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ORIGINAL PAPER

A shift in the paradigm? A male-specific lactone increases the response of both sexes of the olive fruit fly *Bactrocera oleae* to the food lure ammonium bicarbonate

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Abstract

The olive fruit fly *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) is a key pest species of wild and cultivated olive trees worldwide. Contrarily to most tephritid flies, in which males release the sex pheromone, in *B. oleae* the female is the sex responsible of attracting the opposite sex. However, and even though vast research has been done during the last decades, we are still far from understanding the chemical signals involved in the sexual communication of this species, including those produced by males. Here, we report for the first time the presence of two male-specific volatile compounds, namely γ-hexalactone and δ-hexalactone, with the former exerting a significant attraction upon both sexes under laboratory and field conditions. Volatile collections conducted on laboratory-reared virgin individuals of both sexes revealed the presence of these two compounds only in males, regardless of their age. In double-choice behavioral assays, γ-hexalactone resulted to be attractive for virgin males and females (7–14 days old), with no attractiveness reported for δ-hexalactone. Finally, in field assays traps baited with the binary blend of ammonium bicarbonate and γ-hexalactone yielded significantly more catches per week of both sexes than those baited with ammonium bicarbonate and ammonium bicarbonate plus 1,7-dioxaspiro[5.5] undecane, the major sex pheromone component. Altogether, our results shed light on the chemical ecology of the species and represent a promising experimental basis for the development of more innovative and effective mass trapping tools based on the use of γ-hexalactone.

Keywords *Bactrocera oleae* · γ-hexalactone · δ-hexalactone · Food lure · Chemical communication

Key message

Bactrocera oleae virgin males release two lactones, γ-hexalactone and δ-hexalactone.γ-Hexalactone elicits an attraction on virgin mature males and females in laboratory tests.

In field tests, the combination of ammonium bicarbonate and γ-hexalactone is highly attractive.

Our results might represent an innovative approach for improving ammonia-based baits in *B. oleae.*

Introduction

The so-called true fruit flies belonging to the family Tephritidae comprises more than 4000 species from 500 genera, with some of the species representing a serious threat for several agricultural and horticultural crops worldwide (White and Elson-Harris 1992). It has been estimated that direct and indirect economic losses attributable to tephritid flies exceed US $2 billion per year (Souza et al. 2021). In Europe, the Mediterranean fruit fly *Ceratitis capitata* Wiedemann and the olive fruit fly *Bactrocera oleae* (Rossi) are key pests regarded as the most harmful tephritid species in the continent, causing substantial damage (Enkerlin

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and Mumford 1997; Kampouraki et al. 2018). *Bactrocera oleae*, an endemic species to the Mediterranean Basin and Middle East, and currently also present in South and Central Africa, Pakistan, California and Mexico (Nardi et al. 2005), is considered as the most damaging insect to wild and cultivated olive trees worldwide (Daane and Johnson 2010). While adults are polyphagous and feed on different substrates, such as nectar, honeydew, fruit and plant exudates, bacteria and even bird faeces (Christenson and Foote 1960), larvae are mainly monophagous, and they breed and feed on the mesocarp of the fruits of some species within the genus *Olea*, especially on *Olea europaea*, but also on *O. verrucosa* and *O. chrysophylla* (Daane and Johnson 2010). The number of generations per year is variable and suggested to be related to the geographical region, the quality of the fruits, and agronomic and climatic conditions (Malheiro et al. 2015). Specifically, in the Mediterranean temperate areas three to four generations are usually reported, with up to six generations in the warmest Mediterranean areas where summer high temperatures may be a limiting factor for the development of the insect (Pappas et al. 2011). In this sense, *B. oleae* is highly dependent of temperature, with temperatures higher than 35 ℃ negatively affecting the life cycle of the insect (Johnson et al. 2011; Pappas et al. 2011). Similarly, at least four generations per year are observed in California, although an additional generation on fall is suggested (Rice et al. 2003). Gravid females lay their eggs in healthy olives, and upon hatching, larvae feed on the mesocarp. Larval development comprehends three instar stages, and during mid-autumn onwards third-instar larvae of the last generation leave the fruit for pupating in the soil, where they overwinter and emerge in the following spring. Due to the carpophagous feeding habit of larvae, the fruit oil content declines, the chemical composition is altered, and a premature drop of the olives is promoted (Bento et al. 1997; Gómez-Caravaca et al. 2008; Gucci et al. 2012). Additionally, the value of stung olives is reduced for table consumption (Malheiro et al. 2015). Altogether, the incidence of the olive fruit fly drastically affect the quantity and quality of table fruit and oil products, with average yield losses of up to 15% (Malheiro et al. 2015), and even 90% of crop losses may occur in the absence of control measures (Ordano et al. 2015). As a consequence, severe economic impact on olive production is produced. Losses attributable to olive fruit fly infestations are estimated to excess US $1 billion dollars per year in the Mediterranean Basin (van Asch et al. 2015). To cite an example, only in the island of Crete annual damage translates into more than 20 million euros (Kampouraki et al. 2018).

