Diel rhythm of volatile emissions of males and females of the peach fruit fly *Bactrocera zonata*

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ABSTRACT

Fruit flies in the genus *Bactrocera* are among the most destructive insect pests of fruits and vegetables throughout the world. A number of studies have identified volatiles from fruit flies, but few reports have demonstrated behavioral effects or sensitivities of fly antennae to these compounds. We applied a recently developed method of automated headspace analysis using SPME (Solid Phase Microextraction) fibers and GC–MS (gas chromatography mass spectrometry), termed SSGA, to reveal volatiles specific to each sex of *B. zonata* that are emitted in a diel periodicity. The volatiles released primarily at dusk were identified by GC–MS and chemical syntheses as several spiroacetals, pyrazines, and ethyl esters. Solvent extraction of male rectal glands or airborne collections from each sex, followed by GC–MS, showed that certain of the volatiles increase or decrease in quantity sex-specifically with age of the flies. Electroantennographic (EAG) analysis of dose-response indicates differences in sensitivities of male and female antenna to the various volatiles. Our study provides a comprehensive analysis of the volatile chemicals produced and released by *B. zonata* and their antennal responses. The possible pheromone and semiochemical roles of the various volatiles released by each sex and the difficulties of establishing behavioral functions are discussed.

1. Introduction

Fruit flies (Diptera: Tephritidae) include over 4000 described species with many being important agricultural pests throughout the world where they damage a wide range of fruits and vegetables (White and Elson-Harris, 1992; Aluja and Norrbom, 1999). Losses to tephritid flies have been estimated at over 2 billion USD annually (Malavasi, 2014). Many countries enforce quarantine regulations against exporting countries with established invasive tephritid species, or the exported/ imported fruits and vegetables must undergo post-harvest treatment (Hee et al., 2015).

Fruit flies in the genus *Bactrocera* include over 450 species that are notorious worldwide for their destructive impact on agriculture (Drew and Romig, 2013; Doorenweerd et al., 2018). The highly polyphagous genus that earlier distributed throughout tropical Asia, the south

Pacific, and Australia has more recently invaded other regions including South America, USA, the eastern Mediterranean, and most of the African continent (White and Elson-Harris, 1992; Doorenweerd et al., 2018). Adult female fruit flies cause damage by laying eggs in the peel of fruits and vegetables where the resulting larvae feed on decaying plant tissues yielding a soft mush that is not marketable. Infested fruits and vegetables soon become inedible and fall to the ground. Besides the direct damage to the fruit, the substantial economic importance of fruit flies is due to their status as quarantine pests in many countries that preclude importation from those regions (Cobos-Suarez et al., 2010).

Eradication or significant reduction of fruit fly populations has been the aim of numerous integrated pest management (IPM) and area-wide control programs (Steiner et al., 1965; Suckling et al., 2016). In the past, control programs have used (a) chemical control with

conventional insecticide sprays (e.g. organophosphates and pyrethroids) and/or poisoned food–based baits, (b) sterile insect technique (SIT), and (c) male annihilation technique (MAT) employing chemicals attractive to males. The prime example of MAT is the use of methyl eugenol, found naturally in nectars of many plants, as a lure for *B. dorsalis* males (Tan and Nishida, 2012). However, for some species of *Bactrocera* and other fruit flies where a strong male lure is not known, MAT is not a viable option for control or eradication. In addition to the area-wide control measures mentioned above (insecticides, SIT, MAT) control of *Bactrocera* fruit flies and related species throughout Asia and the Pacific includes releases of natural enemies and cultural control methods (Vargas et al., 2012).

In Israel there are two known tephritid flies of economic importance that have been present for at least 100 years: the highly polyphagous Mediterranean fruit fly (medfly), *Ceratitis capitata*, known to oviposit in fruits of over 250 species of plants (McQuate and Liquido, 2017) and the monophagous olive fruit fly, *Bactrocera oleae*, which attacks only olives. Two other species have invaded Israel in recent years: the Ethiopian fruit fly, *Dacus ciliatus*, with limited distribution in the south of Israel (Rempoulakis et al., 2015), and the peach fruit fly *Bactrocera zonata* within the metropolitan area of Tel–Aviv (EPPO 2014; Gazit and Akiva, 2017). *B. zonata* is ranked in the category of most damaging pests that includes widespread invasive polyphagous generalists or highly destructive specialists that have become established outside their native range (Vargas et al., 2012). According to McQuate and Liquido (2017), *B. zonata* causes damage to at least 55 host plants of economic importance. *B. zonata* males are attracted to methyl eugenol that is used for monitoring and in MAT, however, MAT does not provide satisfactory control because females causing the economic loses are not removed.

The type of semiochemicals isolated and identified from *Bactrocera* fruit flies are generally unique to the group (Fletcher and Kitching, 1995). A visible emission from male *Bactrocera* flies was observed in two species at dusk, as a smoky plume by Ohinata et al. (1982). Following this report, a number of pest *Bactrocera* species were found to contain or release various spiroacetals, mostly in females but sometimes in males (Baker and Bacon, 1985; Booth et al., 2006; Levi−Zada et al., 2012). Pyrazines have mainly been found in other fruit flies (Perkins et al., 1990a,b; Baker et al., 1982; Chuman et al., 1987). Ethyl esters have been found in some species of *Bactrocera* fruit flies (Gariboldi et al., 1983; Baker and Bacon, 1985; Perkins et al., 1990b; Canale et al., 2015). The suggestion has been that these volatiles play some role as pheromones in mating systems, but behavioral evidence is scarce due to the semiochemical complexity of both sexes, probable short-range effects of the components, and possible interactions with acoustic communication in leks (mating aggregations) (Fletcher, 1987; Aluja and Norrbom, 1999; Sivinski et al., 2000; Heath et al., 2001; Benelli et al., 2014). There is only one report in the literature that males of *B. zonata* contain a pheromone in their rectal glands that attracts females (Ahmad and Afzal, 1977). However, no volatiles released by males or identified from these glands have been reported.

Our main objective was to observe quantitative and qualitative changes in volatile compounds released by both sexes of *B. zonata* throughout the day as they age after eclosion. A change in amounts of some volatiles with age, sexual maturation, and time of day (i.e., when copulation occurs) would suggest that these compounds may be pheromone components. To explore this we used a recently developed technique that samples headspace volatiles termed SSGA, Sequential SPME (Solid Phase Micro Extraction)/GC–MS (gas chromatography-mass spectrometry) Analysis. Under natural photoperiod conditions, the SSGA method uses the SPME fiber to collect volatiles released by the target insect every 1–2 h by means of an auto-sampler. The absorbed volatiles on the SPME fiber are then injected directly into the GC–MS port, which heats the volatiles off the fiber and cleans it in preparation for another volatile collection by the auto–sampler. Repeating the sampling process for several days reveals the cyclic diel pattern

(circadian rhythm) of pheromone release if such exists (Levi-Zada et al., 2012, 2013, 2014a,b, 2019a,b). Usually, only a few insects are needed with the SSGA method to reveal pheromone components that vary in quantity during the day, as opposed to any contaminants that remain constant or erratic. In addition, SSGA is able to discover unstable components due to faster analysis and avoidance of enzymatic degradation that may occur in solvent extracts of tissues (Byers, 2006). Since we had access to plentiful fruit flies in rearing, we also used common methods of pheromone isolation such as aeration (collecting airborne volatiles on absorbents) and gland extraction in solvent to characterize putative pheromone components.

