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Diel rhythm of volatile emissions from males and females of the olive fruit fly *Bactrocera oleae* using PTR-ToF and GC–MS

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ABSTRACT

The olive fruit fly *Bactrocera oleae*, is the major key pest of olive groves worldwide. As an odor-driven species, its intraspecific communication has been thoroughly investigated, yielding a combination of spiroacetals, esters and hydrocarbons. However, its management with pheromone is still restricted to olean, the major pheromone component. Given the crucial role of circadian rhythm and pheromone blends in mediating flies reproductive behavior compared to single compounds, *B. oleae* headspace chemical profile was carefully examined, through the combination of Proton Transfer Reaction Time of Flight Mass Spectrometry (PTR-ToF) and Gas Chromatography coupled with Mass Spectrometry (GC–MS). This novel approach aimed at continuously investigating the temporal scale of volatilome profile of *B. oleae* individuals, as well as the determination of new candidate sex-borne compounds (particularly those emitted in traces or having low molecular weight), that may be relevant to the fly's chemical communication and were unreported due to limitations of frequently used analytical techniques. Our results describe the dynamics and diversity of *B. oleae* chemical profile, highlighting the emission of 90 compounds, with clear diel rhythm of release, of known pheromone components of *B. oleae* (e.g., olean, alpha-pinene and muscalure) and new candidates. In contrast to ammonia, acetaldehyde and muscalure, which were highly emitted during the afternoon by males and mixed groups, olean was mostly released by mature females and mixed groups, with a peak of emission during early-morning and afternoon. This emission of olean around dawn is reported for the first time, suggesting early-morning mating activity in *B. oleae*. Furthermore, esters, such as methyl tetradecanoate, which had been earlier identified as a pheromone for *B. oleae*, did not exhibit any discernible release patterns. These findings are the first to demonstrate the emission of chemicals, which are only produced when males and females are close to one another, with an emission peak during the afternoon (mating period), and that may have aphrodisiac properties for *B. oleae* males. These results emphasize the relevance of compounds with distinct diel rhythm and address their potential function as intraspecific messengers, according to their source and timing of release.

1. Introduction

The olive fruit fly (*Bactrocera oleae* (Rossi), Diptera), is a tephritid recognized globally as the major pest of olive groves, and mostly managed by chemical insecticides that endanger human health and the environment (Stark et al., 2004; Thomas and Mangan, 2005). As alternatives, natural enemies and semiochemicals (such as plant-born Volatile Organic Compounds (VOCs)) were included in IPM programs to enhance the ecological management of this pest (Benelli et al., 2014; Canale et al., 2013a; Wang et al., 2011). However, the outcomes were not consistently favorable (Daane and Johnson, 2009).

In 1980, the pheromone of *B. oleae* was described and synthesized for the first time by Baker et al., 1980. It was reported to be released by females and composed of a major spiroacetal component, called olean (1,7-dioxaspiro[5.5]undecane) and its two hydrates minor ones (1,7-dioxaspiro[5.5]undecan-4-ol, 1,7-dioxaspiro[5.5]undecan-3-ol) (Baker

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et al., 1980, 1982). Later, a series of investigations focused on the female sex-pheromone resulted in the discovery of 22 additional minor compounds (mainly esters) produced in rectal glands (Canale et al., 2015, 2013b, 2012; Carpita et al., 2012; Fletcher et al., 1992; Gariboldi et al., 1983; Mazomenos and Haniotakis, 1985, 1981). Even though olive fruit fly sexual chemical ecology is driven by a multi-component pheromone, only olean has been thoroughly studied and used for monitoring and management (Canale et al., 2015), being described as the most abundant component with the highest biological activity towards males (Mazomenos and Haniotakis, 1985, 1981). However, the effectiveness of these pheromone-based approaches could be enhanced by incorporating synergistic minor components, alongside the determination of pheromones spatial and temporal distributions, which are difficult to real-time monitor in naturally occurring concentrations (Cardé and Minks, 1995; Valeur et al., 1999).

In Dacine fruit flies, pheromone emission and mating activity are restricted to periods of the day, due to the interaction between internal circadian rhythms and external factors (Fletcher, 1987; Smith, 1979; Tychsen and Fletcher, 1971) that are adapted to different environments or photoperiods (Peschel and Helfrich-Förster, 2011). For instance, light is the most important input factor of the so-called "Zeitgebers" that harmonize the clock neurons with their environment (Dunlap and Loros, 2004; Levine, 2012). For most *Bactrocera*, mating occurs during a narrow species-specific time window of the day, often connected with dusk and less frequently with midday (Clarke, 2019; Fletcher, 1987), at which male sexual activity coincides with female receptivity (Ekanayake et al., 2017) and the peak of pheromone production. In *B. oleae*, the investigation of olean's diurnal emission unveiled that females predominantly release pheromone during the scotophase, with an increase of production just prior to the onset of this period (Levi-Zada et al., 2012).

To investigate the emission of pheromones, VOCs are collected from exocrine glands or the cuticle through headspace sampling techniques (Chin and Yew, 2013; Lin and Zhou, 1991). However, due to their production in minute quantities (picograms to nanograms), the collected samples are often insufficient to conduct many spectroscopic analyses (Kalinová et al., 2006). Their identification is mainly based on gas chromatography coupled to mass spectrometry (GC–MS) analysis (Agelopoulos and Pickett, 1998). In addition, when presented in sufficient amounts, other analytical techniques are used singularly or in combination such as nuclear magnetic resonance (NMR), two-dimensional gas chromatography using a time-of-flight mass spectrometric detector (GC × GC / ToF MS), thin layer chromatography coupled with gas chromatography (TLC × GC–MS) and ultraviolet laser desorption/ionization orthogonal time-of-flight mass spectrometry (UV-LDI-o-ToF-MS) (Chin and Yew, 2013; Kalinová et al., 2006; Rizvi et al., 2021; Yew et al., 2011). This latter is known for its remarkable sensitivity in detecting polar and non-polar lipids also with a high molecular weight (m/z 500–1,000). This sensitivity has enabled the direct identification of the male pheromone of *Drosophila melanogaster* from the external cuticle of flies, without the need for chemical extraction (Yew et al., 2011).

Once identified, pheromones are demonstrated to be biologically active using electrophysiological techniques such as gas chromatography coupled to electroantennography (GC-EAD) and single sensillum recordings (SSR) (Rizvi et al., 2021), followed by behavioral assays to determine their attraction potential of conspecifics (Chin and Yew, 2013). However, frequently, GC-EAD displays activity in chromatogram areas where no chemicals are detected in FID or MS, likely due to the detection limit of these techniques (Svatoš et al., 1999). Furthermore, the temporal resolution of semiochemicals release is often lost because the techniques that are typically used require prior collection of volatiles (Kilpinen et al., 2012). These limitations restrict the exploitation of pest chemical communication necessary for the development of efficient management tools.

Since the better comprehension of chemical compounds profile requires integration of multiple analytical methods, this study has investigated *B. oleae* pheromone combining GC–MS and proton transfer reaction time of flight mass spectrometry (PTR-ToF) techniques.

PTR-ToF is an analytical technique that enables rapid and highly sensitive real-time monitoring of VOCs (Biasioli et al., 2011). It has been applied in different research fields including food and flavor science, atmospheric chemistry, and plant science (Blake et al., 2009). In chemical ecology, it has been used for the monitoring of herbivore induced plant volatiles (HIPVs), odor plume tracking, stimuli characterization for insect electrophysiology as well as the real-time measurement of insect released VOCs (Kilpinen et al., 2012; Riffell et al., 2008; Schaub et al., 2010; Tasin et al., 2012).

The current study aims to monitor emission of volatiles from *B. oleae* males and females and identify new volatile compounds of low molecular weight or low concentrations that may be relevant to its chemical communication. The use of both techniques (GC–MS and PTR-ToF) in parallel can unveil the diel rhythm of compounds identified with GC–MS, while also exploring additional potential inferences from literature (Joó et al., 2010). This is particularly significant as closely related species may share certain pheromone components that elicit responses in their conspecifics only when released in species-specific ratios and unique blends (Cardé and Haynes, 2004; Fine et al., 2004).

2. Materials and methods

2.1. Insects

Pupae of *B. oleae* were collected from an olive mill in Tuscana region, Italy. They were singularly isolated in glass vials (20 ml) to facilitate sex segregation and prevent interaction between males and females. After emergence, a mixed group of males and females was moved to a Bug-Dorm insect cage (30 × 30 × 30 cm), while other groups of males and females were kept separate. The objective of this separation was to investigate whether the presence of individuals of the opposite sex would affect the emission of volatile compounds.