Over the last four decades, the management of *B. oleae* populations has been based on the use of wide-spectrum organophosphate insecticides and pyrethroids (Manousis and Moore 1987; Margaritopoulos et al. 2008). Drawbacks derived from the overuse of pesticides, such as residues prevalence (Cavanna and Molinari 1998; Amvrazi and Albanis 2009), development of insect resistance (Skouras et al. 2007; Kakani et al. 2010; Kampouraki et al. 2018), and potential side effects on beneficial insects (Pinheiro et al. 2020), have led to seeking more effective and eco-friendly approaches subjected to an Integrated Pest Management framework. These alternative strategies, with not satisfactory results in many of the cases, include the development of novel and safer insecticides (Canale et al. 2013a; Rizzo et al. 2020), research on natural enemies and entomopathogenic organisms as biological control tools (Daane and Johnson 2010; Shaurub 2023), the Sterile Insect Technique (Ant et al. 2012), symbiotic control strategies targeting its endosymbiont bacterium *Candidatus Erwinia dacicola* (Sinno et al. 2020), and mass trapping and attract-and-kill programs based on the use of food lures alone or in combination with the sex pheromone of the species. (Broumas et al. 2002; Mazomenos et al. 2002; Speranza et al. 2004; Yasin et al. 2014). In this sense, food lures, such as sugar and yeast baits, protein hydrolysates, and ammonium salts (e.g. ammonium phosphate, biammonium phosphate, ammonium carbonate, ammonium acetate, and ammonium sulphate), have been long time recognized as relevant attractants for true fruit flies, including *B. oleae* (for a historical review, see Epsky et al. 2014). Even though both sexes of many tephritid species are attracted to these kind of baits, field catches tend to be female-biased (Yokoyama et al. 2006; Martinez et al. 2007; Vázquez et al. 2022), since they require a protein source to complete egg maturation (Hagen and Finney 1950). The ammonia released from these baits is suggested to be the agent mediating this attraction, and therefore, the efficacy of different ammonium salts as an ammonia source has been tested on fruit flies for several decades (Bateman and Morton 1981; Mazor et al. 1987; Katsoyannos et al. 2000; Thomas et al. 2008). There is not still a consensus about the performance of ammonium salts in comparison to protein hydrolysates, since they have been referred to elicit a higher (Bateman and Morton 1981; Varikou et al. 2021) or lower (Haniotakis et al. 1998; Varikou et al. 2014) attraction than the latter, or even enhance the effect of hydrolyzed protein baits (Piñero et al. 2015, 2017, 2020). In the case of *B. oleae*, the co-release of ammonium bicarbonate and the major sex pheromone compound 1,7-dioxaspiro[5.5]undecane (= olean) (Baker et al. 1980; Mazomenos and Haniotakis 1981; Gariboldi et al. 1983) significantly increases male, and strikingly, female catches in comparison to ammonium bicarbonate alone (Haniotakis and Vassiliou-Waite 1987; Broumas and Haniotakis 1994). Nevertheless, male catches with this food lure-pheromone combination are often reported to exceed those of females in fall season, when the crop is

more susceptible (Haniotakis and Vassiliou-Waite 1987; Yokoyama et al. 2006; Burrack et al. 2008), and therefore, the elucidation of novel chemical cues involved in the sexual communication of the species would allow to improve the performance of ammonia-based baits during this season.

The semiochemistry of the mating system of tephritid flies involves complex intraspecific interactions (Scolari et al. 2021), in which male- and female-produced compounds are involved in the attraction of either one (Kobayashi et al. 1978; Haniotakis et al. 1986; Landolt et al. 1988; Carpita et al. 2012) or both sexes (Perdomo et al. 1976; Baker et al. 1990; Hee and Tan 1998; Būda et al. 2020). In contrast to other tephritid genera, in which pheromones are known to be released by males (comprehensively reviewed in Scolari et al. 2021), in some species of the genus *Bactrocera* Macquart chemical communication is more particular, with both sexes producing pheromone components (Haniotakis et al. 1986; Noushini et al. 2020), and females involved in pheromone communication in few species (Mazomenos and Haniotakis 1981; Zhang et al. 2019; Noushini et al. 2019, 2021a, b). Even though the characterization of the sex pheromone blend of the olive fruit fly was accomplished four decades ago (Baker et al. 1980; Mazomenos and Haniotakis 1981; Gariboldi et al. 1983), and the additional research conducted on the intraspecific chemical communication of the species (Baker et al. 1982b; Gariboldi et al. 1982, 1983; Carpita et al. 2012; Canale et al. 2013b, 2015; Fusini et al. 2018), the chemical ecology of the species still remains far from being completely understood. In the olive fruit fly the sex pheromone is released by females, and comprises a four-component blend, composed by the aforementioned olean, nonanal, ethyl dodecanoate, and α-pinene (Mazomenos and Haniotakis 1981, 1985). The emission of olean is produced close to the onset of the scotophase (Levi-Zada et al. 2012), in accordance with the time window in which mating occurs (Loher and Zervas 1979). Both sexes are reported to release olean since the first day after emergence, although quantitative and age-related differences are detected between sexes (Canale et al. 2012; Levi-Zada et al. 2012). In young males the maximal productivity is reached in few days after emergence (5–8 days), and the production ceases by the 11th day (Canale et al. 2012; Levi-Zada et al. 2012), in marked contrast to females, in which the emission peaks in the first 18 days and continues up to 35–45 days after emergence (Canale et al. 2012; Levi-Zada et al. 2012). In addition, the amount of olean released from females is ca. 1000 ng/h at the onset of scotophase, significantly exceeding that emitted by males (ca. 15 ng/h) (Levi-Zada et al. 2012). The role of male-released olean is still unknown. Benelli and coworkers discarded the emission of olean from young males as a chemical mimicry, suggesting that it may benefit them by keeping away older males, thus avoiding mating competence (Benelli et al. 2013). In addition to chemical signals, intrasexual communication is also mediated by additional sensory cues. Indeed, courtship and successful mating on males seem to be tightly linked to male wing vibration and associated behavior (f.i., abdomen rubbing with hind tarsus) (Benelli et al. 2012; Canale et al. 2013b), which in turn is related to the size of males (Benelli et al. 2016). Recently it has been determined that *B. oleae* males emit intermittent pulses of highly variable duration, at a frequency of ca. 350 Hz, and this wing vibration trait is suggested to be involved not only on courtship behavior, but also on male-male interactions (Terzidou et al. 2022). Similarly, the sexual communication of other tephritid species relies on a multimodal process based on the interaction of chemical and physical cues, highlighting the complexity of the mating system and courtship behavior of this dipteran family (extensively reviewed in Benelli et al. 2014).