The antenna of both sexes of *B. zonata* contain numerous trichoid and basiconic sensilla that are likely to respond to volatiles (Awad et al., 2015). Thus, a second objective of the present work was to use electroantennographic (EAG) analysis to explore the antennal sensitivities of males and females to the volatile chemicals released in a diel rhythm by each sex of *B. zonata*. Information on the identities and quantities of circadian-released volatiles and their excitation of antennae of each sex could ultimately be employed in behavior-based control strategies.

2. Materials and methods

2.1. Insects

B. zonata adults were obtained from rearing chambers in the quarantine facility of the Plant Protection and Inspection Services of the Ministry of Agriculture and Rural Development of Israel, located at Volcani Center (Gazit and Akiva, 2017). Photoperiod in quarantine was 14:10 (L:D), onset 7:00, dark 21:00. Males were separated from the females immediately after eclosion. Individuals were removed from quarantine and analyzed with SSGA or EAG. Males for gland dissections were caught alive in Tel-Aviv urban area (N 32°3′47.011″, E 34°47′40.695″) by a trap baited with methyl eugenol (ME). Inside the trap there was a small vial with water and a cut date fruit in order to sustain the flies alive until brought to the laboratory and frozen to await chemical analysis.

2.2. Chemicals

Three chiral spiroacetal standards: (S,S)-, (R,R)- and (R,S)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (meso form) were contributed by Prof. Wittko Francke of Hamburg University. Racemic E,E spiroacetals for EAG were synthesized according to Phillips et al. (1980). (E,E)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (DMSU) containing the 2S,8S and 2R,8R forms was synthesized from δ-caprolactone and 4-pentyl-2-ol, and the yield of the two isomers (2S,8S and 2R,8R) was 17% with chemical purity of 98%. (E,E)-2,8-ethylmethyl-1,7-dioxaspiro[5.5]undecane (EMSU) was synthesized from δ-caprolactone and 5-hexyn-3-ol, and yield of two isomers was 10% (chemical purity 98%). (E,E)-2-Propyl-8-methyl-1,7-dioxaspiro[5.5]undecane (PMSU) was synthesized from octanolactone and 4-pentyl-2-ol, and yield of the two isomers was 12% (chemical purity 98%). 2-Ethyl-3,5,6-trimethylpyrazine standard was contributed by Prof. Jeroen S. Dickschat of Rheinische Friedrich-Wilhelms-University, Bonn. Later this pyrazine was synthesized by us (Dickschat et al., 2010; Bohman et al., 2012) with yield of 2% and chemical purity of 97%. N-Isopentylacetamide was synthesized by the known Schotten-Baumann acetylation reaction starting from isopentylamine (15% yield, 99% chemical purity). Tetrahydrofuran (THF) and ethyl ether were pre-distilled and then dried by sodium-benzophenone mixture. All commercially available chemicals and solvents (chemical purity > 97%) were purchased from Sigma-Aldrich and used without further purification.

2.3. Analytical instrumentation

Analyses by a nonpolar RTX-5SilMS (Restek, Bellefonte, PA, USA) column (30 m × 0.25 mm ID × 0.25 μm film) were performed on an Agilent 6890 N GC instrument interfaced with an Agilent 5973 MS detector equipped with a commercial auto-sampler (MPS2-Twister, Gerstel, Germany) that was programmed by Maestro software 1.4.8.14 (Gerstel, Germany). The column was kept at 50 °C for 5 min, then programmed at 10°/min to 230 °C and held for 10 min. Analyses on chiral RT-βDEXsm (Restek, Bad Homburg, Germany) column (30 m × 0.25 mm ID × 0.25 μm film) were performed using an Agilent 7890A GC interfaced with an Agilent 5975C MS detector and flame ionization detector (FID) with a commercial auto-sampler (GC-sampler 80, Agilent Technologies, Switzerland) operated by MassHunter GC–MS acquisition software (B.07.02.1938, Agilent, USA). The column was kept at 60 °C for 0 min, then programmed at 15°/min to 170 °C and held for 10 min. The flow of the polar column was split to both detectors equally by an Agilent purged two-way effluent splitter, enabling qualitative and quantitative analyses simultaneously. Quantification was performed using calibration curves of the corresponding compounds: 1,7-dioxaspiro[5.5]undecane (olean, the olive fruit fly sex pheromone) for spiroacetal components, tetramethyl pyrazine for pyrazine components, and ethyldodecanoate for ethyl ester components. Analyses on both machines were performed in the splitless mode with the split valve opened after 1 min and an MS m/z range of 40–400 a.m.u. Helium flow through the column was 1.5 mL/min and the GC–MS inlet temperature was kept at 230 °C. A 0.75 mm ID glass inlet liner was used for Solid Phase Microextraction (SPME) injections. The liner was replaced with a 4 mm ID liner for liquid injections. SPME syringes with a 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber (Supelco, Bellfonte, PA) were used for headspace analyses with desorption time of 6 min in splitless mode. A 10 μL syringe was used for liquid analyses. Identification of the compounds was done by comparing their mass spectra to Wiley08 and NIST MS databases, literature information, and to authentic standards.

2.4. Analysis of pheromone release by sequential SPME/GC–MS

SSGA used the auto-samplers mentioned above to expose the SPME fiber for 2 h to collect volatiles released by groups of four to six females ($N = 29$) or males ($N = 22$) placed inside a cylindrical 10 mL glass vial. The groups contained adult flies of ages 0–5, 5–10, 10–15, 15–20, and 20–25 days. After each 2-h collection period the fiber was injected into the GC–MS port to desorb the volatiles and clean the fiber. The fiber was cleaned by baking out in the needle heater unit of the autosampler for at least 10 min at 240 °C prior to each sequential collection period. This collection and injection of the fiber was repeated throughout the day and night for several days until the fly died or stopped release of volatiles. The cylindrical 10 mL glass vials (45 mm high × 20 mm ID) with septum caps had a small hole (~2 mm ID) in the bottle neck for ventilation. Inside the vial, a strip of clean filter paper (Whatman, No 1) was placed diagonally to provide better footing for the flies and a piece of dental roll was impregnated with 50 μL of 10% sucrose solution. The auto-sampler and GC–MS were located close to a window, exposing the flies to the natural photoperiod. In addition, lights in the room were turned off at 20:00 PM and turned on the next day at 5:00 AM to simulate a natural daylight cycle (15L:9D).

2.5. Airborne collections of volatiles from virgin males and females

Groups of 200–250 males or females in the ages of 0–5, 5–10, 10–15, 15–20, 20–25, 25–30 days ($N = 4$) after eclosion were placed in a glass tube (17 cm long × 3 cm diam.) along with a cotton roll disc impregnated with 10% fructose solution. Air was passed through an activated charcoal filter into the glass tube containing the flies and their volatiles were passed through and collected by SuperQ adsorbent (Alltech Associates Inc., Deerfield, IL, USA) inside a small tube (4 mm I.D. × 11 cm long). After about 2 days of collection, the adsorbent tube was washed with a mixture of pentane (1 mL) and n-hexane (100 μL). A separate final wash of the adsorbent tube was done with 1 mL of dichloromethane that was analyzed by GC to verify no residues of other compounds.

