± SE) to five concentrations are shown for each synthetic candidate compound: R-limonene, (E/Z)-β-ocimene (green), β-caryophyllene (cyan), and α-humulene (magenta). The responses for 0-μg/μl doses correspond to n-hexane, the solvent used for dilutions. All responses were normalized to the male antennal response to 1 μg/μl 1-hexanol.

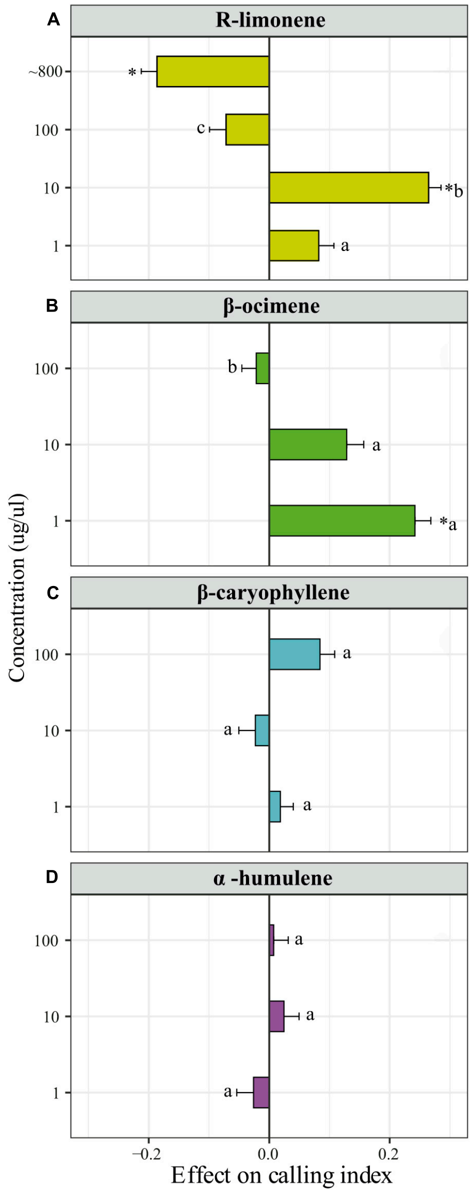

FIGURE 3
Dose-dependent effect of candidate compounds on *A. fraterculus* male calling behavior. Mean (±SE) effect on the male calling index (the calling index of exposed males minus the calling index of control males) is shown for males exposed to different concentrations of **(A)** R-limonene, **(B)** (E/Z)-β-ocimene, **(C)** β-caryophyllene, and **(D)** α-humulene. The calling index stands for the proportion of calling males (number of calling-males/number of assayed males). Control males were exposed to n-hexane. *Indicates significant differences between exposed and control males ($P < 0.05$, Tukey test) while letters indicate significant differences across treatments ($P < 0.05$, Tukey test) except for R-limonene at ~800 μg/μl for which a separated assay was performed.

compounds, not detected here, could be shared between GF and GEO. This is an experimental limitation of our approach.

The effect of R-limonene exposure on male courtship behavior is in agreement with Ruiz et al. (2021), who showed that *A. fraterculus* males previously exposed to this compound exhibit higher mating success. Also, R-limonene is the major volatile compound of citric essential oils that were found to increase the sexual performance of *A. fraterculus*, *A. ludens*, and *C. capitata* (Shelly T. et al., 2004; Shelly, 2009; Morató et al., 2015; Kouloussis et al., 2017; Ruiz et al., 2021). However, male exposure to R-limonene as a pure compound did not affect male mating success in *C. capitata* (Juan-Blasco et al., 2013). E-β-ocimene, which is a major component of *A. fraterculus* sex pheromone blend (Bachmann, 2016), had not been directly implied in the stimulation of male calling behavior. Nonetheless, it was used in the past as part of a synthetic blend that proved to increase male mating success in this species (Bachmann et al., 2015). For some compounds, insects may respond differentially to enantiomers (Flath et al., 1994; Giatropoulos et al., 2012; Borrego et al., 2021). Given that Z-β-ocimene isomer was also reported in the *A. fraterculus* sex pheromone blend (Bøízová et al., 2013) and that we tested the effect of β-ocimene using a mix of isomers (E and Z), further studies are necessary to determine the effect of each isomer alone. The other two compounds we evaluated, β-caryophyllene and α-humulene, did not affect *A. fraterculus* male calling behavior when exposed to only one of these compounds. The potential of β-caryophyllene to stimulate tephritid fruit flies' sexual behavior has had not been assayed before, although this compound was found to attract