In spite of all these advances on the intraspecific communication of the species, the identification of biologically active male-borne chemical cues is limited to some extent. To the best of our knowledge, only one male-specific active compound has been identified so far. Carpita and coworkers reported the presence of the unsaturated hydrocarbon (*Z*)-9-tricosene ("muscalure") in rectal glands of mature males, and this compound resulted to be attractive for virgin females at close-range (Carpita et al. 2012). However, the efficacy of (*Z*)-9-tricosene on field has not been evaluated yet, and probably its low volatility makes unfeasible to lure conspecific females at a long-range. Hence, we questioned whether males may produce and release overlooked volatile cues that may be active on females, and therefore potentially strengthen the attractiveness mediated by an ammonium salt. To achieve our goal, we first focused on the volatile profile of sexually mature individuals, and determined the behavioral activity of two male-specific lactones on both sexes under laboratory conditions. Finally, the attractiveness of the binary blend composed by γ-hexalactone and ammonium bicarbonate was determined in field assays.

Materials and methods

Insects

Volatile collections and behavioral assays were conducted on insects from a permanent laboratory colony maintained at the installations of the Institute for Advanced Chemistry of Catalonia (Barcelona, Spain) since 2016. The parental generation for establishing this colony was obtained from a long-term colony (ca. 10 generations per year) cultured at the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture (Vienna, Austria) since 2008. For egg production, larvae rearing and adult maintenance, previously

described methodologies based on artificial oviposition and larvae-developing substrates were followed (Estes et al. 2012 and references therein). To obtain virgin individuals for being further tested, the presence of pupae was daily checked, and upon emergence, adults were sorted by sex, and those of the same sex were pooled in cubic Bugdorm© cages (30 × 30 × 30 cm). Adults were fed on a mixture of sugar, yeast hydrolysate, and egg yolk (75:19:6) (Tsitsipis and Kontos 1983), and water was provided ad libitum, by wetting a sponge strip. Both food and water were replaced every two days. All the developmental stages were kept at 24 ± 1 ℃, 55 ± 5% (relative humidity), and a L:D photoperiod of 16:8.

Chemicals

Racemic γ-hexalactone (98%) (hereafter referred to as γ-hexalactone) was purchased from Alfa Aesar (Heysham, United Kingdom), while racemic δ-hexalactone (98%) (δ-hexalactone) was acquired from Thermo Fisher Scientific (Madrid, Spain). Commercial suppliers of racemic olean (98%) and ammonium bicarbonate (98%) were Cymit Química S.L. (Barcelona, Spain) and Barcelonesa de Drogas y Productos Químicos S.A.U. (Barcelona, Spain), respectively. n-Hexane of GC purity (SupraSolv®, Merck, Darmstadt, Germany) was used as solvent for preparing the serial dilutions to be tested in behavioral assays. Kovats retention indices (KI) were calculated using a commercial series of saturated n-alkanes (C_7–C_{40}, Merck-Sigma Aldrich, Madrid, Spain).

Headspace collection and analysis

Volatile collection from *B. oleae* adults of both sexes was performed by solid phase microextraction (SPME), with a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coated fiber (50/30 µm; Supelco, Merck-Sigma Aldrich). First, a comparison of the volatile profiles released from males and females (7–14 days old) was conducted. A total of $n = 6$ collections per sex were performed. In each volatile collection, ten virgin individuals, either males or females, were introduced in a 40 mL screwed-cap vial (Supelco, Merck Sigma Aldrich), and left exposed to the SPME fiber for 6 h deprived of any food or water source. Next, virgin males of different ages (1, 5, 14, 19 and 23 days old) were sampled under the same conditions to determine whether the release of the specific compounds followed an age-dependent pattern. Two samples per age category were performed, each containing ten males. Prior to being used for the first time, SPME fibers were conditioned by inserting them into a GC injection port at 270 ℃ for 30 min. All the collections were done from 10:00 am to 18:00 pm at room temperature.

After each collection, the fiber was immediately injected at 270 ℃ in splitless mode (5 min) into a Thermo Finnigan Trace 2000 GC system coupled to a Trace MS quadrupole mass spectrometer (Thermo Fisher Scientific). A non-polar TR-5MS column (30 m × 0.25 mm I.D. × 0.25 µm; Thermo Fisher Scientific) was used, and the following temperature program was set up: an initial temperature of 40° hold for 5 min, followed by an increase of 5 °C/min to 180 °C, and finally raised 15 °C/min to 300 °C, with a hold time of 5 min. The MS was used in the electron impact mode at 70 eV. The MS system was operated in the scan mode, from 40 to 500 amu, at 1.0 scan/s. Compound identification was achieved by comparison of mass spectra with those of synthetic standards (only in the case of γ- and δ-hexalactone) and a mass spectral library (The National Institute of Standards and Technology-NIST Mass Spectral Database), and by comparison of calculated KI values with those in literature, when available.

Behavioral activity

Walking response of virgin *B. oleae* males and females (7–14 days old) in response to γ-hexalactone (1, 10, and 100 µg) and δ-hexalactone (1 and 10 µg) was determined in a double-choice glass olfactometer (main arm 10 cm long × 18 mm I.D., arms 8 cm long × 1.8 mm I.D., angle between arms 90°) set in vertical position and suspended by a retort stand. In each trial, hexane-diluted γ-hexalactone or δ-hexalactone was confronted against n-hexane as solvent control. Testing quantities of each lactone were obtained from serial dilutions in n-hexane, and 10 µL of the corresponding dilution was loaded onto a filter paper disc (Whatman, 2.5 cm diameter, Merck-Sigma Aldrich), while 10 µL of hexane were loaded on another filter paper as solvent control. An incoming charcoal-filtered airflow at 350 ml/min was set for each arm. Filter papers were renewed every five insects, and the position of the arms were also switched to avoid any directionality. All the system was homogenously illuminated using a table lamp with white light (60 W) placed on a shelf ca. 30 cm above the olfactometer junction, providing a light intensity of approximately 500 lx. Prior to the beginning of each trial, flies were individually isolated in 15 ml Falcon tubes, and allowed to acclimate to room conditions for 1 h. A total of 40–80 flies of each sex were tested for each compound and amount, and each insect was used only once. A positive response was considered if the fly entered any arm at least 2 cm beyond the arm junction. If an insect did not make a choice after 5 min, it was considered as non-responding, and discarded for further statistical analysis.