2.6. Extraction of male rectal gland contents for quantification of pyrazines

Sixty males were caught alive with ME-baited traps in Tel-Aviv urban area and taken to the laboratory where they were frozen at −32 °C until use. The caught males did not contact the ME baits but could have ingested ME from plants in nature (lab-reared males do not have access to ME). Each male's rectal gland was dissected into phosphate buffered saline (PBS) solution (NaCl 0.008%, NaH_2PO_4 0.00034%, Na_2HPO_4 0.00121% m/v). The gland was then inserted into a 200 μL vial that contained 10 μL n-pentane and 10 μL methanol, shaken briefly by Vortex and left for 20 min, then the solution was separated from the gland and used for further analysis by GC–MS ($N = 60$). Quantification of pyrazines in glands was done by GC–MS analysis with a calibration curve of tetramethylpyrazine.

2.7. EAG responses of fly antennae

EAG responses were recorded with an IDAC-2 system (Syntech, Kirchzarten, Germany). Each individual head was dissected at the neck and fixed between two electrodes filled with Ringer solution (NaCl 0.0008%, KCl 0.0004%, $CaCl_2$ 0.0002% m/v). The indifferent electrode was inserted into the base of the head, and the recording electrode was put in contact with the tip of an antenna. Gold wires were used to maintain the electrical continuity between the antennal preparations. A stimulus controller unit, (type CS-55, Syntech, Kirchzarten, Germany) was used to maintain constant purified air (charcoal filter) at a flow of 0.1 L/min that was humidified (through a water flask). Air (3.5 L/min) was delivered onto the antennal preparation by a 2 mL plastic syringe (BD Plastipak, Madrid, Spain) with its plunger removed and top exit connected to the water flask by a pipe and the bottom tip exit directed toward the antenna. The plastic syringe was perforated with 1 mm hole to insert a glass Pasteur puffing pipette. Puffs of 0.1 sec each on fly antennae were done by placing a piece of filter paper (Whatman #3, 0.3 × 1.5 cm) impregnated with the appropriate solution inside a different Pasteur pipette. The antenna was tested (puffed) with 300 ng methyl eugenol (ME, in n-hexane) on filter paper before each test. The antenna was not used if the antenna response to ME was lower than 2 mV. The data were recorded and analysed by data acquisition software (GC-EAD 2012 V1.2.4 Syntech, Germany). Solutions for subtractive tests contained in total 30 μg of the mixture found in males/females in n-hexane (3 μL per one test, $N = 10$). In the dose-response tests, the chemicals were dissolved in n-hexane, except for pyrazines that were dissolved in methanol ($N = 5$). The EAG response to the solvents were also recorded and compared with the response to each test solution.

2.8. Statistics

Electrophysiology tests (EAG) were analyzed by statistical software (JMP 9.0.1, SAS, Cary, NC, USA) using standard least squares analysis of variance (ANOVA) and Wilcoxon matched pairs signed-rank tests (Wilcoxon). Dose-response curves for single compounds were analyzed for significance within and between sexes with one-way and two-way ANOVA, respectively. Differences in EAG response of each sex to compound mixtures and differences between the sexes to each mixture were analyzed by Wilcoxon tests.
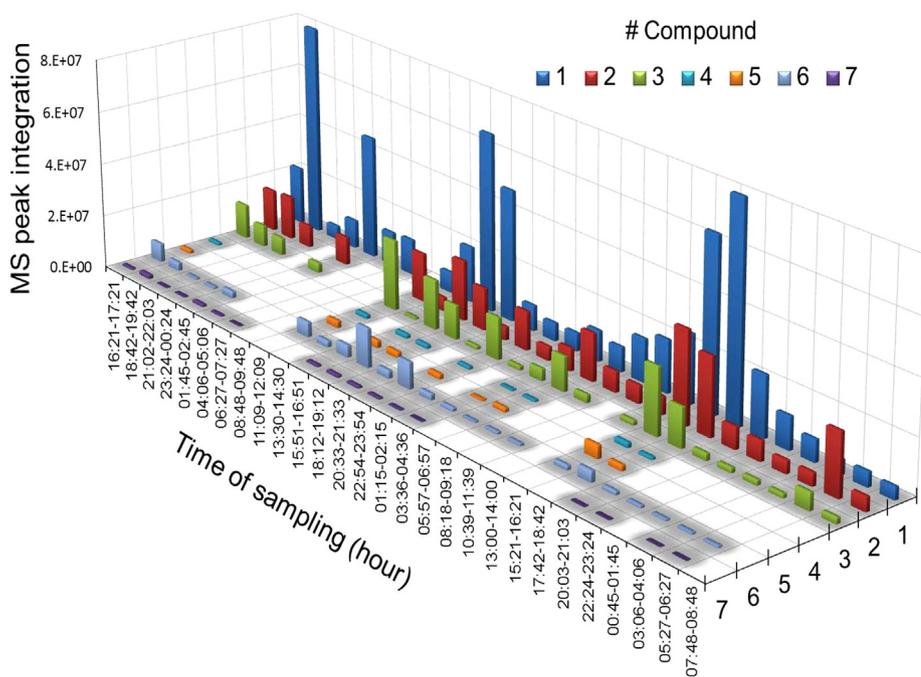
Fig. 1. Diurnal release of volatiles from four *B. zonata* females of 0–5 days age. The time of sampling represents the hours of the peak release on each day of the 3 days of sequential analysis. The bars represent the MS abundance of the main components. The compound numbers represent: 1. N-isopentylacetamide, 2. DMSU, 3. EMSU, 4. EMMSU, 5. PMSU, 6. 12EE, and 7. 14EE.

3. Results

3.1. Periodicity of volatile release by sequential SPME/GC–MS analyses

SSGA analyses of *B. zonata* females (0–5 days, Fig. 1) and males (5–10 days, Fig. 3) revealed volatile chemicals that are released in a diurnal rhythm in each sex. Other ages of each sex (0–25 days age) showed similar periodicities (data not shown), although amounts differed according to age (presented subsequently). In all SSGA, a maximum emission was observed near dusk, the time period that has been well associated with the mating of these tephritids (Bateman, 1972; Wee and Tan, 2005), but sometimes also in the early mornings, perhaps associated with turn on of the lab light. Volatiles identified in SSGA analyses of females were (numbers refer to those in Figs. 1 and 2): 1. N-isopentylacetamide, 2. DMSU, 3. EMSU, 4. (*E,E*)-2-ethyl-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (EMMSU) that was found in small amounts (< 1%) also in *Bactrocera tryoni* (Booth et al., 2006), 5. PMSU, 6. ethyl dodecanoate (12EE (ethyl ester)), 7. ethyl tetradecanoate (14EE), and traces amounts of 8. ethyl hexadecanoate (16EE, not included in Fig. 1).