All groups were maintained under ambient temperature (around 25 °C) and LD 14:10 photoperiod and fed on a sugar and water solution. *B. oleae* individuals were arranged in four groups: young females (1–5 days), sexually mature females (5–10 days), males (5–10 days), and a mix of mature females and males. Sexual maturity of individuals was defined at the age of 5 days after emergence, as reported by Malheiro et al., 2015.

2.2. Experimental setup

2.2.1. GC–MS

Insects were placed in 250 ml Pyrex™ glass bottles (Thermo Fisher Scientific, Inc., Waltham, MA, USA) previously rinsed with ethanol and dichloromethane (Merck KGaA, Darmstadt, Germany) and VOCs were collected from the four groups (20 individuals/group) using closed loop stripping analysis (CLSA) (Kunert et al., 2009). The collection occurred three times in two periods of the day (light period: (9:00 h-12:00 h; 15:00 h-18:00 h) and dark period: 21:00 h-02:00 h) from the same bottles for three hours. The experiment was repeated for four days with one replicate every day for each group. Every day at 9:00 h am, a new batch of insects was introduced to the bottles for VOCs collection. The headspace samples were collected using an adsorbent trap (glass tube, 6.5 × 0.55 × 0.26 cm^3, loaded with 1.5 mg activated charcoal; CLSA filter LR-type; Brechbühler AG, Schlieren, Switzerland), according to the protocol described by Caselli et al., 2022. VOCs were collected also from an empty bottle as negative control. Using a Teflon tube, the trap was attached to a 12-V graphite vacuum pump (Fürgut, Tannheim, Germany) that circulated air at a rate of ca. 1 L min^{-1} inside the VOCs bottle. VOCs were eluted from filters with 100 µl dichloromethane and injected (2 µl) into GC/MS (7890, Agilent Technologies, Santa Clara, USA) equipped with a mass selective detector-MS (5975C, Agilent Technologies). The separation was ensured by a nonpolar HP-5 MS

column (Agilent Technology, 30 m × 0.25 mm × 0.25 μm film thickness) with helium as carrier gas at a flow of 1.2 ml/min. Injection was in splitless mode at 250 °C, while the oven temperature at injection was 50 °C for 1.5 min, then steadily increased of 7.5 °C/min until 250 °C, where it was held for 5 min. VOCs tentative identification was achieved by comparing calculated linear retention indexes (LRIs) (van den Dool and Kratz, 1963) and mass spectra with Nist20 library (National Institute of Standards and Technology) using MassHunter program ("Agilent MassHunter WorkStation - Qualitative Analysis for GC/MS (RRID: SCR_016657)"), NIST MS Search program (("Nist Mass Spectral Search Program, v 2.4, 2020")) and Kovats extractor app (Larsson Herrera, 2022). In addition, the identification of some compounds was confirmed by running available standards.

2.2.2. PTR-ToF

PTR-ToF was used to monitor VOCs emission from the listed insect groups. For each group volatiles were collected from 30 individuals (15:15 ratio for mixed group) placed in 250 ml Pyrex™ glass bottles (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and kept in a climate cabinet (Climacell 707, BMT Medical Technology, Brno, Czech Republic) for one day prior to the experiment for insect acclimatization. To maintain the survival of flies throughout the experiment, a piece of cotton was soaked with a water-sugar solution (1:1) and provided to each group. The climate cabinet was set up with the optimal parameters for *B. oleae* activity: 14-hour photoperiod, temperature of 25 °C, and 60 % relative humidity (Solinas et al., 2001). Each group was connected with the PTR-ToF via three capillary tubes: a perfluoroalkoxy (PFA) tube providing a constant flow of humidified air to the VOC-bottle, a second PFA tube removing the overflow air, and a polyether ether ketone (PEEK) capillary tube (ca. 1.5 m length × 1.01 mm ID, temperature: 110 °C, flow: 40 sccm) sampling the VOC-bottle air into the PTR-ToF. In parallel, volatiles were monitored as well from an empty VOC-bottle (blank) and a VOC-bottle containing cotton with water-sugar solution (food) serving as control and connected to the PTR-ToF with the same tubing system described above. The monitoring of VOCs was run in real-time for three days. Throughout the recordings, an automated inlet switching system enabled PTR-ToF to cycle between the VOC-bottles every 2 min, allowing for the cyclical analysis of the air in each sample. The PTR-ToF (mod. 8000, Ionicon Analytik, Innsbruck, Austria equipped with a time-of-flight detector from Tofwerk AG, Thun, Switzerland) was set to operate in H_3O^+ mode and a gas calibration unit (GCU) instrument (Ionicon Analytik, Innsbruck, Austria) was continuously providing each VOC-bottle with 5 l/h of humidified (50 % relative humidity) zero air.

2.3. Data collection and analysis

The analysis of GC–MS and PTR-ToF data was performed using RStudio software version 4.4.2 (Team, 2020). The GC–MS peak areas were analyzed to compare compound abundances across insect groups and sampling periods. Non-parametric Kruskal Wallis and Mann-Whitney tests were utilized to investigate significant differences in VOCs emission between insect groups and sampling periods, respectively, because the normality of distribution and equality of variances were invalid. Dunn post-hoc test with Bonferroni adjustment was used for pairwise comparisons. The analysis and visualization of figures were conducted using ggstatsplot package (Patil, 2021), with mean and standard deviation indicated in Table 1.

Using ggplot2 package (Wickham et al., 2016), the dynamics of release for PTR-ToF data were displayed over three days, highlighting the beginning and conclusion of light and dark periods. Using the equation reported below, the emission rates of each ion mass (in pmol/min) were computed from raw data emissions based on the flow of each inlet and transmission coefficients. We refer to (Giacomuzzi et al., 2017) for further details. Additionally, to emphasize the trend of emissions between insect groups, data was smoothed across time by calculating mean emissions every four hours of the sampling period.

$$Emission\ rate\ (pmol/min) = \frac{\text{Emission rate (ppbv)} \times \text{flow}}{\text{Transmission} \times 22.414}$$

PTR-ToF peaks were tentatively identified, following an approach based on the sharedness of sex-borne volatiles amongst closely related species. In fact, several studies have noted that species that exhibit a degree of relatedness may employ similar chemical cues to perform their biological functions (Cardé and Haynes, 2004; Fine et al., 2004; Fletcher and Kitching, 1995; Francke and Kitching, 2001). For instance, many spiroacetals (such as olean and 1,7-dioxaspiro[5.5]undecanols) are shared between several *Bactrocera* species (e.g., *B. oleae*, *B. cacuminatus*, *B. distincta* and *B. tryoni*), whereas their relevance is determined by their proportions that may vary for each species (Booth et al., 2009; Fletcher et al., 1992). Similarly, a fatty acyl ((*Z*)–non-3-en-1-ol) was reported to be shared among four *Anastrepha* species (Diptera, Tephritidae) (*A. fraterculus*, *A. ludens*, *A. obliqua* and *A. suspensa*), eliciting behavioral responses in all of them (Scolari et al., 2021).

In this context, a database was developed from all potential candidates whose protonated *m/z* matches with one of the ion masses obtained from PTR-ToF measurements (Table 1, Supplementary material). The database contains molecules, selected for their biological activity in a descending order of importance, starting from *Bactrocera* genus, followed by Tephritidae family, then Diptera order and additional insect and floral species. These compounds were classified depending on whether they were emitted from closely related species or distant ones, and arranged according to their function as kairomones, pheromones, allomones, or attractants for these species (El-Sayed, 2023). Numerous candidates from various species matched for each protonated *m/z*, and the correctness of information reported in each candidatés original paper was examined, and inaccurate material was removed. In addition, a thorough investigation was conducted to search for the occurrence of database candidates (via molecular ion search) in *B. oleae* headspace VOC collections. This database was populated using Pherobase (a database for insect semiochemicals) (El-Sayed, 2023), Glovocs (a platform for PTR-ToF molecules) (Yáñez-Serrano et al., 2021), and other substances available in literature.

The classification of all tentatively identified compounds was obtained according to NPC classifier (Kim et al., 2021), using chemodiv package (Petrén et al., 2023).