Field tests

To test the efficacy of γ-hexalactone enhancing the attractiveness of ammonium bicarbonate under natural conditions, three field assays were carried out. In the first one, hereafter referred to as Assay 1, we assessed the attractiveness of the binary blend composed of ammonium bicarbonate (AB, 40 g) and γ-hexalactone (200 mg) in comparison to AB (40 g) alone. This assay was deployed in an olive orchard (41.45751, 1.81742; var. Arbequina) sited in Sant Llorenç d´Hortons (Catalonia, Spain), from 29th August to 28th November of 2018. In a second set of assays, namely Assays 2 and 3, the efficacy of the binary blend composed by AB (40 g) and olean (200 mg) was compared against the blend of AB (40 g) and γ-hexalactone (200 mg). The Assay 2 was conducted in the same olive orchard as Assay 1, and it started on 17th October and ended on 13th December 2019. On the other hand, the Assay 3 was conducted from 13th July to 23rd November 2021 in an olive orchard (43.81803, 4.05009; var. Picholine) located in Aspères (France). Both sampling sites were selected due to reported *B. oleae* attacks in previous years and their management by organic farming.

For all field trials, the same experimental design and methodology were followed. Cone yellow traps (FLYPACK®DACUS, SEDQ Healthy Crops, Spain) were baited with the corresponding lure, and hung up in the leaf canopy at a height of 1.4–1.8 m from ground level. Three trap blocks were deployed per assay, and each block contained one trap for each attractant tested. The dispenser for releasing olean and γ-hexalactone was a 2 mL-polyethylene capsule, which provides a constant release rate of approximately 0.80 and 0.14 mg/day respectively, while AB was released from a polyethylene-cellulose envelope, affording a release rate for ammonia of ca. 5 mg/day. All the release rates were estimated in a wind tunnel at 40 °C and an incoming airflow of 2 m/s during 169 days (Supplementary Figures S1 & S2). A minimum distance of 25 m between traps and blocks was set. Trap catches were weekly checked, and rotated clockwise within each block after fly counting and sex determination.

Statistical analysis

Walking response of males and females in the double-choice olfactometer was subjected to a Chi-square goodness-of-fit test, to test if the proportion of flies making a choice differed from a 50:50 distribution. The performance of the baits tested in each field assay was analyzed by comparing the mean catches of both sexes of *B. oleae* per week with the Mann–Whitney *U* nonparametric test. All the statistical procedures were subjected to a significance level of $\alpha = 0.05$, and performed using SPSS Statistics 17.0 software (SPSS, Chicago, IL, USA).

Results

Headspace collection

Analysis of the volatile profiles from males and females of 7–14 days old revealed the presence of γ-hexalactone and δ-hexalactone only in males, while no traces were detected in females (Fig. 1, Table 1). Apart from these two lactones, (*Z*)-9-tricosene and ethyl (*Z*)-9-octadecenoate (= ethyl oleate) were also detected, although the latter was also present in all the headspace collections from females. When comparing the volatile profiles from males of different ages, both lactones were detected in all sampling groups, i.e. from one to 23 days old (Fig. 2).

With regard to females, volatile collections included the major sexual pheromone component olean, its unsaturated form l,7-dioxaspiro[5.5]undec-4-ene, and tentatively identified hydroxy derivatives, viz. 3-hydroxy-1,7-dioxaspiro[5.5]undecane and 1,6-dioxaspiro[4.5]decan-2-methanol (Table 1). Six additional esters were also identified from females: ethyl decanoate, ethyl dodecanoate, ethyl tetradecanoate, ethyl hexadecanoate, ethyl (*Z*)-9-hexadecenoate, and the abovementioned ethyl (*Z*)-9-octadecenoate (Table 1).

Behavioral activity

Both males and females showed a positive response to γ-hexalactone at 1 µg (males, 71% of attraction, $\chi^2 = 3.857$, df = 1, $p = 0.050$; females, 69% of attraction, $\chi^2 = 4.235$, df = 1, $p = 0.040$), and 10 µg (males, 69% of attraction, $\chi^2 = 3.846$, df = 1, $p = 0.050$; females, 65% of attraction $\chi^2 = 3.930$, df = 1, $p = 0.047$) (Fig. 3), whereas the amount of 100 µg did not exhibit an attractive effect on neither of the sexes (males, $\chi^2 = 1.385$, df = 1, $p = 0.239$; females, $\chi^2 = 0.053$, df = 1, $p = 0.819$) (Fig. 3).

On the other hand, none of the sexes showed a positive response towards δ-hexalactone at neither 1 µg (males, 53% of attraction, $\chi^2 = 0.118$, df = 1, $p = 0.732$; females, 62% of attraction $\chi^2 = 1.882$, df = 1, $p = 0.170$) nor 10 µg (males, 50% of attraction, $\chi^2 = 0.0$, df = 1, $p = 1.0$; females, 54% of attraction, $\chi^2 = 0.154$, df = 1, $p = 0.695$) (Fig. 4).