SSGA of *B. zonata* males revealed that the compound type released

in a diurnal rhythm depends on the age of the male. However, males at all ages release the following compounds: 1. n-isopentylacetamide, 3. DMSU, 9. 2,3,5-trimethylpyrazine, 10. 3-ethyl-2,5-dimethylpyrazine, 11. 2,3,5,6-tetramethylpyrazine, and 12. 2-ethyl-3,5,6-trimethylpyrazine (Figs. 3 and 4). The release happens mostly during dusk, but sometimes also in the morning.

3.2. Effect of age on quality and quantity of volatiles released by B. Zonata males and females determined by airborne collections

Since SPME is not appropriate for compound quantification, the amounts and ratios of the identified components in each sex were demonstrated by airborne volatile collection (aeration). The results show a change of chemical profile with age in each sex of *B. zonata* (Fig. 5). Interestingly, males and females of *B. zonata* share a few volatile components that are released in a diurnal rhythm, but amounts between sexes greatly differ. Males at early ages release the same four spiroacetals that were found in females: DMSU, EMSU, EMMSU, and PMSU (Fig. 5). However, in general the spiroacetal amounts released by males are much smaller than those released by females. While the major spiroacetal component DMSU in females is released for over 30 days of adult life in the range of ∼30–150 ng per individual per day, males released < 10 ng per male per day and only up to the period 10–15 days of age, when sexual maturity occurs. In addition, EMSU, the major spiroacetal found in males, is only released in quantities of up to 18 ng per day. Males show a constant reduction of the released amounts of the four spiroacetals from day one to about 15–20 days of age, in contrast, females increase the release of EMSU and PMSU spiroacetals throughout adult life and up to the age of 30 days (Fig. 5).

Aeration analyses shows that *B. zonata* males release only two major pyrazines (SSGA is more sensitive to small amounts of other pyrazines): 2,3,5,6-tetramethylpyrazine and 2-ethyl-3,5,6-trimethylpyrazine. The amounts of these pyrazines increase and reach a peak at age 15–25 days, when sexual maturity occurs, but then decrease (Fig. 6A). These pyrazines were absent in females. Aeration analyses also showed that both sexes release ethyl esters: i.e. ethyl dodecanoate, ethyl tetradecanoate and minor amounts of ethyl hexadecanoate (Fig. 6). The release of ethyl dodecanoate in females starts from the first day and increases with age up to ∼0.5 μg per female per day (Fig. 6B), following the pattern of release of spiroacetals (Fig. 5). Males release these ethyl
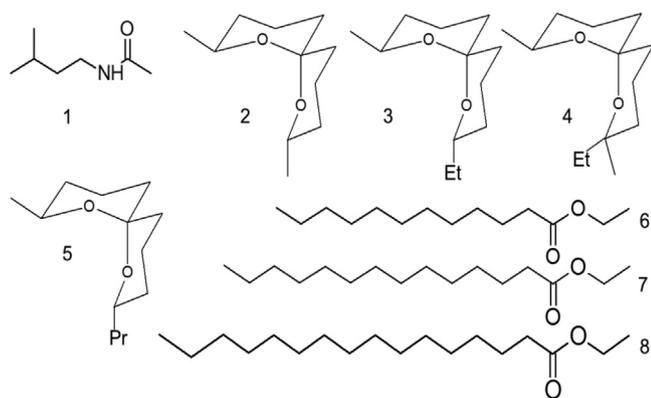

Fig. 2. Molecular structure of female *B. zonata* volatile components that were released in a diel rhythm and identified in SSGA: 1. N-isopentylacetamide, 2. DMSU, 3. EMSU, 4. EMMSU, 5. PMSU, 6. 12EE, 7. 14EE, and 8. 16EE.
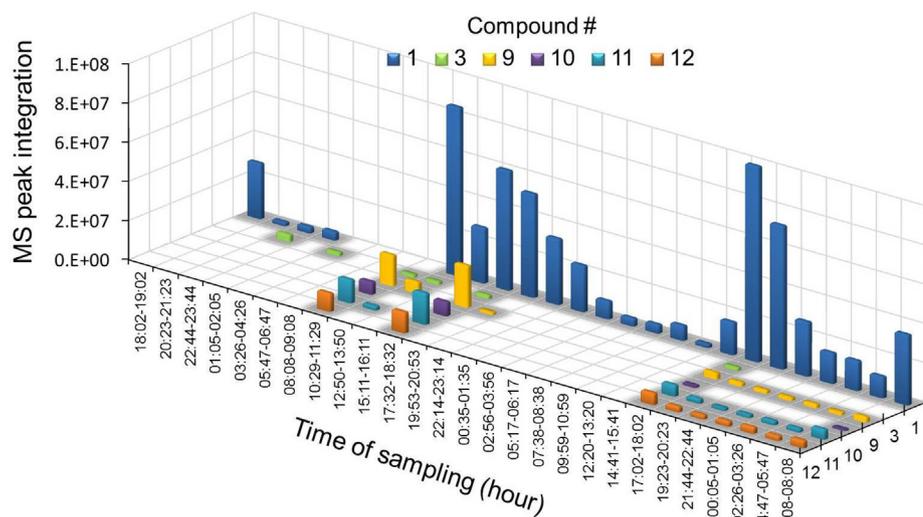
Fig. 3. Diurnal release of volatiles from six *B. zonata* males of 5–10 days age. The time of sampling represents the hours of the peak release on each day of the 3 days of sequential analysis. The bars represent the MS abundance of the six main components: 1. N-isopentylacetamide, 3. EMSU, 9. 2,3,5-tri-methylpyrazine, 10. 3-ethyl-2,5-dimethylpyrazine, 11. tetramethylpyrazine, and 12. 2-ethyl-3,5,6-tri-methylpyrazine (described in Fig. 4).
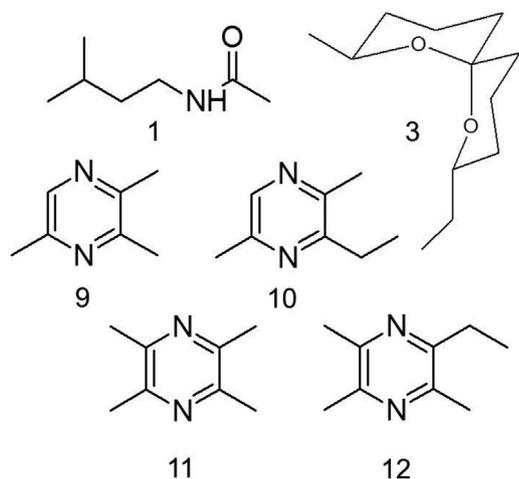

Fig. 4. Male *B. zonata* volatile components that were released in a diel rhythm and identified in SSGA: 1. N-isopentylacetamide, 3. EMSU, 9. 2,3,5-tri-methylpyrazine, 10. 3-ethyl-2,5-dimethylpyrazine, 11. tetramethylpyrazine, and 12. 2-ethyl-3,5,6-trimethylpyrazine.
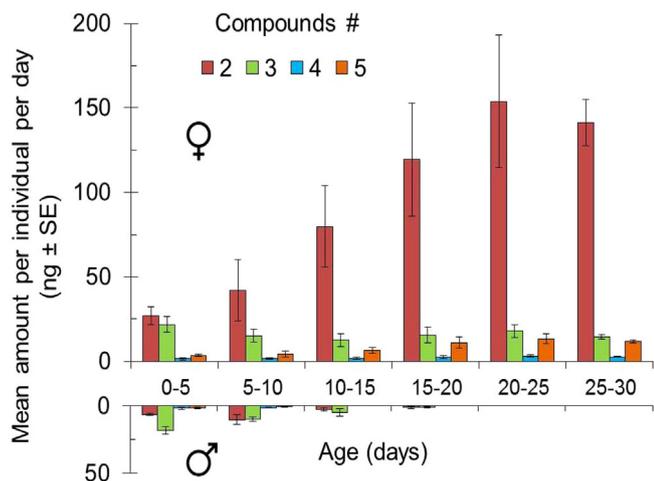