3. Results

3.1. GC–MS

The investigation of *B. oleae* sex-borne VOCs yielded a total of 49 compounds which spanned various chemical classes, of which 15 are unknown (Table 1). The emission of VOCs and their abundance were qualitatively and quantitatively different among olive fruit fly groups. In fact, some compounds were exclusively emitted from a particular group or during specific time interval, whereas others were more shared temporally and between groups. For instance, spiroacetals were released by nearly every group during light and dark periods. In particular, olean (**1**) was the most abundant compound in all extracts, with the highest total-ion current in mature females (184 ± 133; mean ± std) and mix (111 ± 88) groups during light period (c^2Kruskal − Wallis = 12.86, $p < 0.01$). However, during the dark period, there was no significant difference in its release among the groups (c^2Kruskal − Wallis = 7.79, $p = 0.05$). Surprisingly, the emission of this compound did not vary significantly between light and dark periods for all groups (**young females:** *W*Mann − Whitney = 21.00, $p = 0.44$; **mature females:** *W*Mann − Whitney = 25.00, $p = 0.15$; **males:** *W*Mann − Whitney = 21.00, $p = 0.44$**; mix:** *W*Mann − Whitney = 25.00, $p = 0.15$).

In addition, six spiroacetals ((**2a, 2b**), (**3a, 3b**), (**4a, 4b**)) with a hydroxy group were consistently observed in each extract, except for

Table 1
Bactrocera oleae sex-borne VOCs. Summary table of the total-ion current (TIC) of *B. oleae* headspace VOCs collected from males, young females, mature females, and mix, during light and dark periods of the day. The results are displayed as "mean area $\times 10^{-5} \pm$ SD". Each sample was collected from 20 individuals for three hours, and four replicates were performed for each group. Compounds were tentatively identified by mass spectrometry and comparison of experimental linear retention indexes LRI (exp) with those found in literature LRI (lit), as well as by laboratory standards when available (compounds with "‡"). Pairwise comparisons were run between insect groups for each collection period, and significant differences are emphasized by different letters. Compounds with symbol "*" were tentatively identified using PTR-ToF as well.

Compound	CAS	LRI (lit)	LRI (exp)	Light				Dark			
				Young females	Mature females	Males	Mix	Young females	Mature females	Males	Mix
Alcohols											
2-Heptanol	543-49-7	900	899	2.13 ± 1.74 **ab**	2.08 ± 1.18 **ab**	0.0 ± 0 **a**	6.46 ± 5.36 **b**	10.75 ± 9.40	12.07 ± 7.84	0.0 ± 0	14.49 ± 11.60
1-Heptanol	111-70-6	970	964					0.0 ± 0	0.22 ± 0.16	0.0 ± 0	0.42 ± 0.16
Aldehydes											
Decanal ‡	112-31-2	1204	1207	0.0 ± 0 **b**	0.11 ± 0.1 **ab**	0.14 ± 0.07 **ab**	0.23 ± 0.09 **a**	0.11 ± 0.06	0.14 ± 0.03	0.09 ± 0.02	0.16 ± 0.07
Branched fatty acids											
Palmitic acid	57-10-3	1972	1958	0.0 ± 0 **b**	0.07 ± 0.04 **ab**	0.08 ± 0.03 **ab**	0.09 ± 0.04 **a**	0.0 ± 0	0.0 ± 0	0.08 ± 0.02	0.07 ± 0.02
Esters											
2-Ethylhexyl acetate	103-09-3	1159	1152	0.0 ± 0 **a**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.18 ± 0.11 **b**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.26 ± 0.07 **b**
2-Ethylhexyl acrylate	103-11-7	1220	1232	0.0 ± 0 **a**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.38 ± 0.04 **b**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.57 ± 0.39 **b**
Methyl dodecanoate	111-82-0	1526	1527	0.6 ± 0.5 **b**	0.16 ± 0.08 **ab**	0.09 ± 0.11 **ab**	0.0 ± 0 **a**	0.02 ± 0.04	0.31 ± 0.38	0.0 ± 0	0.0 ± 0
Ethyl dodecanoate *	106-33-2	1597	1595	0.0 ± 0 **a**	0.51 ± 0.63 **b**	0.0 ± 0 **a**	0.0 ± 0 **a**				
Methyl tetradecanoate *	124-10-7	1724	1726	2.27 ± 1.42 **b**	0.59 ± 0.40 **ab**	0.0 ± 0 **a**	0.58 ± 0.86 **ab**	0.27 ± 0.28	0.73 ± 0.73	0.0 ± 0	0.0 ± 0
Isobutyl laurate	37811-72-6	1745	1746	0.0 ± 0	0.61 ± 0.52	0.0 ± 0	0.17 ± 0.11	0.0 ± 0 **a**	0.23 ± 0.15 **b**	0.0 ± 0 **a**	0.0 ± 0 **a**
Ethyl tetradecanoate *	124-06-1	1795	1795	0.0 ± 0	1.13 ± 1.11	0.0 ± 0	0.64 ± 0.97	0.0 ± 0	0.08 ± 0.08	0.0 ± 0	0.0 ± 0
Isobutyl myristate	25263-97-2	1932	1946	0.0 ± 0 **a**	0.48 ± 0.20 **b**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.31 ± 0.26 **b**	0.0 ± 0 **a**	0.0 ± 0 **a**
Ethyl palmitoleate	56219-10-4	1970	1975	0.0 ± 0	0.11 ± 0.13	0.0 ± 0	0.0 ± 0				
Ethyl palmitate	628-97-7	1995	1995	0.0 ± 0 **a**	0.36 ± 0.28 **b**	0.0 ± 0 **a**	0.0 ± 0 **a**				
1-Hexadecanol, acetate	629-70-9	2010	2010	0.0 ± 0 **b**	0.0 ± 0 **b**	0.81 ± 1.11 **a**	0.0 ± 0 **b**	0.0 ± 0 **b**	0.0 ± 0 **b**	1.33 ± 1.63 **a**	0.0 ± 0 **b**
Ethyl oleate	111-62-6	2175	2176	0.0 ± 0	0.0 ± 0	1.21 ± 1.58	0.99 ± 1.27	0.0 ± 0	0.0 ± 0	0.47 ± 0.51	0.37 ± 0.45
Z-13-Octadecen-1-yl acetate	60037-58-3	2200	2185	0.0 ± 0	0.0 ± 0	0.32 ± 0.45	0.12 ± 0.21	0.0 ± 0	0.0 ± 0	0.39 ± 0.45	0.2 ± 0.18
Hydrocarbons											
Tetradecane ‡	629-59-4	1400	1400	0.0 ± 0 **b**	0.1 ± 0.09 **ab**	0.16 ± 0.07 **ab**	0.28 ± 0.06 **a**	0.0 ± 0 **b**	0.16 ± 0.03 **ab**	0.09 ± 0.02 **ab**	0.27 ± 0.08 **a**
Heptadecane ‡	629-78-7	1700	1700	0.0 ± 0 **b**	0.0 ± 0 **b**	0.08 ± 0.03 **a**	0.0 ± 0 **b**	0.0 ± 0 **b**	0.0 ± 0 **b**	0.08 ± 0.03 **a**	0.0 ± 0 **b**

(*continued on next page*)

Table 1 (*continued*)