Field tests

Overall, 2,468 *B. oleae* were trapped in Assay 1, with an equal sex ratio for both baits (♂:♀ in AB, 0.98:1; AB plus γ-hexalactone, 1.02:1). Traps baited with the binary blend of AB and γ-hexalactone significantly lured more individuals of both sexes per week than those traps baited

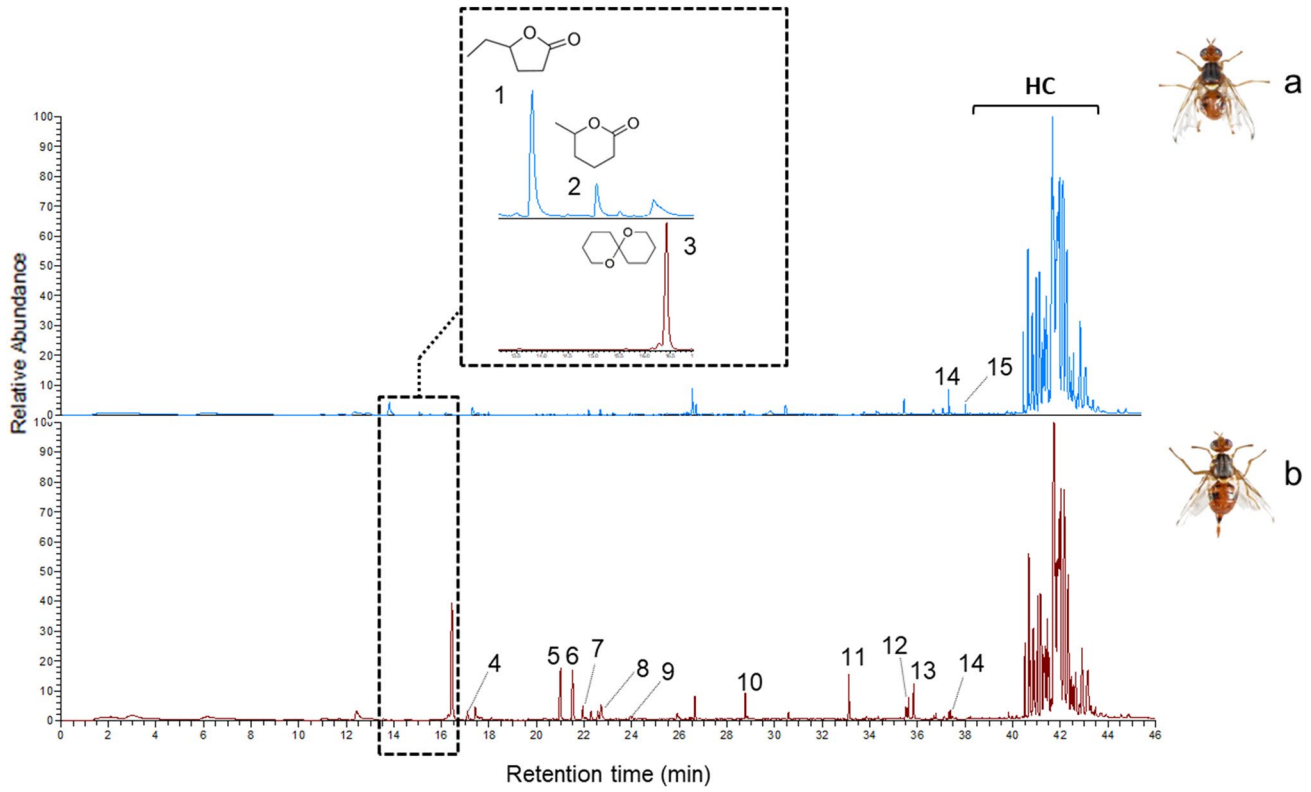

Fig. 1 Representative total ion chromatogram from SPME headspace volatiles of laboratory-reared *B. oleae* (7–14 days old) virgin males **(a)** and females **(b)**. Inset depicts the elution of male-specific compounds γ-hexalactone (1) and δ-hexalactone (2). HC = hydrocarbons. Numbers above each peak match with the numbers of the compounds in Table 1

only with AB (males, $U = -23.0$ $z = -2.043$, $p = 0.043$; females, $U = 22.5$, $z = -2.080$ $p = 0.035$) (Fig. 5).

In both Assays 2 and 3, the mean number of males and females per week of traps co-releasing AB and γ-hexalactone significantly differed from those traps baited with the blend AB and olean (Fig. 6). Specifically, in Assay 2 a total of 1,112 flies were trapped (♂:♀ in AB plus γ-hexalactone, 1.58:1; AB plus olean, 1.38:1), and the binary blend of AB and γ-hexalactone resulted to be more attractive than the combination of AB and olean (males, $U = 12.5$, $z = -2.051$, $p = 0.038$; females, $U = 11.5$, $z = -2.155$, $p = 0.028$) (Fig. 6a). The same response pattern was detected in Assay 3 (771 *B. oleae* trapped, ♂:♀ in AB plus γ-hexalactone, 1.60:1; AB plus olean, 0.75:1), being the binary blend AB plus γ-hexalactone the most attractive bait for both sexes (males, $U = 72.0$, $z = -3.176$, $p = 0.001 < 0.$; females, $U = 86.0$, $z = 2.765$, $p = 0.005$) (Fig. 6b).

Discussion

In this work we report for the first time the presence of two male-specific lactones, namely γ-hexalactone and δ-hexalactone, in *B. oleae* virgin males, with the former exhibiting a significant attraction on virgin males and females in double-choice assays, and enhancing the attractiveness of the food bait ammonium bicarbonate upon both sexes in field tests. In contrast, the role of δ-hexalactone is still unclear, since no biological activity was observed in laboratory behavioral assays. Nonetheless, further research would be needed to determine whether the insect is capable of detecting the compound (i.e. by mean of electroantennographic and electropalpographic assays), and, if so, compare the biological activity of the compound alone and together with the additional male specific-chemical cues, viz. γ-hexalactone and (*Z*)-9-tricosene, in order to determine any possible synergism.