Fig. 5. Mean amounts (ng ± SE) of different spiroacetals released per individual per day that were analyzed in aerations of *B. zonata males* and females in relation with their age. Compound numbers are 2. DMSU, 3. EMSU, 4. EMMSU and 5. PMSU as shown in Fig. 2.
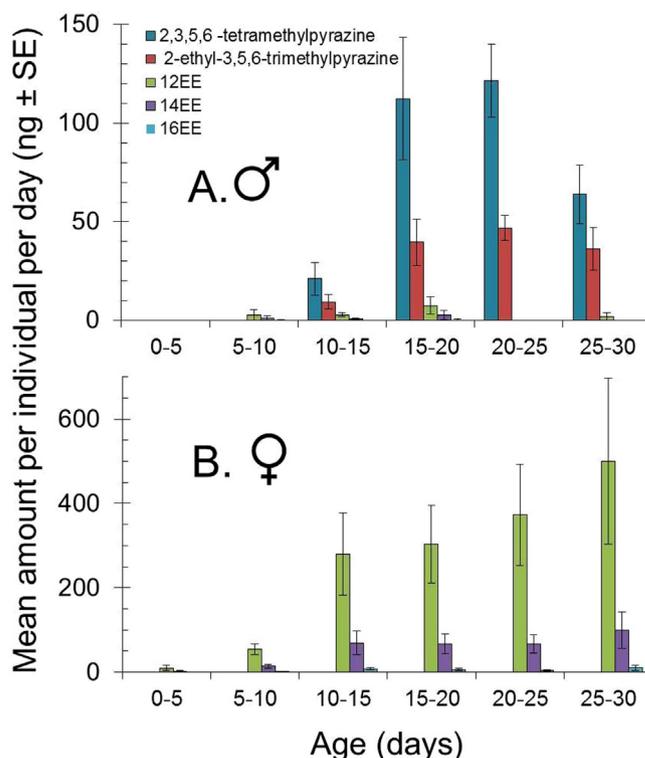

Fig. 6. Mean amounts (ng ± SE) of pyrazines and ethyl esters released per individual per day in airborne collections of *B. zonata* males (A) and females (B) according to their age (days).

esters from day five but in much lower quantities than females (Fig. 6). These esters were not found in SSGA of males.

3.3. Determination of pyrazine quantities in the male's rectal gland

In the airborne volatile collections of males reared in the lab we found a maximum of 122 ± 19 ng of 2,3,5,6-tetramethylpyrazine and 47 ± 6 ng of 2-ethyl-3,5,6-trimethylpyrazine per male per day at age 20–25 days (Fig. 6) but none of the other two pyrazines, 2,3,5-tri-methylpyrazine and 3-ethyl-2,5-dimethylpyrazine, that were identified in SSGA. Since airborne collections failed to detect all the pyrazines that were found in SSGA, male rectal glands were extracted from 60 wild males (age not known) and the amounts of pyrazine components were
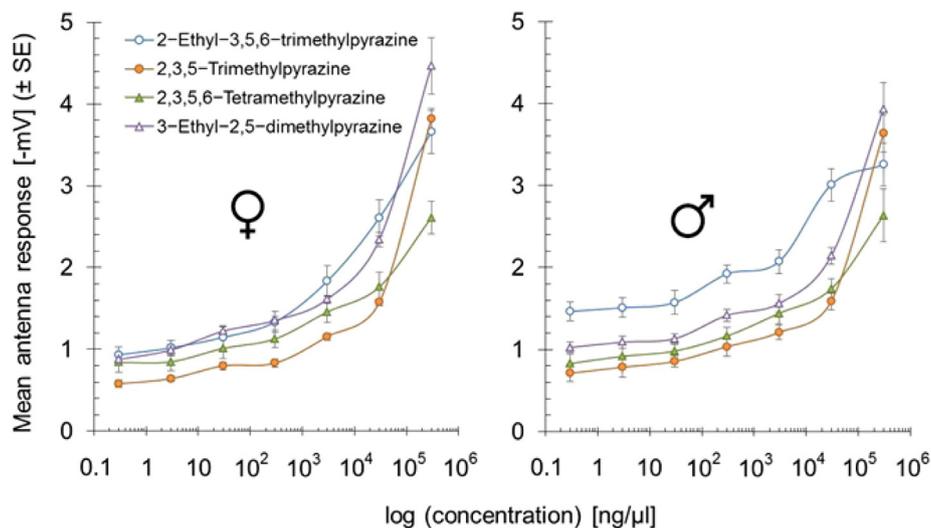
Fig. 7. Mean antenna responses of 15–21 days old males and females to synthetic pyrazines identified in SSGA of males ($N = 5$).

determined. The amounts found in the glands per wild male were: 1544 ± 343 ng of 2,3,5,6-tetramethylpyrazine, 1679 ± 480 ng of 2-ethyl-3,5,6-trimethylpyrazine, 29 ± 13 ng of 2,3,5-trimethylpyrazine, and 13 ± 8 ng of 3-ethyl-2,5-dimethylpyrazine.

3.4. EAG responses of fly antennae

3.4.1. Testing single synthetic major components of male airborne volatiles on antennae of both sexes

Dose-EAG responses of male and female antennae (Fig. 7) were performed with synthetic pyrazines identified as those released by males in a diurnal rhythm according to SSGA. Antennae of each sex increasingly responded to increasing doses of 3-ethyl-2,5-dimethylpyrazine (ANOVA: male $F_{6,63} = 50.73$, $P < 0.0001$; female $F_{6,63} = 75.34$, $P < 0.0001$) and there was no significant difference between the sexes ($F_{1,126} = 1.08$, $P = 0.30$). Antennae of each sex responded increasingly to increasing dosage of 2,3,5,6-tetramethylpyrazine, one of the major pyrazine components (male $F_{6,63} = 16.23$, $P < 0.0001$; female $F_{6,63} = 20.26$, $P < 0.0001$), with no significant difference between the sexes ($F_{1,126} = 0.008$, $P = 0.93$). Dose-response curves of a minor component 2,3,5-trimethylpyrazine increased significantly for each sex (male $F_{6,63} = 66.31$, $P < 0.0001$; female $F_{6,63} = 94.8$, $P < 0.0001$), and there was no significant difference between the sexes ($F_{1,126} = 0.84$, $P = 0.36$). Another major pyrazine component, 2-ethyl-3,5,6-trimethylpyrazine, shows an increasing response with dose in both sexes (male $F_{6,63} = 19.69$, $P < 0.0001$; female $F_{6,63} = 34.96$, $P < 0.0001$), but this pyrazine elicits significantly higher responses in the male's antenna than in the female's ($F_{1,126} = 13.14$, $P = 0.0004$; Fig. 7).