Compound	CAS	LRI (lit)	LRI (exp)	Light				Dark			
				Young females	Mature females	Males	Mix	Young females	Mature females	Males	Mix
(Z)-9-Tricosene (Muscalure) *	27519-02-4	2291	2275	0.0 ± 0	0.0 ± 0	0.23 ± 0.29	0.19 ± 0.19	0.0 ± 0	0.0 ± 0	0.05 ± 0.09	0.0 ± 0
Lactones											
Gamma-hexalactone *	695-06-7	1056	1055	0.0 ± 0	0.0 ± 0	1.41 ± 1.04	0.77 ± 0.69	0.0 ± 0	0.0 ± 0	1.91 ± 1.28	0.64 ± 0.59
Oxygenated hydrocarbons											
2-Heptanone	110-43-0	892	892	1.19 ± ± 1.09 **ab**	0.0 ± 0 **a**	0.0 ± 0 **a**	3.47 ± 1.61 **b**	3.52 ± 2.95	4.34 ± 3.13	0.0 ± 0	5.2 ± 3.98
Phenylpropanoids											
Methyleugenol	93-15-2	1402	1407	0.16 ± 0.03 **b**	0.02 ± 0.03 **ab**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.48 ± 0.65	0.1 ± 0.17	0.0 ± 0	0.0 ± 0
Spiroacetals											
Olean *	180-84-7	1143	1136	96.46 ± 60.87 **ab**	183.87 ± 133.13 **b**	17.4 ± 13.04 **a**	110.68 ± 88.33 **ab**	48.69 ± 12.89	58.63 ± 38.45	11.28 ± 13.22	25.92 ± 16.29
(2S,5S) 1,6 dioxaspiro[4.5]decane-2-methnaol/ (2R,5R) 1,6-dioxaspiro[4.5]decane-2-methnaol *		–	1291	2.32 ± 1.71	5.97 ± 4.88	0.79 ± 0.47	2.54 ± 2.37	1.05 ± 0.44 **ab**	2.37 ± 1.16 **b**	0.17 ± 0.10 **a**	1.34 ± 0.93 **ab**
(2R,5S) 1,6-dioxaspiro[4.5]decane-2-methnaol/ (2S,5R) 1,6-dioxaspiro[4.5]decane-2-methnaol *		–	1311	1.91 ± 1.53 **ab**	6.24 ± 4.12 **b**	0.59 ± 0.33 **a**	3 ± 2.52 **ab**	0.83 ± 0.48 **ab**	2.08 ± 1.14 **b**	0.12 ± 0.08 **a**	1.15 ± 0.78 **ab**
(3S,6S) 1,7-dioxaspiro[5.5]undecan-3-ol/(3R,6R) 1,7-dioxaspiro[5.5]undecan-3-ol *		–	1354	3.49 ± 2.78	5.88 ± 4.01	1.02 ± 0.52	2.65 ± 2.52	2.15 ± 1.03 **ab**	3.25 ± 0.71 **b**	0.35 ± 0.25 **a**	1.88 ± 0.79 **ab**
(3R,6S) 1,7-dioxaspiro[5.5]undecan-3-ol/(3S,6R) 1,7-dioxaspiro[5.5]undecan-3-ol *		–	1327	3.26 ± 2.31 **ab**	6.65 ± 4.08 **a**	1.14 ± 0.35 **ab**	0.0 ± 0 **b**	1.86 ± 0.45 **ab**	3.09 ± 0.71 **b**	0.0 ± 0 **a**	1.78 ± v0.46 **ab**
(4S,6S) 1,7-dioxaspiro[5.5]undecan-4-ol/(4R,6R) 1,7-dioxaspiro[5.5]undecan-4-ol *		–	1350	2.44 ± 1.89	4.77 ± 3.74	1.08 ± 0.52	2.29v2.18	1.58 ± 0.73	2.73 ± 2.10	0.3 ± 0.19	2.01 ± 1.23
(4R,6S) 1,7-dioxaspiro[5.5]undecan-4-ol/(4S,6R) 1,7-dioxaspiro[5.5]undecan-4-ol *		–	1314	0.0 ± 0 **a**	1.19 ± 0.71 **b**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.0 ± 0	0.71 ± 0.17	0.0 ± 0	0.0 ± 0
Terpenoids											
Caryophyllene * ‡	87-44-5	1465	1430					0.21 ± 0.32	0.05 ± 0.08	0.0 ± 0	0.0 ± 0
Unknowns											
unknown1		–	903	2.16 ± 2.01	0.0 ± 0	0.0 ± 0	6.86 ± 4.52	10.49 ± 7.77	10.21 ± 6.39	0.0 ± 0	13.56 ± 11.85
unknown2		–	932					0.0 ± 0 **b**	0.0 ± 0 **b**	0.25 ± 0.16 **a**	0.0 ± 0 **b**
unknown3		–	1075	4.74 ± 2.13 **ab**	5.63 ± 1.79 **ab**	0.0 ± 0 **a**	8.94 ± 1.77 **b**	3.76 ± 1.55	3.66 ± 1.66	0.0 ± 0	0.0 ± 0
unknown4		–	1101	13.96 ± 12.71	16.99 ± 14.68	12.59 ± 11.89	9.15 ± 10.51	24.97 ± 11.34	27.68 ± 14.35	15.31 ± 7.98	35.5 ± 24.21
unknown5		–	1164	0.0 ± 0	2.19 ± 1.53	0.0 ± 0	1.66 ± 0.38	1.28 ± 0.36 **ab**	1.48 ± 0.21 **b**	0.0 ± 0 **a**	0.92 ± 0.39 **ab**
unknown6		–	1173	0.0 ± 0	2.12 ± 1.31	1.34 ± 0.59	3.15 ± 1.87	3.03 ± 0.68	3.53 ± 2.41	1.12 ± 0.77	3.41 ± 2.69
unknown7		–	1242					0.0 ± 0 **a**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.07 ± 0.02 **b**
unknown8		–	1444	0.19 ± 0.06	0.0 ± 0	0.0 ± 0	0.18 ± 0.05				
unknown9		–	1562					0.0 ± 0 **b**	0.0 ± 0 **b**	0.1 ± 0.06 **a**	0.0 ± 0 **b**
unknown10		–	1579	0.0 ± 0	0.0 ± 0	0.07 ± 0.04	0.0 ± 0	0.0 ± 0 **a**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.13 ± 0.04 **b**
unknown11		–	1612	0.0 ± 0 **b**	0.0 ± 0 **b**	0.25 ± 0.06 **a**	0.0 ± 0 **b**	0.0 ± 0 **b**	0.11 ± 0.01 **ab**	0.18 ± 0.05 **a**	0.0 ± 0 **b**
unknown12		–	1644	0.0 ± 0 **b**	0.0 ± 0 **b**	0.08 ± 0.03 **a**	0.0 ± 0 **b**				
unknown13		–	1722	0.0 ± 0 **b**	0.0 ± 0 **b**	0.13 ± 0.08 **a**	0.0 ± 0 **b**				
unknown14		–	1757	0.0 ± 0 **a**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.21 ± 0.17 **b**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.14 ± 0.03 **b**
unknown15		–	1800	0.0 ± 0 **a**	0.12 ± 0.05 **b**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.0 ± 0 **a**	0.1 ± 0.02 **b**

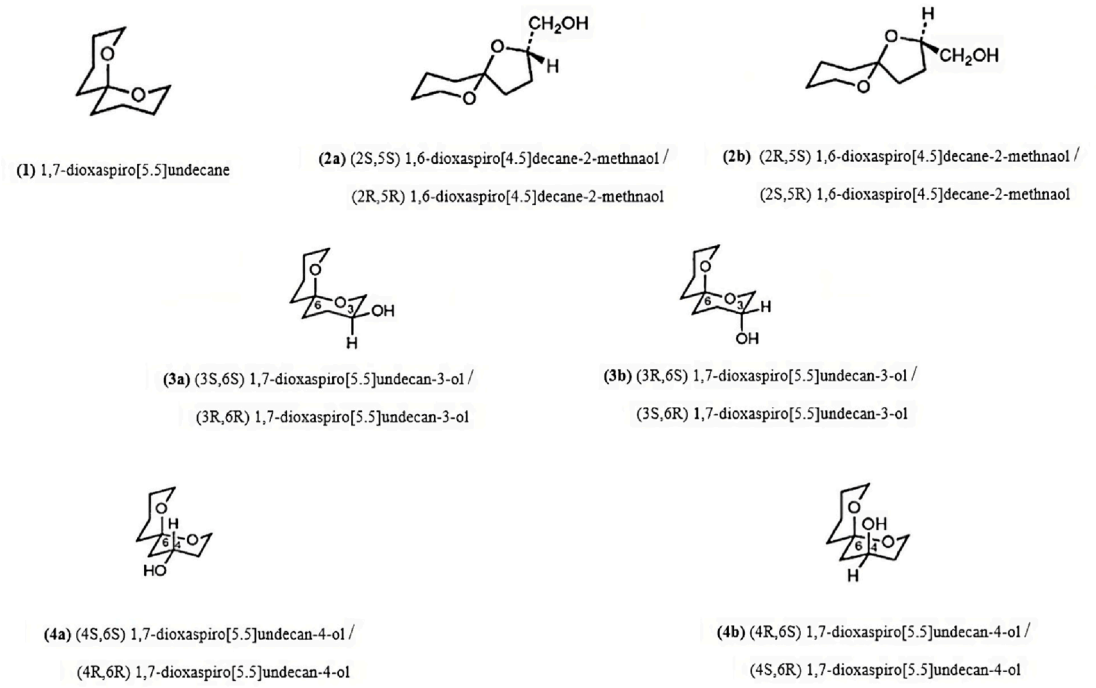

Fig. 1. *Bactrocera oleae* spiroacetals tentatively identified from males and females headspace VOCs collections, according to (Fletcher et al., 1992). These compounds are presented in pairs with their possible enantiomers, differenciated only on a chiral column. They were found in almost all extracts, including females, males and their mixuture.