The relevance of the lactone motif in the intraspecific chemical communication of insects (Schulz and Hötling 2015), and specifically on tephritid genera, has previously been described, with the general pattern of males being the sex involved in their production and emission. In *Rhagoletis* spp., short-chain lactones have been reported as relevant intraspecific cues. For instance, males of *Rhagoletis batava* Hering release (-)-δ-heptalactone, and it results attractive for both sexes, therefore acting as an aggregation pheromone (Būda et al. 2020). Males of the walnut husk fly *Rhagoletis completa* Cresson also release δ-hexalactone, along with

Table 1 Chemicals identified in SPME headspace collections from laboratory-reared *Bactrocera oleae* virgin males and females (7-14 days old).

ID	Compound	KI[a]	Identification[b] (Lib., KI, Ss)	Males	Females	Refs[c]
1	*γ*-hexalactone	1067	Lib, KI, Ss			1
2	*δ*-hexalactone	1107	Lib., KI, Ss			1
3	l,7-dioxaspiro[5.5]undecane (=olean)	1139	Lib., Ss			2,3,4
4	1,7-dioxaspiro[5.5]undec-4-ene[d]	1162	Lib			1
5	1,6-dioxaspiro[4.5]decan-2-methanol[d]	1304	Lib			5
6	1,6-dioxaspiro[4.5]decan-2-methanol[d]	1322	Lib			5
7	3-hydroxy-1,7-dioxaspiro[5.5]undecane[d]	1341	Lib			5
8	3-hydroxy-1,7-dioxaspiro[5.5]undecane[d]	1373	Lib			5
9	Ethyl decanoate	1400	Lib, KI			6
10	Ethyl dodecanoate	1600	Lib, KI			4,6,7
11	Ethyl tetradecanoate	1800	Lib, KI			4,6
12	Ethyl (*Z*)-9-hexadecenoate	1983	Lib, KI			4[e],8
13	Ethyl hexadecanoate	1999	Lib, KI			4,6
14	Ethyl (*Z*)-9-octadecenoate	2183	Lib, KI			6,9
15	(*Z*)-9-tricosene	2279	Lib, KI			9

[a]Calculated Kovats retention indices (KI) on a TR-5MS column (30 m × 0.25 mm I.D. × 0.25 µm)

[b]Compound identification achieved by comparison of their mass spectra with those in a mass spectral library (Lib.), literature-reported Kovats retention indices (KI), and synthetic standards (Ss).

[c]References in which the presence of the compound is detected in *B. oleae*: 1: Current work; 2: (Baker et al. 1980); 3: (Mazomenos and Haniotakis 1981); 4: (Gariboldi et al. 1983); 5: (Baker et al. 1982b); 6: (Canale et al. 2015); 7: (Mazomenos and Haniotakis 1985); 8: (Fusini et al. 2018); 9: (Carpita et al. 2012).

[d]Tentatively identified

[e]Double bond position not determined

δ-heptalactone, and this binary mixture is highly attractive in field tests, although no information of the sex-ratio of the catches was provided by the authors (Sarles et al. 2018). With regard *to Anastrepha* spp., more complex lactones are suggested to be part of the sex pheromone blend. In three

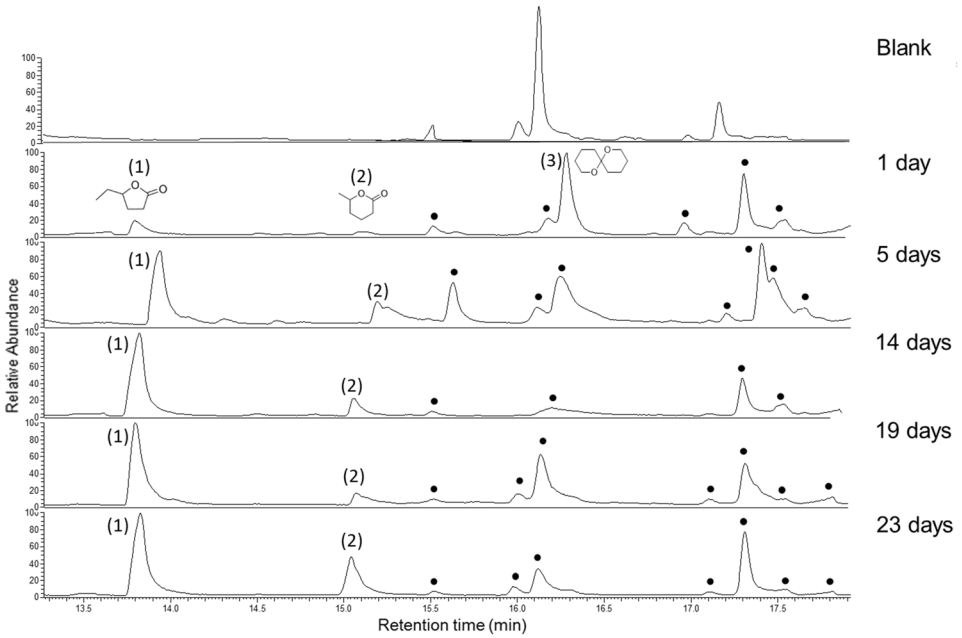

Fig. 2 Zoomed-in region of SPME headspace collections from laboratory-reared *Bactrocera oleae* virgin males of different ages (1, 5, 14, 19 and 23 days old). Eluting peaks of *γ*-hexalactone (1), *δ*-hexalactone (2), and olean (3) are shown. Those peaks highlighted with a black dot match with those in the SPME fiber blank (upper trace)

Anastrepha species, namely *Anastrepha ludens* (Loew), *Anastrepha fraterculus* (Wiedemann), and *Anastrepha suspensa* (Loew), the trivially-known lactones suspensolide, epianastrephin and anastrephin have been identified as components of the male sex pheromone (Battiste et al. 1983; Stokes et al. 1983; Chuman et al. 1988; Lima et al. 2001; Milet-Pinheiro et al. 2015).