3.4.2. Testing single synthetic major components of female airborne volatiles on antennae of both sexes

The dose-EAG responses of male and female antennae to DMSU and EMSU (identified in both male and female airborne volatiles) as well as to PMSU (that was in negligible amounts in airborne volatiles of males) were tested (Fig. 8). The dose-response curve for EMSU increased slowly until above 3 μg in both sexes (male $F_{5,54} = 11.07$, $P < 0.0001$; female $F_{5,54} = 16.34$, $P < 0.0001$). Both sexes also responded to increasing doses of DMSU (male $F_{6,63} = 12.8$, $P < 0.0001$; female $F_{6,63} = 34.3$, $P < 0.0001$), however, at the two highest dosages, antennae of females were more responsive than males ($F_{1,36} = 13.43$, $P = 0.0008$). Antennae of each sex increased response slightly over the dosage range of PSMU, but the trends were not significant in either sex (male $F_{5,54} = 0.93$, $P = 0.47$; female $F_{5,54} = 1.4$, $P = 0.24$). The

antenna of both sexes appeared to reach saturation with DMSU dosage over 10^5 ng/μL. EMSU and PMSU were not tested at this higher dosage range due to low stock of these standards.

Both sexes increased their antenna response with dosage of ethyl esters 12EE (male $F_{5,54} = 10.1$, $P < 0.0001$; female $F_{5,54} = 19.4$, $P < 0.0001$) and 14EE (male $F_{5,54} = 26.6$, $P < 0.0001$; female $F_{5,54} = 19.4$, $P < 0.0001$) (Fig. 8). The male antenna appears more responsive to 14EE than does the female antenna ($F_{1,108} = 40.5$, $P < 0.0001$). These ethyl esters were found mostly in female airborne collections (Figs. 1 and 6). The female antenna responds significantly more to 12EE than to 14EE ($F_{1,108} = 8.49$, $P = 0.0043$, Fig. 8). In contrast, the male antenna is more responsive to 14 EE than to 12EE ($F_{1,108} = 19.44$, $P < 0.0001$, Fig. 8).

3.4.3. Testing male and females airborne volatile mixtures by EAG

The goal of these tests was to see which components of *B. zonata* volatiles are responsible for the antenna response. For male volatiles we prepared a solution in which the ratio of the components was 49% 2,3,5,6-tetramethylpyrazine, 49% 2-ethyl-3,5,6-trimethylpyrazine, 1% 2,3,5-trimethylpyrazine, and 1% 3-ethyl-2,5-dimethylpyrazine (according to wild male gland contents). Since there is no known attractive bait that catches females in the field, we used the synthetic mixture of major components (those above 1%) that was determined from airborne collection of lab-reared 10–30 day old mature female volatiles (Figs. 5 and 6B): 21% DMSU, 3% EMSU, 2% PMSU, 61% 12EE, and 13% 14EE. The two mixtures above were tested for dose-response on male and female antenna and the results (Fig. 9) show that at the highest dose (30 μg) there is an increase in antenna response, and that the responses of antennae of both sexes over the dosage range of each mixture were similar. Therefore, this approximate dosage was used in the next series of EAG tests with the subtractive-combination method (Byers, 1992).

The subtractive method with EAG tests the total volatile mixtures of males and females compared to the same mixtures each excluding a different component. In addition, the antenna responses to ME and the solvent were compared. The results show that generally in tests with male volatiles the female antennal responses were consistently lower than the male antennal response (Fig. 10A). In addition, the antennal response decreased significantly in both sexes when 2-ethyl-3,5,6-trimethylpyrazine was removed from the male's total mixture. When 2,3,5-trimethylpyrazine was removed from the total mixture only the antennal response of males was reduced (Fig. 10A). In a second experiment (Fig. 10B), 2-ethyl-3,5,6-trimethylpyrazine alone gave the same male antennal response as the total mixture, but the response of
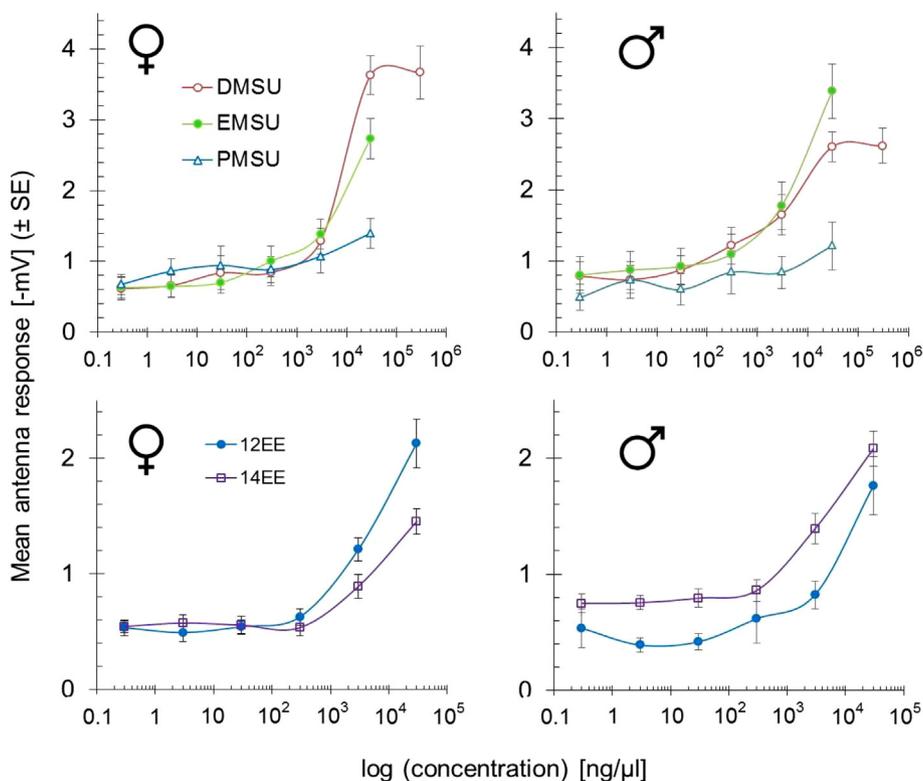
Fig. 8. Mean antenna responses of 15–24 days old males and females to synthetic components identified in female airborne collections ($N = 5$).

the female antennae was significantly less than to the total mixture. In addition, females responded significantly less than males to 2-ethyl-3,5,6-trimethylpyrazine, ME, or methanol alone (Fig. 10B).

The subtractive EAG tests of the female total mixture show that when either DMSU or 12EE was removed, the antennae responses decreased in both sexes, while subtraction of other components (EMSU, PMSU, or 14 EE) had no significant effect (Fig. 11A). DMSU or 12EE tested alone elicited a significantly lower response in both sexes than did the total mixture (Fig. 11B). Testing DMSU and 12EE together gave the same response as to the total mixture (Fig. 11B).

4. Discussion

Much effort has been invested in the last 40 years to identify the pheromones of species in the *Bactrocera* genus (Tan et al., 2014).