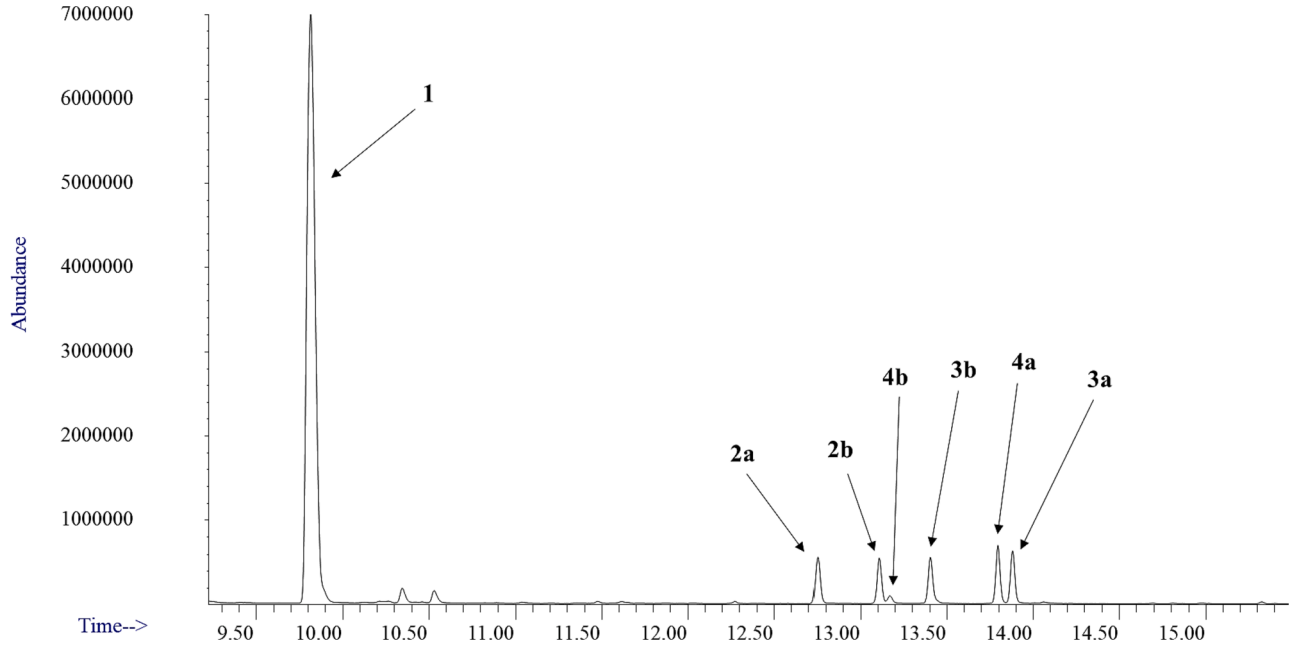

Fig. 2. Gas chromatogram of the headspace of *Bactrocera oleae* female spiroacetals on a non-polar column (HP5-MS column), during light period. The headspace volatiles were collected for three hours using closed-loop stripping analysis (CLSA). The tentative identification of absolute stereochemistry of these alcohol spiroacetals was performed according to (Fletcher et al., 1992)(**1**) Olean (racemic), (**2a**) (2S,5S) 1,6-dioxaspiro[4.5]decane-2-methnaol /(2R,5R) 1,6-dioxaspiro[4.5]decane-2-methnaol, (**2b**) (2R,5S) 1,6-dioxaspiro[4.5]decane-2-methnaol /(2S,5R) 1,6-dioxaspiro[4.5]decane-2-methnaol, (**4b**) (4R,6S) 1,7-dioxaspiro[5.5]undecan-4-ol /(4S,6R) 1,7-dioxaspiro[5.5]undecan-4-ol, (**3b**) (3R,6S) 1,7-dioxaspiro[5.5]undecan-3-ol /(3S,6R) 1,7-dioxaspiro[5.5]undecan-3-ol, (**4a**) (4S,6S) 1,7-dioxaspiro[5.5]undecan-4-ol /(4R,6R) 1,7-dioxaspiro[5.5]undecan-4-ol, (**3a**) (3S,6S) 1,7-dioxaspiro[5.5]undecan-3-ol /(3R,6R) 1,7-dioxaspiro[5.5]undecan-3-ol.

(**4b**) that was only emitted by mature females in both periods. These compounds were tentatively identified based on the suggestions of NIST library and the comparison of mass spectra using Spectrabase (John Wiley & Sons) as well as according to Fletcher et al., 1992 research work on the asymmetry of these alcohols and their natural occurrence for *B. oleae*. These compounds are summarized in Figs. 1 and 2 in pairs along with their potential enantiomers that we were not able to separate on a non-chiral column. Regarding their emission, all groups were releasing these compounds during both periods.