Concerning subfamily Dacinae, in which genus *Bactrocera* is included, scant records of male-produced lactones are known (Ohinata et al. 1982; Baker et al. 1985; Ono et al. 2020). (*E*)-5-(3,6-heptadienyl)dihydro-2(3*H*)-furanone was identified from the volatile bouquet released by *Bactrocera cucurbitae* (Coquillett) males (Ohinata et al.

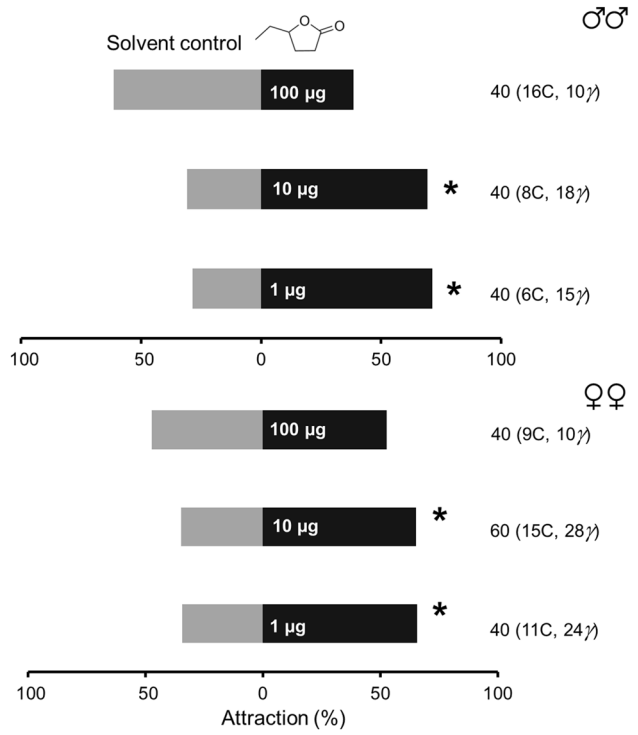

Fig. 3 Behavioral response (expressed as percentage of attraction) of laboratory-reared virgin *B. oleae* (7–14 days old) males and females to γ-hexalactone (1, 10 and 100 µg). Asterisks denote a significant preference towards γ-hexalactone (Chi-square goodness-of-fit, at $\alpha = 0.05$). The number beside each bar indicates the total number of flies tested, and the number of flies making a choice for the control arm (C) and γ-hexalactone (γ) is indicated within parentheses

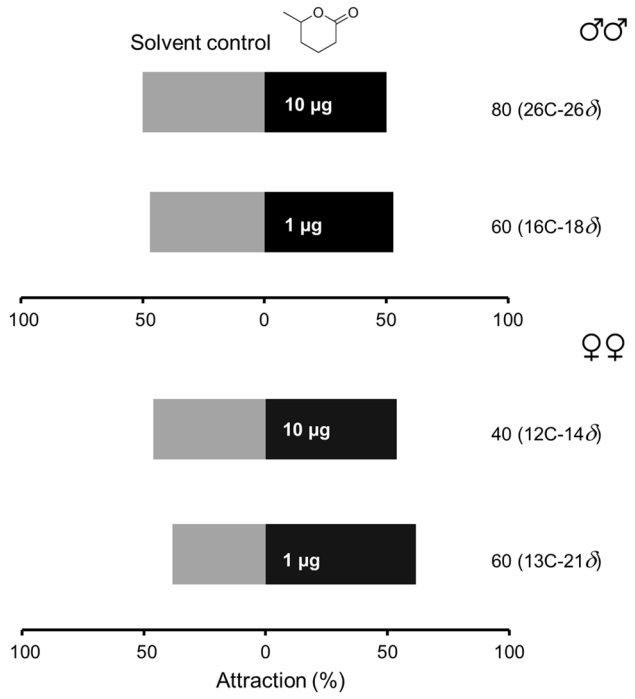

Fig. 4 Behavioral response (expressed as percentage of attraction) of laboratory-reared virgin *B. oleae* (7–14 days old) males and females to δ-hexalactone (1 and 10 µg). No significant preference was detected for neither of the olfactometer arms (Chi-square goodness-of-fit test, at $\alpha = 0.05$). The number beside each bar indicates the total number of flies tested, and the number of flies making a choice for the control arm (C) and δ-hexalactone (δ) is indicated within parentheses

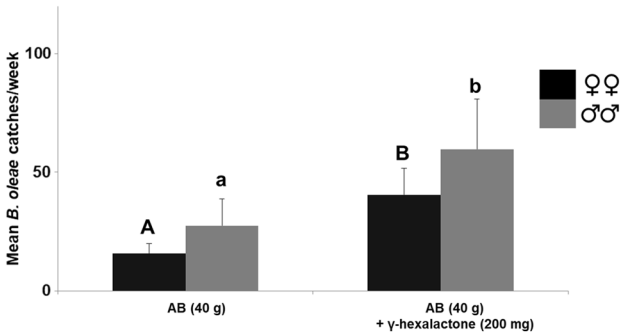

Fig. 5 Field Assay 1: mean number (+SEM) of *B. oleae* females and males trapped per week (n = 13 weeks) (29th August to 28th November 2018) in FLYPACK®DACUS traps baited with AB (ammonium bicarbonate, 40 g) and AB (40 g) plus γ-hexalactone (200 mg). Columns headed with different letters within a sex (capital letters for females, lowercase letters for males) are statistically different (Mann–Whitney *U* test, at $\alpha = 0.05$)

1982). Recently, Ono and coworkers detected 3-hydroxy-decalactone in the rectal gland of both sexes of *Bactrocera tsuneonis* (Miyake), although the amount of compound detected in mature males was significantly higher than in immature males and females (Ono et al. 2020). However, no biological activity of any of the abovementioned compounds has been determined so far. Similarly, some lactones from host plants are reported as biologically actives for true fruit flies. In *Bactrocera dorsalis* (Hendel) and *Bactrocera tryoni* (Froggatt), γ-octalactone, a mango-released compound, is recognized as a strong oviposition stimulant (Pagadala Damodaram et al. 2014; Kempraj et al. 2019). Additionally, an aggregative response in *B. tryoni* is induced by this compound (Kempraj et al. 2019). Moreover, gamma- and delta-lactones of diverse chain length (C_4-C_{12}) from host fruits elicit electroantennographic responses on *C. capitata* (Light et al. 1988). Therefore, our findings represent the first report within this subfamily of a male-produced lactone biologically active on conspecifics.