However, the identified volatiles from the various fly species have not been reported to attract either sex in the field except for the female olive fruit fly, *B. oleae*, sex pheromone olean that attracts males in olive groves (Mazomenos and Haniotakis, 1985) and has been in commercial use to date. Our previous SSGA results for the olive fruit fly revealed that olean is released by females mostly at dusk (Levi−Zada et al., 2012). A circadian rhythm of pheromone volatiles was shown for the Mexican fruit fly *Anastrepha ludens* (Heath et al., 2001). These studies support our hypothesis that one or more of the chemicals released in a diel rhythm by *B. zonata* could be components of a pheromone. Our SSGA analyses for *B. zonata* showed that volatiles were released mainly in the late afternoon and dusk but also in the early morning. Regarding the early morning emissions, Heath et al. (2001) suggested that turning on or off artificial light in quarantine facilities (photoperiod 14:10, onset 7:00, dark 21:00) may trigger a brief flurry of male calling that
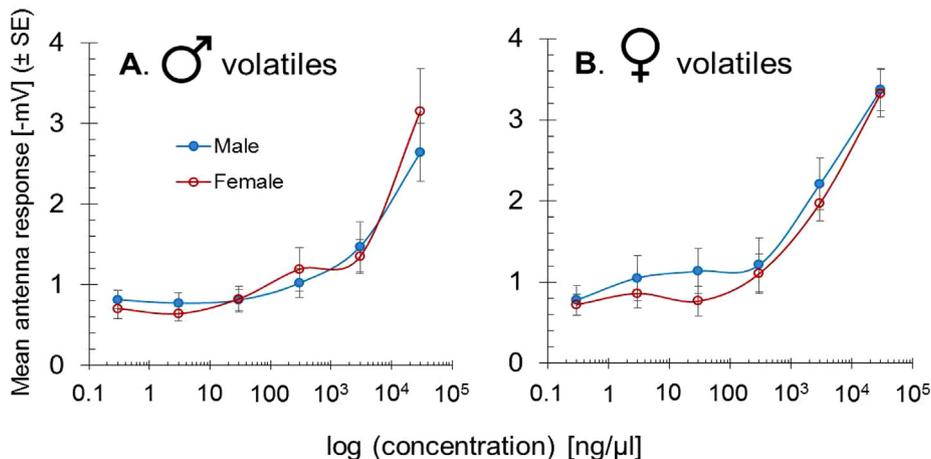

Fig. 9. Mean antenna responses of *B. zonata* males and females to mixtures of synthetic components identified in: A. extraction of wild male rectal glands and B. lab-reared female airborne collections ($N = 5$). The concentration refers to the total mixture.
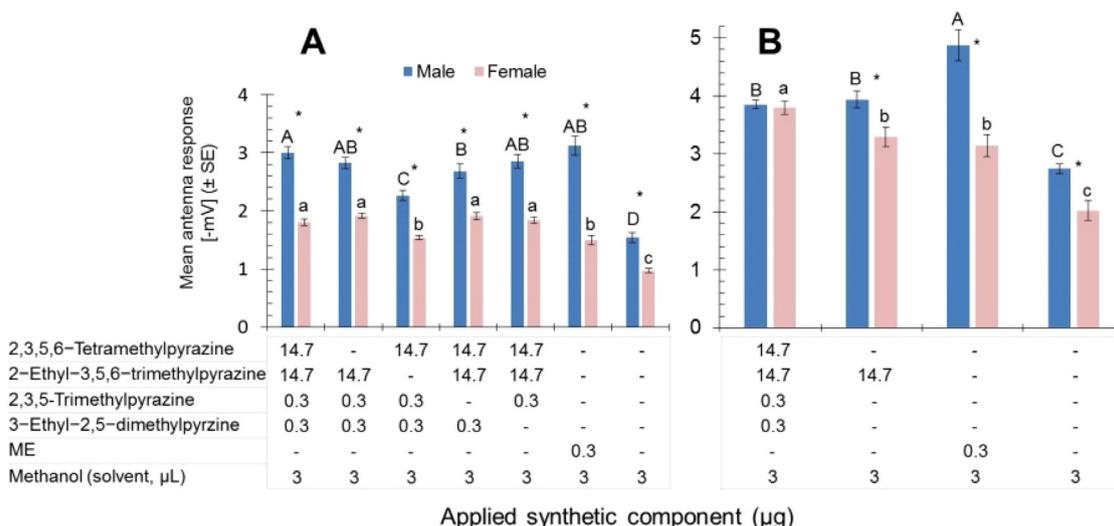
Fig. 10. Effect of subtraction of synthetic male components from full mixture on antenna responses of *B. zonata* males and females: A. subtraction of single components from the mixture compared to ME and solvent, B. Subtraction of all components, except 2-ethyl-3,5,6-trimethylpyrazine, compared to ME and solvent alone. Bars with the same letters are not significantly different (Wilcoxon α = 0.05; uppercase for males and lowercase for females). Asterisks represent a statistical difference ($P < 0.0001$) in antenna response between sexes within a treatment (Wilcoxon tests, $N = 10$ flies, age 20 days).

affects pheromone release. We found that the diel periodicities of emitted volatiles occurred at all ages but generally increased with age and sexual maturity. Similar to males of the olive fruit fly (Levi−Zada et al., 2012; Canale et al., 2013), spiroacetals are found in young males of *B. zonata* but begin to disappear at sexual maturity. Females of *B. zonata* then continue to increase the production of their specific spiroacetals unlike olive fruit fly (Levi−Zada et al., 2012).

We identified a complex variety of volatiles from *B. zonata* males and females such as spiroacetals, pyrazines, ethyl esters and amides, just as in *B. dorsalis* (Baker and Bacon, 1985; Perkins et al., 1990a). It seems that some of these volatile chemicals appear in greater amounts when the individual is sexually mature as determined by our observations of courting behavior and mating (data not shown). In *B. zonata* the release of n-isopentylacetamide appeared mostly at dusk and some in