Besides spiroacetals, *B. oleae* mature females were leading in esters

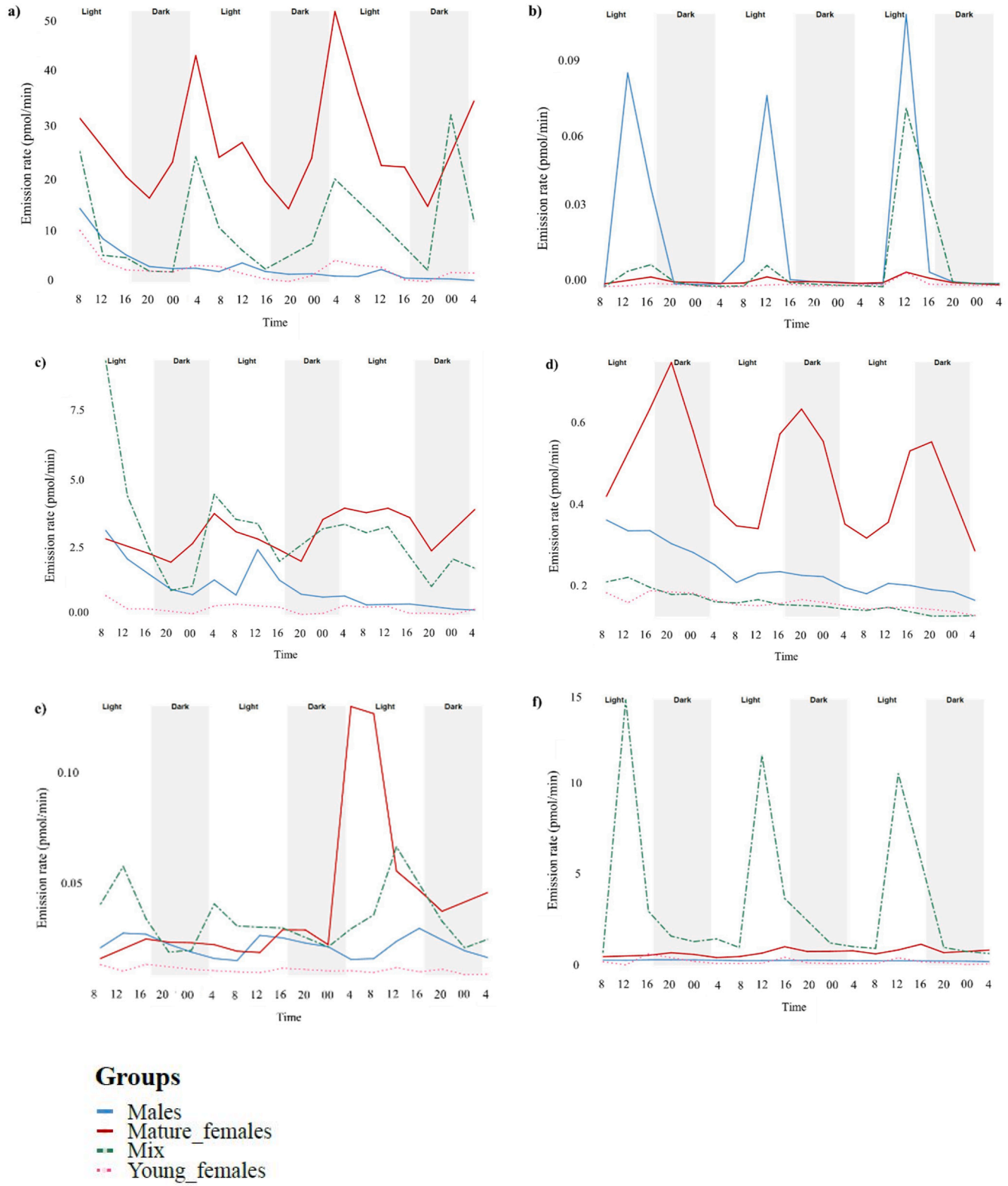

Fig. 3. Dynamics of release of *Bactrocera oleae* (young females, mature females, males, and mix) sex-borne volatiles using PTR-ToF, for three days over light and dark periods. **a)** Olean (m.e 22.89 pmol/min; $m/z = 157.123$), **b)** Muscalure (m.e 0.06 pmol/min;; $m/z = 323.367$), **c)** *B. oleae* spiroacetals: 2a, 2b, 3a, 3b, 4a, 4b (m.e 8.52 pmol/min; $m/z = 173.118$), **d)** Alpha-pinene (m.e 0.06 pmol/min; $m/z = 137.133$), **e)** Methyl tetradecanoate (m.e 0.08 pmol/min; $m/z = 243.231$), **f)** unknown_mix (mix-specific, m.e 8.09 pmol/min, $m/z = 89.025$), **g)** Rhododendrol (m.e 0.33 pmol/min; $m/z = 167.109$), **h)** unknown_male (male-specific, m.e 44.93 pmol/min; $m/z = 86.104$), **i)** Gamma-hexalactone (m.e 9.22 pmol/min; $m/z = 115.076$), **j)** Ammonia (m.e 7469 pmol/min; $m/z = 18.034$). **a), b), c), d), e)** ion masses were reported as *B. oleae* pheromone, **g)** and **j)** were tentatively identified from literature, whereas **i)** has been identified from the males and mix headspace VOCs collections using GC–MS.

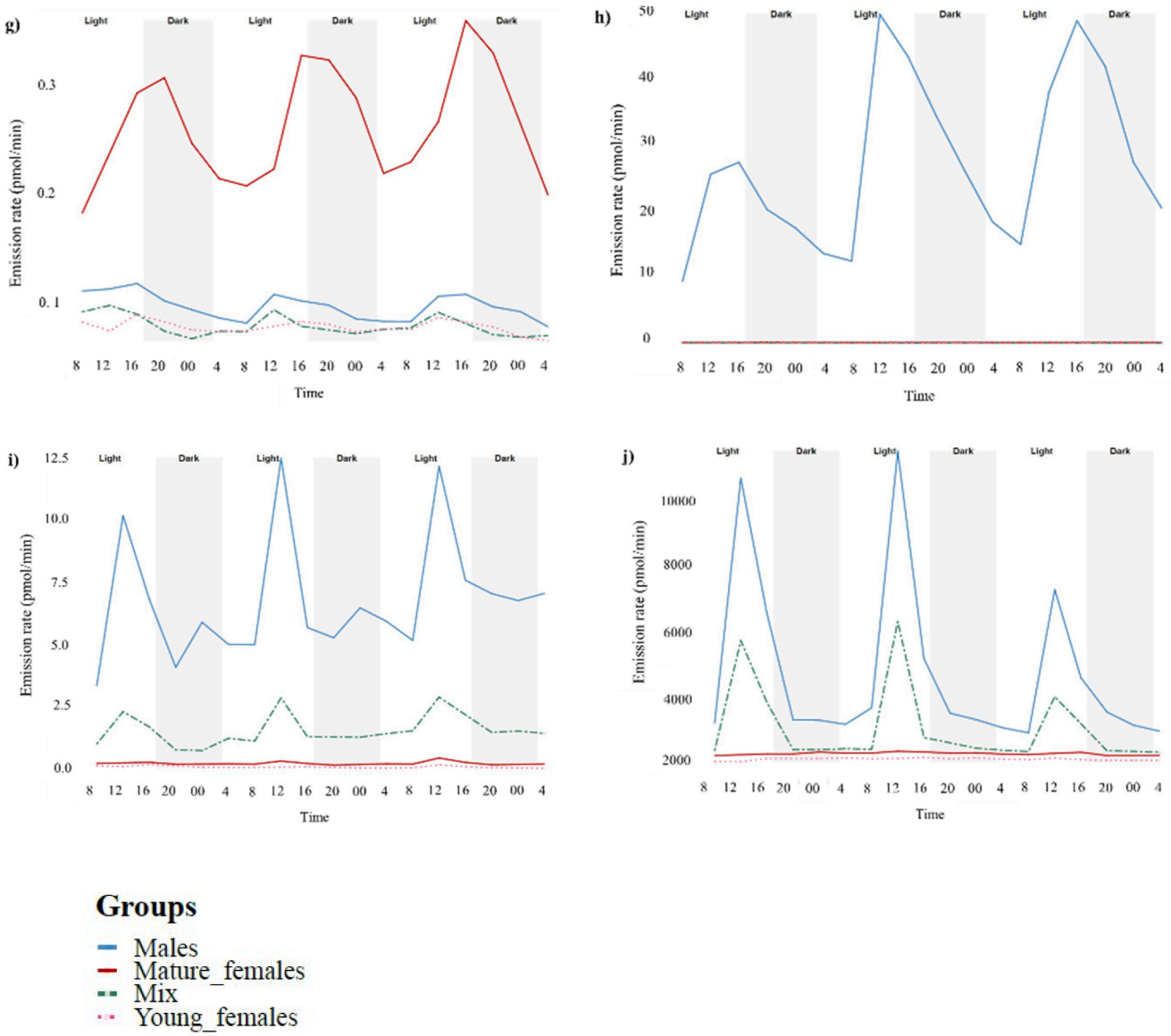

Fig. 3. (*continued*).

release, demonstrating significant differences with other groups in compound abundance, diversity, and timing (Table 1). In fact, 8 of the 13 identified esters were either solely emitted by mature females (e.g., ethyl dodecanoate and ethyl tetradecanoate) or shared with young ones and mix (e.g., methyl tetradecanoate and isobutyl laurate). Male-specific esters were either discovered to be only present in males (1-hexadecanol, acetate) or to be also present in mix extracts (ethyl oleate**;** *Z*-13-octadecen-1-yl acetate), whilst the origin of mix-specific esters remains unknown (2-ethylhexyl acetate, 2-ethylhexyl acrylate). Comparatively, esters abundance in all groups was relatively very low in comparison to spiroacetals abundance, with the exception of young females emitting methyl tetradecanoate (2.3 ± 1.4), a sex pheromone component of *B. oleae* females (Canale et al., 2015).

Additionally, males and mix groups were also releasing muscalure, (*Z*)-9-tricosene (**light:** c^2Kruskal − Wallis = 5.23, p = 0.16; **dark:** c^2Kruskal − Wallis = 3.00, p = 0.39) and gamma-hexalactone (**light:** c^2Kruskal − Wallis = 8.19, p = 0.04; **dark:** c^2Kruskal − Wallis = 8.19, p = 0.04) during light and dark periods, with no discernible variations in their abundance or emission time.

3.2. PTR-ToF

PTR-ToF recordings revealed the emission of 554 ion masses between *m/z* 15 and 322, with an emission rate ranging from 4.5×10^{-5} to 8.85×10^{3} pmol/min. The peaks were selected and classified according to the source and pattern of emission. The dynamics of each peak were smoothed and visualized over a span of three days, with data points taken every four hours (Fig. 3). In total, 90 peaks showed a clear release pattern from single or multiple groups. According to the protocol described in material and methods, 41 ion masses were tentatively identified as candidate compounds emitted by *B. oleae* individuals, while 49 remain unknown. In addition, 25 ion masses of unclear pattern were also selected for their great importance in the chemical communication of tephritids in general (20 ion masses), and the olive fruit fly in particular (5 ion masses).

The initial analysis of PTR-ToF peaks concerned 13 compounds that have been previously identified using GC–MS, for which ion masses were matching with those detected by PTR-ToF. These compounds are highlighted in Table 1.