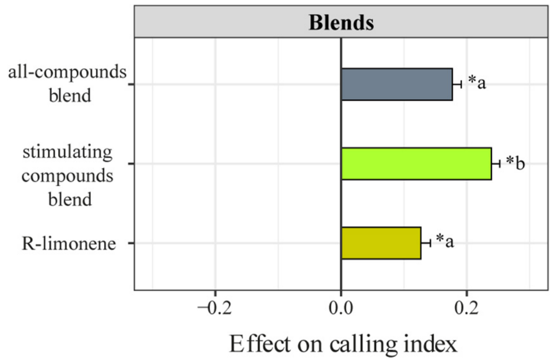

FIGURE 4
Effect of candidate compounds blends on *A. fraterculus* male calling behavior. Mean (± SE) effect on male calling index (calling index of exposed males minus calling index of control males) is shown for males exposed to R-limonene (yellow), the stimulating compounds blend (i.e., a blend of R-limonene and (E/Z)-β-ocimene) (green), the all-compounds blend (i.e., a blend of R-limonene (E/Z)-β-ocimene, β-caryophyllene, and α-humulene) (gray). Calling index stands for the proportion of calling males (number of calling-males/number of males assayed). All candidate compound dilutions were made using n-hexane as solvent. * Indicates significant differences between exposed and control males (P < 0.05, Tukey test) while letters indicate significant differences across treatments (*P* < 0.05, Tukey test).

flies of several tephritid species (Kamiji et al., 2018; Wee et al., 2018; Jaleel et al., 2019). Exposure to α-humulene was tested on *C. capitata* males by Shelly and Nishimoto (2015), who found a negative effect on male mating performance.

The effects of limonene and (E/Z)-β-ocimene were dependent on the exposure dose. R-limonene enhanced male calling behavior at 10 μg/μl while no effects were observed for the lower dose of 1 μg/μl. Moreover, a detrimental effect was observed for pure R-limonene (∼800 μg/μl). Toxic effects of limonene and EOs containing high proportions of limonene were previously reported for tephritid species, including *A. fraterculus* (Salvatore et al., 2004; Papachristos et al., 2009; Ruiz et al., 2014; Papanastasiou et al., 2017; Oviedo et al., 2018, 2020), where survival, emergence, and fecundity were affected by exposure to these phytochemicals. In agreement, Ruiz et al. (2021) have found that only the EO of lemon, as opposed to the EOs of orange and grapefruit, was able to stimulate *A. fraterculus* males. Among these three EOs, lemon EO had the lowest R-limonene concentration (Ruiz et al., 2015; González-Mas et al., 2019). Ruiz et al. (2021) also found that males exposed to pure R-limonene achieved more matings during the assay than non-exposed males. While Ruiz et al. (2021) exposed flies for one hour, in the present work the exposure lasted four hours. Therefore, the time of exposure (together with exposure dose) may likely modulate the treatment response. On the other hand, (E/Z)-β-ocimene did not reduce male calling index at any evaluated dose, although we did not evaluate concentrations higher than 100 μg/μl for this compound. Nonetheless, (E/Z)-β-ocimene only stimulated calling males at the lowest tested dose of 1 μg/μl, again showing a dose-dependent effect. Similarly, in *C. capitata*, the stimulating effect of GRO exposure on male mating success is maximized at low doses (Paranhos et al., 2013).