the morning during the release of the other volatiles, but the amide's release was sporadic, therefore it was not tested further. Amides are considered products of protein metabolism (Weston et al., 1997). Spiroacetals, on the other hand, have been found in other insects (Perkins et al., 1990b; Francke and Kitching, 2001), while in fruit flies they have been reported in females of *B. cucurbitae* (Baker and Bacon, 1985) and *B. tryoni* (Booth et al., 2006), and in males and females of *B. dorsalis* (Perkins et al. 1990a; Baker and Bacon, 1985). *B. oleae* males release the olean spiroacetal on the order of 100 times less than do the females (Levi−Zada et al., 2012). A similar trend is seen in *B. zonata* males that also stop releasing the spiroacetals after a certain age (~15–20 days by which time they have reached sexual maturity), unlike the females that increase the release of spiroacetals (and ethyl ester) as they age. Conversely, males of *B. zonata* increase the release of
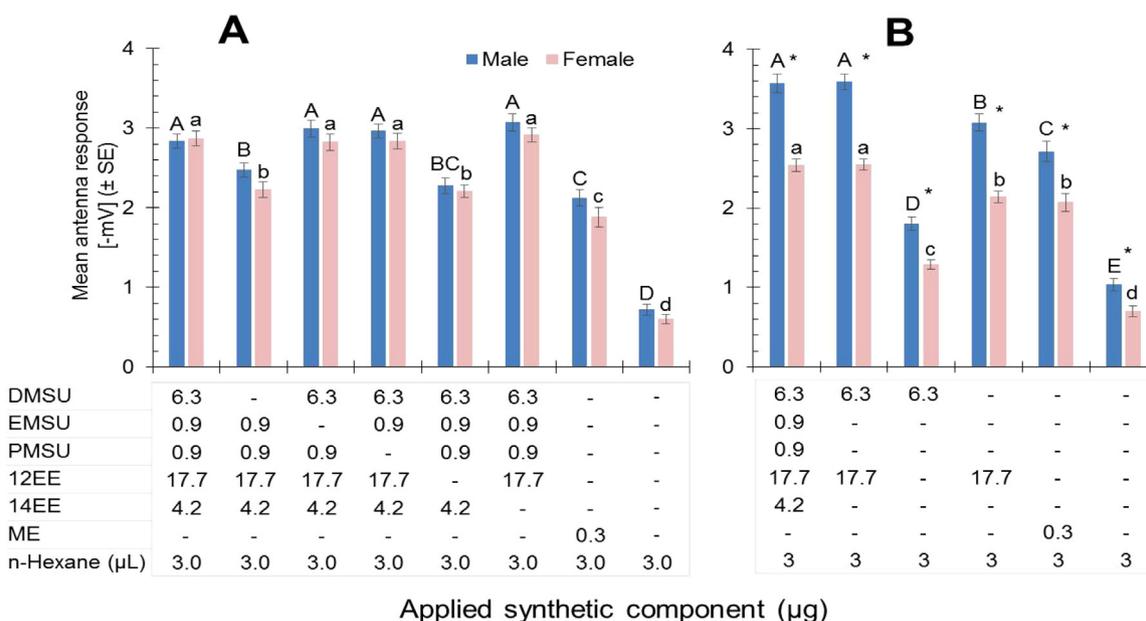

Fig. 11. Effect of subtraction of synthetic female components from full mixture on antenna responses of *B. zonata* males and females: A. subtraction of single components from the total mixture compared to ME and solvent, B. Subtraction of all components, except DMSU and 12EE, compared to ME and solvent alone. Bars with the same letters are not significantly different (Wilcoxon α = 0.05, uppercase for males and lowercase for females). Asterisks represent a statistical difference ($P < 0.0001$) in antenna response between sexes within a treatment (Wilcoxon tests, $N = 10$, flies age 18 days).

pyrazines with age after reaching sexual maturity. Pyrazines have been found in other fruit flies (Baker et al., 1982; Chuman et al., 1987; Perkins et al., 1990a,b), but their biological role has not been yet determined (Robacker et al., 2009). Alkyl esters have been found in other fruit flies (Gariboldi et al., 1983; Baker and Bacon, 1985; Perkins et al., 1990b,) but there are no reports about their behavioral role except for Canale et al. (2015). Canale et al. found, in a two-choice bioassay conducted in a still-air test arena, that females of the olive fruit fly are attracted to ethyl decanoate while males are attracted to methyl hexadecanoate.

Although methyl eugenol (ME) is not produced by any *Bactrocera* fly and thus is not a pheromone, several studies have investigated the attraction of *Bactrocera* males, especially of *B. dorsalis*, to methyl eugenol (ME) and their feeding behaviour on flowers containing ME. After ingesting ME, males subsequently convert it to derivatives that are reported to be attractive to females in the laboratory (Tan et al., 2014). The volatiles released by *B. dorsalis* are similar to those from *B. zonata*, though they are different species (Tan et al., 2011). Interestingly, however, Kobayashi et al. (1978) found that females of *B. dorsalis* were significantly attracted to dissected rectal glands of lab-reared males which had not fed on ME. The attraction of females to dissected male rectal glands was not significantly different from the attraction of females to live males. Ohinata et al. (1982) also used lab-reared males, which had not fed on ME, and describe a "smoke" emanating from males at dusk that is attractive to females in a wind tunnel. These studies suggest that there are volatile chemicals from males not related to ME that are attractive to females. The derivatives of ME may enhance/synergize the attraction of females to males. Thus, it is possible that the male *B. zonata* release pyrazines within the lek that attracts females to males. Additionally, the pyrazines of males may also stimulate males to find and remain in the lek. The female-released spiroacetals and ethyl esters may also attract nearby males inside the lek to orient toward females and mate. Our findings with respect to the female volatiles are similar to chemicals in the female olive fruit fly that releases a sex pheromone spiroacetal that attracts males in the field (Mazomenos and Haniotakis, 1985), suggesting a possible role in sexual attraction also in *B. zonata*.

Our EAG tests examined the various volatiles that were released in circadian-like patterns from males and females of *B. zonata* and suggest that in the males the component that elicits the strongest response is 2-ethyl-3,5,6-trimethylpyrazine, while in the females the most important components are DMSU and 12EE. The behavioral role of these quantitatively sex-specifically released volatiles is obscure. This may be due to their possible function in close-range sexual communication, which is difficult to characterize due to the lack of a bioassay incorporating acoustic cues, lekking behavior, and host plant involvement conducive to mating. There have been many chemical and behavioral studies regarding *B. dorsalis* (Shelly, 2010) and other fruit flies, but there is little evidence in the literature that any of these non-ME derived chemicals (Tan et al., 2014) are attractive over several meters to the males or females, either in laboratory bioassays or in the field. The lack of conclusive evidence for many species may be related to weak responses in bioassays that are difficult to detect and quantify, or the fact that it is difficult to recreate the complex pattern of behavioral (acoustic, visual, chemical) expressions that elicit response under natural conditions (Benelli et al., 2012). Another contributing factor could be the fact that ME is highly attractive to *Bactrocera* males, is commercially available, and good enough for practical aspects of monitoring and male annihilation technique.

As mentioned above, the literature on *Bactrocera* flies in general is not definitive on the role of each sex in attraction and mating. It is known that males of *Bactrocera*, and specifically *B. dorsalis*, form leks on the undersides of leaves; and females are attracted from some unknown distance to the leks and may produce pheromones that initiate close-range attraction, mounting and copulation (Sivinski et al., 2000). We tested flies in cages and in the field for their attraction to male gland

contents and to different combinations of synthetic components identified in our study. However, none of the individual chemical stimuli we used appeared attractive to males or females (except for ME that attracts males). Perhaps the components must be presented in appropriate blends and dosages as well as under certain environmental conditions to elicit behavioral responses related to reproduction.

Knowing the components that are released in a diel rhythm and elicit antenna response may be one step forward to understand the chemical communication system of the peach fruit fly *B. zonata*. The role of non-ME compounds in the mating systems of *Bactrocera* is still largely unknown, but further investigation based on the volatiles we identified may provide new paradigms on the sexual communication of Tephritidae, which could open new applications for the control of these pests.

Competing interests

The authors have declared that no competing interests exist.

Author contributions

Conceived and designed the experiments: ALZ, JAB, AL, PR, DN, YG and BY. Synthesized chemicals: ALZ, SS. Collected and analyzed the data: ALZ, DF, AL, PR. Wrote the manuscript: ALZ.

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