Olean (**1**) ($C_9H_{16}O_2$, *m/z* 157.122) was primarily emitted by mature females (max of emission: m.e. = 41.64 pmol/min) and mix groups (m.

e. = 22.89 pmol/min), displaying a consistent rhythm characterized by the first significant peak at the onset of the light period (early in the morning), and a second peak in the afternoon (Fig. 3). Its emission from young females and males was very low, yet its pattern of release was still visible. Young females emitted olean in the early morning, whereas males' emission occurred in the afternoon. For all groups, the lowest release of this compound was observed during the evening.

As *B. oleae* pheromone is composed of other spiroacetals, the ion masses of compounds detected with GC–MS were searched to determine their pattern of release. However, since all these alcohols have the same molecular weight, their dynamics of release were visualized as the sum of their emission rate. The compounds **2a**, **2b**, **3a**, **3b**, **4a** and **4b** were represented by one ion mass ($C_9H_{16}O_3$, *m/z* 173.118) whose emission pattern was relatively similar to olean (high emission at early morning and low at the evening). The emission of these alcohols was mainly from mature females (m.e. = 8.52 pmol/min) and mix groups. However, the sum of different emissions limits the ability to determine the real rhythm of each of these compounds.

In contrast to the spiroacetals, three esters including ethyl dodecanoate ($C_{14}H_{28}O_2$, *m/z* 229.214), methyl tetradecanoate ($C_{15}H_{30}O_2$, *m/z* 243.231) and ethyl tetradecanoate ($C_{16}H_{32}O_2$, *m/z* 257.245) did not follow the olean release pattern, and did not have a clear pattern at all. Moreover, their emission rate was very low (m.e. = 0.09 pmol/min; m.e. = 0.08 pmol/min; m.e. = 0.08 pmol/min; respectively) with young females having the lowest rates. These compounds were tentatively identified by GC–MS and reported as sex pheromone components of *B. oleae* (Canale et al., 2015). Interestingly, ethyl myristoleate ($C_{14}H_{28}O_2$, *m/z* 229.214), another ester, was mainly released by mature females in a clear pattern with a peak of emission during evening. This compound has been reported as a sex pheromone for *B. tryoni* (Mazomenos and Haniotakis, 1981) and follows the same release pattern of alpha-pinene ($C_{10}H_{16}$, *m/z* 137.133), a sex pheromone component of *B. oleae* (Mazomenos and Haniotakis, 1985). These two compounds had a low emission rate, reaching m.e. 0.06 pmol/min and m.e. 0.65 pmol/min, respectively.

In addition to alpha-pinene and ethyl myristoleate, 11 compounds were identified for mature female emissions, following the same pattern of release with a peak of emission throughout the evening. These compounds belong to different classes and have been linked to a variety of biological functions. Benzothiazole ($C_7H_5N_S$, *m/z* 136.023) is a kairomone for *Bactrocera dorsalis*, 4,8-dimethylnona-1,3,7-triene ($C_{11}H_{18}$, *m/z* 151.149) is an attractant for *Rhagoletis zephyria*, rhododendrol ($C_{10}H_{14}O_2$, *m/z* 167.109) is a male attractant for many *Bactrocera* species, while caryophyllene ($C_{15}H_{24}$, *m/z* 205.196) was described as a pheromone for *Bactrocera correcta* and identified in *B. oleae* extracts (GC–MS) (Cha et al., 2017; Katte et al., 2020; PD et al., 2014; Tokushima et al., 2010).

Mature females were also seen to release particular compounds in the afternoon, such as acetamides, among which N-(3-methylbutyl) acetamide ($C_7H_{15}NO$, *m/z* 130.126) could be a candidate given that it has been noted to function as a pheromone for several *Bactrocera* species (Bellas and Fletcher, 1979; Perkins et al., 1990; Wee and Tan, 2005; Zhang et al., 2019).

In contrast to emissions from females, males and mix groups showed the most consistent and clear release patterns for numerous compounds. With only a few exceptions, their emissions mostly occurred during the afternoon and evening, and at extremely high rates. In fact, aldehydes such as acetaldehyde (C_2H_4O, *m/z* 45.033), isobutyraldehyde (C_4H_8O, *m/z* 73.065) and (*E*)-4-oxo-2-hexenal ($C_6H_8O_2$, *m/z* 113.060) were primarily released during the afternoon, whereas crotonaldehyde was emitted early in the morning, similarly to olean. Moreover, many additional unknown ion masses followed the same pattern and are assumed to be nitrogen compounds. For instance, ammonia (H_3N, *m/z* 18.034), the ion mass with the greatest emission rate (m.e. 7469 pmol/min) among all the peaks, was exclusively released and surely identified from males and mix emissions, showing a peak during the afternoon. This compound has been claimed to function as an attractant for many tephritid species (Kendra et al., 2005; Robacker and Flath, 1995; Stelinski and Gut, 2004).

Furthermore, muscalure, (*Z*)-tricos-9-ene ($C_{23}H_{46}$, *m/z* 323.367), and gamma-hexalactone ($C_6H_{10}O_2$, *m/z* 115.076) were only present in males and mix of *B. oleae*, displaying a clear release pattern. The former, which was identified as the male pheromone of *B. oleae* (Carpita et al., 2012), had a peak of emission in the afternoon, whereas the latter, which was recently reported as a male-specific lactone of the same species (López et al., 2023), showed a first peak in the afternoon and a second one in the evening. Earlier, gamma-hexalactone was identified from *B. oleae* headspace VOC collections using GC–MS. These compounds are thought to be male-specific and their occurrence in the mix is explained by the fact that male individuals are present in this group as well. However, ten unknown ion masses (such as *m/z* 86.104) were regularly and solely emitted by males without being recorded in the mix (Fig. 3). These ion masses showed various emission rates ranging from m.e. 0.91 pmol/min to m.e. 267.48 pmol/min, during early morning and afternoon. Interestingly, the presence of females may have prevented the production/release of certain compounds.

On the other hand, the mix group principally exhibited two ion masses (*m/z* 89.0257, *m/z* 91.039) related to unidentified compounds, with the peak of emissions (m.e. 8.09 pmol/min, m.e. 20.25 pmol/min, respectively) occurring during the afternoon. Females followed a similar pattern, emitting this compound at low emission rates, while males consistently exhibited minimal emissions. In reality, these compounds are presumed to be initially released by females, with their emissions being amplified in the presence of males, because females and mix groups displayed similar release pattern with significant difference in emission rate.

4. Discussion

This study investigated the chemical profile of the olive fruit fly *B. oleae* and its dynamics of release combining GC–MS and PTR-ToF analytical techniques. The headspace collections of VOCs and their real-time monitoring yielded the tentative identification and dynamics of various compounds. These include known sex pheromones for this species, as well as new potential candidates, whose concentration or structural features prevented their detection by commonly used analytical techniques. In fact, several ion masses (found using PTR-ToF) exhibited a distinctive pattern during light and dark periods, following a diel rhythm, and thus emphasizing their potential involvement in completing and enhancing biological activities, such as pheromone emissions and mating.

In *B. oleae*, many researchers have reported that mating and pheromone release predominantly occur in the late afternoon, within the last 3–4 h of the photophase (Clarke, 2019; Fletcher, 1987; Haniotakis and Pittara, 1994; Loher and Zervas, 1979), and quickly conclude with the onset of darkness (Fletcher, 1987). When employing sequential SPME-GCMS, the monitoring of *B. oleae* female-borne volatiles at two-hour intervals revealed that the pheromone is mostly released during the scotophase and reaches its highest emission level at the beginning of this period (Levi-Zada et al., 2012). Conversely, within our research (where emitted VOCs were monitored in real-time), racemic olean showed two peaks of release from mature females, with a greater emission rate early in the morning than in late afternoon. In addition to olean, the 1,7-dioxaspiro[5.5]undecanols (minor components of *B. oleae* pheromone) (Fletcher et al., 1992) also had higher emissions during the photophase. These results partially contradict literature that claimed *B. oleae* only emits its pheromone during late afternoon/dusk (Clarke, 2019; Fletcher, 1987, Levi-Zada et al., 2012). For another *Bactrocera* species like *B. zonata,* the emission of pheromone that was mostly observed near dusk, has also been documented during early morning hours, where both males and females have occasionally emitted the pheromone within this period (Levi-Zada et al., 2020). However, these early

morning emissions from *B. zonata* were suggested to be associated with the activation of laboratory lighting, rather than the natural timing of pheromone release. Moreover, mature females released additional molecules with a peak of release in the early morning and late afternoon, exhibiting a rhythmic pattern and releasing at lower emission rates compared to olean. On the other hand, males emitted VOCs in a clear release pattern during late afternoon. The functions of these sex-borne compounds remain unknown, however, they may potentially be engaged in pre-mating and mating processes and may fulfill a variety of purposes such as attracting/repelling conspecifics and communicating fitness information (Fletcher, 1987). In fact, some of these molecules may play a dual role in attraction and repellency depending on the receiver, as has been reported for *D. melanogaster* male pheromone *cis*-vaccenyl acetate, which acts as an aphrodisiac for females and a repellent for males (Kurtovic et al., 2007).