Individual volatile compounds response often depends on the volatile context in which they are perceived (Schröder and Hilker, 2008; Beyaert et al., 2010; Bruce and Pickett, 2011; Conchou et al., 2019). Thus, the combination of some compounds could activate synergistic or antagonistic behavioral responses on the flies (Bruce and Pickett, 2011; Conchou et al., 2019). For instance, compounds that alone do not trigger any behavioral response may play a key role when perceived in a blend (Beyaert et al., 2010). For this reason, we tested two blends of our candidate compounds: one of them made only with the two stimulating compounds (i.e., R-limonene and β-ocimene) and the other made of all our candidate compounds (i.e., R-limonene, E/Z β-ocimene, β-caryophyllene, and α-humulene). Males exposed to the blend of the stimulating compounds exhibited a higher calling index than those exposed to R-limonene alone and those exposed to the complete blend (all five compounds). Therefore, limonene and β-ocimene seem to have partially additive effects on male calling behavior while β-caryophyllene and α-humulene might be interacting antagonistically with stimulating compounds. In accordance to our results, *A. fraterculus* males increased their mating success when exposed to a synthetic blend containing limonene, β-ocimene, and α-humulene among other synthetic compounds (Bachmann et al., 2015). While Bachmann et al. (2015) used the same concentration for all compounds, in our blends, the concentration of each compound was the one for which the compound showed the highest stimulating effect when tested alone. However, the relative abundance of the compounds we evaluated in both blends do not represent those of the guava fruit, to which males could be exposed in nature [although in nature males could be stimulated by other structures of guava trees, as reported for *C. capitata* males by Shelly and Villalobos (2004)]. Aromas with different proportions of the same compounds could be interpreted by insects as different messages (Conchou et al., 2019). Thus, exploring combinations with proportions found in natural resources may help understanding the ecological relevance of these compounds and find a blend that maximizes the male calling activity.

Anastrepha fraterculus is a cryptic species complex, in which distinct populations differ in the composition of the volatiles released by calling-males (Lima et al., 2001; Cáceres et al., 2009; Bøízová et al., 2013; Vaníèková et al., 2015). In agreement with previous studies on flies that derived from the same population as the ones we used (Cáceres et al., 2009; Bachmann, 2016), we confirmed the presence of E-β-ocimene as one of the major compounds and limonene as a minor compound of the volatile blend released by *A. fraterculus* sp. 1 calling-males. In fact, we only found traces of limonene among the volatile compounds recovered from calling males. However, Bøízová et al. (2013) found higher proportions of limonene in the volatile compounds released by calling-males of the same *A. fraterculus* morphotype fed on a different diet and bred under different conditions. As it was found in *C. capitata*, diet and breeding conditions may affect the composition of the sex pheromones blend (Merli et al., 2018). In addition, we found that both compounds, limonene and β-ocimene, were able to elicit an electrophysiological response on virgin mature male antennae. Curiously, E-β-ocimene but not limonene triggered an electrophysiological response in virgin mature females' antennae, when they were exposed to the volatile blend collected from *A. fraterculus* sp. 1 calling-males (Bachmann, 2016), which suggests that E-β-ocimene, but not limonene, is a sex pheromone. This is particularly relevant in the context of the mating system of this species, in which males aggregate near/on the host to release the pheromone and increase the signal intensity. Albeit not a sex pheromone compound, limonene from the hosts may be an aggregation cue in a sexual context. Still, the role of these compounds must be tested through behavioral studies involving males and females.

The differences in limonene and β-ocimene concentrations among volatile compounds released by calling males were proposed as a mechanism of pre-mating reproductive isolation between *A. ludens* and *A. suspensa* that may has evolved during the initial stages of speciation (Rocca et al., 1992). Unlike *A. fraterculus* morphotype sp. 1, calling males of *A. fraterculus* sp. 3 release limonene as a major compound and β-ocimene as one of the less abundant ones (Bøízová et al., 2013; Milet-Pinheiro et al., 2015). Also, *A. fraterculus* sp. 3 virgin females are able to detect limonene from calling-males' volatile blends (Milet-Pinheiro et al., 2015). Therefore, the relative amount of limonene and β-ocimene in the sex pheromone blend may also play a role as a reproductive barrier between *A. fraterculus* morphotypes. Additional studies are nonetheless needed in order to further test this hypothesis and separate the genetic and environmental components that determine the sex pheromone composition.

Females of many tephritid species are more attracted to ripening fruit than to immature fruit, and they also prefer ripening fruit for oviposition (Cunningham et al., 2016; Cortés-Martínez et al., 2021). During the ripening process of guava, an ancestral host of *A. fraterculus* complex (Hernández-Ortiz et al., 2019), the release of limonene and β-ocimene by the fruit increases (Soares et al., 2007). Therefore, it would not be surprising that the male sexual stimulation caused by the exposure to these compounds constitutes a mechanism useful to synchronize energetic investment in courtship with the availability of host fruit. Although the physiological mechanisms underlying tephritid male sexual stimulation after phytochemical exposure are poorly understood (Segura et al., 2018), they are likely associated with enhanced energy metabolism (Kumaran et al., 2014). In *C. capitata*, for instance, the male energy reserves (glycogen, lipids, and proteins) are reduced during lek participation (Yuval et al., 1998), when males display a set of energetically expensive courtship behaviors (Kaspi et al., 2000). These reserves drop is probably to maintain a high level of sugar available for muscle cells (Warburg and Yuval, 1997; Yuval et al., 1998). The mobilization of insect energy reserves is controlled by the adipokinetic hormone (AKH), which is synthesized in corpora cardiac cells under energy-demanding conditions (Van der Horst et al., 2001; Van der Horst, 2003; Arrese and Soulages, 2010; Nagata and Zhou, 2019). At least in *D. melanogaster* and *B. dorsalis*, the expression of AKH receptors (AKHR) is necessary for starved males to display courtship behavior at the same frequency as fed flies do (Lebreton et al., 2016; Hou et al., 2017). Also, in *D. melanogaster* starved flies, the AKH expression levels and circulating sugar concentration increased when the flies are exposed to food odor, likely to provide fuel to support food search activity (Lushchak et al., 2015). Considering that the stimulating effect of guava volatiles exposure on *A. fraterculus* male calling activity is only detected in flies fed on a low-quality diet (Belliard et al., 2022), it is possible that such an effect is modulated through the AKH pathway. Gene expression analysis based on RNA-seq and RNA interference studies will help identify the genes involved in the guava exposure effect and test this hypothesis.

In the present study, we identified two compounds, limonene and β-ocimene, which a part of the volatiles released by guava fruit and guava essential oil, that stimulated calling behavior in *A. fraterculus* males. In addition, we showed that (E/Z)-β-ocimene exposure requires lower doses than limonene treatment to generate the same effect on male sexual performance, although the use of a combination of both compounds could significantly improve the results. Furthermore, we found that other compounds released by guava fruit, which are also present in the guava essential oil, reduced the effect of limonene and β-ocimene, probably indicating some negative interaction among compounds. Our results also support the previously proposed use of GEO, LO, and limonene to improve the sexual performance of *A. fraterculus* sterile males in a SIT context (Ruiz et al., 2021; Belliard et al., 2022). However, since the male response is modulated by the exposure dose, the time of exposure, and the nutritional status of the flies (Belliard et al., 2022), the implementation of aromatherapy with limonene and β-ocimene may require careful tuning to avoid unwanted effects. In addition, searching for these compounds in non-tested EOs' volatile profiles may help find cheaper and easy-to-obtain resources that can be employed in the context of pest management through the SIT, as in the case of *C. capitata* and the effective use of GRO to boost sterile males' sexual competitiveness.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

PCF and MTV contributed the material. SAB and GEB conducted the bioassays. SAB and JH conducted the statistical analyses. SAB and DFS wrote the manuscript. All authors conceived and designed the research and reviewed and approved the manuscript for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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