Besides spiroacetals, *B. oleae* female pheromone was reported to be composed of nine esters, including ethyl dodecanoate, methyl tetradecanoate, ethyl tetradecanoate, ethyl oleate, ethyl decanoate, methyl dodecanoate, *n*-butyl dodecanoate, methyl hexadecanoate and ethyl hexadecanoate (Canale et al., 2015; Gariboldi et al., 1983; Kamala Jayanthi et al., 2014; Mazomenos and Haniotakis, 1981). The first four esters were found in female extracts analyzed using GC–MS, and their patterns of release were monitored using PTR-ToF. However, neither the compounds we found, nor the ones previously mentioned in literature exhibited a consistent release pattern or were exclusive to any particular insect group. They were all emitted from both the food and the control (PTR-ToF analysis) and reported to be perceived by both sexes (Canale et al., 2015). In fact, these findings raise doubts about the validity of these compounds to serve as sex pheromones for *B. oleae*. They may serve as carrier of pheromone (e.g., trisodium and potassium phosphate roles in *B. cucurbitae* and *B. dorsalis* male pheromones) (Ohinata et al., 1982), or be related to inter-specific chemical signaling, rather than eliciting innate olfactory responses and influencing flies' behavior.

Furthermore, in addition to olean, our headspace VOC collections revealed a richness of female extracts with six abundant spiroacetals and their potential enantiomers (Fletcher et al., 1992), surpassing the abundance of ester compounds. These findings disagree with those of Canale et al., 2015, reporting the abundance of esters in the female pheromone. These variations might be explained by the employment of different sampling methods, duration, and column features (Scolari et al., 2021), as well as by other factors including time of sampling (Nation, 1990; Tumlinson, 1988), diet of the study subjects (Aluja et al., 2020; Merli et al., 2018) and size of *B. oleae* sample. In reality, the sex-borne VOCs of *B. oleae* were thoroughly investigated mainly by dissecting rectal and urotergal glands of males and females (Baker et al., 1980; Benelli et al., 2013; Fletcher et al., 1992; Gariboldi et al., 1983), and less frequently with headspace VOC collections. However, additional pheromone components such as alpha-pinene and nonanal were discovered in the headspace samples of *B. oleae* pheromone, which are presumed to be released by parts of the insect body other than the glands (Mazomenos and Haniotakis, 1981). Ítś interesting to note that alpha-pinene and nonanal were reported to have a synergistic effect on olean (Mazomenos and Haniotakis, 1985).

Alpha-pinene is a monoterpene whose dynamics of release were mainly observed from mature females, with a peak of emission in the evening. Similarly, ethyl myristoleate (a sex pheromone component of *Bactrocera kraussi* (Noushini et al., 2021)) and other unidentified molecules also followed the same pattern of release. These compounds were not emitted from the mixed group, raising questions about the potential role of males in preventing their release. Furthermore, the tentatively identified rhododendrol also had a peak of emission during the evening following the same pattern and source of release. This floral compound has been demonstrated to attract a wide variety of *Bactrocera* males, including *B. albistrigata*, *B. carambolae* and *B. tau* (Katte et al., 2020).

The current study has also illustrated the dynamics of release of muscalure, (*Z*)-tricos-9-ene, the male sex pheromone of *B. oleae* (Carpita et al., 2012) that was emitted from males and mix groups with an afternoon emission peak. This hydrocarbon is electrophysiologically active for this species and attracts *B. oleae* females. Additionally, gamma-hexalactone, which was discovered in *B. oleae* headspace VOCs (using GC–MS), and whose pattern followed the one of muscalure could be involved in male–female communication, especially as this compound has been claimed earlier to serve as pheromone and attractant for many Coleoptera and Lepidoptera (Greenblatt et al., 1977; Tian et al., 2008). Similarly, the highest rates of emission of ammonia and acetaldehyde from males and mix during the afternoon, suggests their potential involvement in chemical signaling of *B. oleae* males. In actuality, *B. oleae* mating strategy is often referred to as a lekking system, in which males congregate late afternoon on host trees and present a marked male-male aggression to guard their leaves (Benelli et al., 2014; Clarke, 2019; Fletcher, 1987). In most *Bactrocera* species, males are the emitters of sex pheromones, thus they aggregate late afternoon to call the females (Clarke, 2019; Wee et al., 2007). However, since *B. oleae* courtship is mainly manipulated by female-produced olean, perhaps these compounds are not necessarily evoking sexual attraction in females, but rather luring them to the host, where mating has been observed to occur in the afternoon. In addition, even though ammonia has only been reported in rectal glands of a single *Anastrepha* species (*A. suspensa*) (Kendra et al., 2005), many Tephritids demonstrate attraction to this compound (Kendra et al., 2005; Robacker and Flath, 1995; Stelinski and Gut, 2004). o-xylene is another intriguing compound that is mostly released by males in the afternoon and that has been described as an attractant for *B. oleae* females from the olive tree (Scarpati et al., 1993).

As with males, mature females also emitted certain compounds in the afternoon, such as N-(3-methylbutyl) acetamide. This compound is one of the most frequently reported chemicals in *Bactrocera* genus, and has been recorded in ten species, including *B. dorsalis*, *B. carambolae*, *B. tryoni* and *B. correcta* (Bellas and Fletcher, 1979; Perkins et al., 1990; Wee and Tan, 2005; Zhang et al., 2019).

For mix-specific compounds, the afternoon emissions peak may also support the occurrence of mating activity during this period, as claimed previously by numerous researchers (Clarke, 2019; Fletcher, 1987; Loher and Zervas, 1979). Based on our findings, we hypothesize that male presence stimulates the release of these compounds by females. We speculate that these compounds could function as a male "excitation stimulant" that females release to confirm their receptivity, or as a result of mating activity.

Furthermore, these molecules that have been tentatively identified from *B. oleae* males and females may serve as interspecific cues for interactions between this species and various components of its ecosystem, rather than intraspecific ones.

5. Conclusion

Overall, using a combination of PTR-ToF and GC–MS analytical techniques, our study describes the dynamics and diversity of *B. oleae* chemical profile released by young and mature females as well as males in the presence and absence of the opposite sex. Our findings provide the first real-time dynamics of release of previously described *B. oleae* pheromone components, including spiroacetals (e.g., olean), esters (e.g., methyl tetradecanoate), monoterpenes (e.g., alpha-pinene) and hydrocarbons (e.g., muscalure). These results showed that, contrary to what has been reported in the literature for the past 40 years, mature females produce sex-borne VOCs at several crucial times throughout the day. Of particular note is olean, which is emitted at a very high rate in the early morning. Additionally, this research revealed the emission of at least 90 compounds with regular release pattern, discussed their potential functions and tentatively identified some of them based on headspace collections of VOCs and literature, confirming the commonality of sex-borne compounds across related species. From these compounds, ammonia and acetaldehyde were identified from males and mix groups, with an afternoon emission peak, suggesting their potential role as

aggregation pheromones for *B. oleae* species. This study also highlighted the relatively low pheromone emissions from young females, confirming the significance of age in pheromone emission. This research work is the first to demonstrate in *Bactrocera* species the presence of chemicals that are only produced when males and females are in the proximity of each other, with an emission peak during the afternoon. The tentatively identified candidates require confirmation and further investigation to determine their electrophysiological activity and behavioral relevance to both males and females of *B. oleae*. These findings may serve as a springboard for further investigation into pheromones in the *Bactrocera* genus as well as the design of novel management tools of *B. oleae* in olive groves.

Conflicts of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Chaymae Fennine: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Riccardo Favaro:** Methodology, Writing – review & editing. **Iuliia Khomenko:** Conceptualization, Methodology, Writing – review & editing. **Franco Biasioli:** Writing – review & editing. **Luca Cappellin:** Conceptualization, Writing – review & editing. **Sergio Angeli:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data is shared as supplementary material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2023.104596.

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