Analytical procedures on the volatile fraction of *B. oleae* allowed to detect both lactones exclusively in virgin males, and although the stereochemistry was not determined, both sexes positively responded to the racemic mixture of γ-hexalactone. In a similar vein, racemic δ-hexalactone and δ-heptalactone are effective attracting *R. completa* (Sarles et al. 2018). Nonetheless, discrepancies in the response level related to enantiomeric composition are commonly reported.

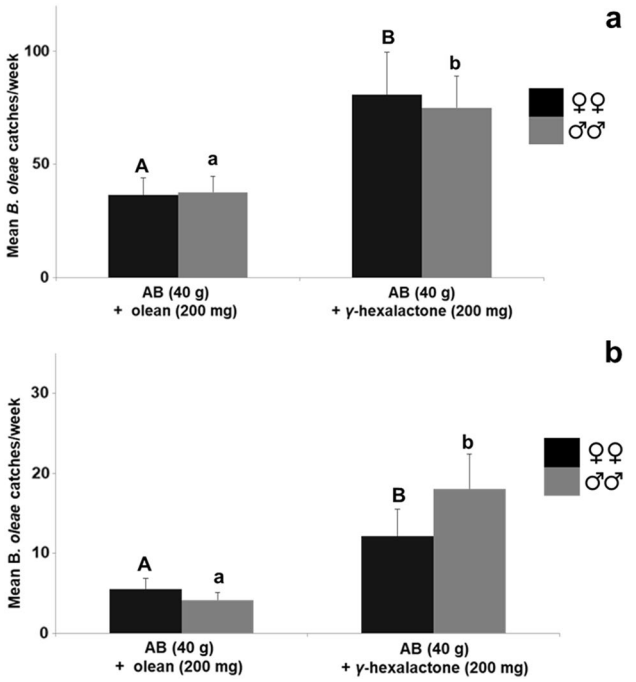

Fig. 6 Field Assays 2 & 3: mean number (+SEM) of *B. oleae* females and males trapped per week in FLYPACK®DACUS traps baited with AB (ammonium bicarbonate, 40 g) plus olean (200 mg) and AB (40 g) plus γ-hexalactone (200 mg). Columns headed with different letters within a sex (capital letters for females, lowercase letters for males) are statistically different (Mann–Whitney *U* test, at $\alpha = 0.05$); **a** Field assay 2, conducted from 17th October to 13th December 2019 (n = 8 weeks) in Sant Llorenç d´Hortons (Spain); **b** Field assay 3, conducted from 13th July to 23rd November 2021 (n = 19 weeks) in Ásperes (France)

In fact, olean is produced as racemate by both *B. oleae* sexes (Haniotakis et al. 1986; Levi-Zada et al. 2012), and while sexually mature males are attracted to the (*R*)-(-) enantiomer in laboratory and field assays, females only respond to (*S*)-(+)-olean under laboratory conditions (Haniotakis et al. 1986). Conversely, males of *R. batava* only emit (-)-δ-heptalactone, and antennae from both sexes strongly responded to it, while anosmia was reported when stimulated with the opposite enantiomer (Būda et al. 2020). In this sense, as earlier stated, none of the sexes of *B. oleae* responded to δ-hexalactone in our laboratory assays, and a possible antagonistic effect between enantiomers may be masking any biological activity. Therefore, the determination of its enantiomeric composition and further biological tests would provide valuable foundation to assess the true role of the compound.

The emission of γ-hexalactone was detectable in all the sampling ages considered (from one to up to 23 days old), which suggests that the biological relevance of the compound may prevail throughout males´ lifespan. It is worth noting that the presence of γ-hexalactone was also detected in 7- and 14-day-old feral males reared from infested olive fruits, while only traces were found in samples from feral males of less than one week old (Supplementary Figure S3). With regard to δ-hexalactone, it was clearly detectable in feral males of 14 days old, while younger males showed either traces (7 days old) or lacked the compound (< 7 days old) (Supplementary Figure S3). Thus, these findings consistently demonstrate that the volatile profile of laboratory-reared individuals is similar to that of feral males. Different factors have been demonstrated to quantitatively and qualitatively modify the pheromone production in long-established laboratory insect colonies (Raina et al. 1989; van Bergen et al. 2013; Merli et al. 2018). For instance, major and minor sex pheromone components of *C. capitata* males are altered under different larvae diets (Vaníčková et al. 2012; Merli et al. 2018). Nevertheless, our volatile collections from feral males demonstrated that both lactones are naturally produced, and a potential influence of the artificial diet should be discarded. Further research on age-related production and release of γ-hexalactone in feral males would be of great aid to gain a better understanding of its natural role.

Both laboratory and field trials confirmed that the male-specific γ-hexalactone is attractive for males and females, either when singly presented (laboratory assays) or when co-released with ammonium bicarbonate (field trials). Indeed, the combination of γ-hexalactone with the food lure increases the number of catches of both sexes compared to the performance of ammonium bicarbonate and olean. Even though the male to female ratio in ammonium bicarbonate and γ-hexalactone bait was male-biased (ca. 1.6:1, with the exception of Assay 1), the number of females trapped was significantly increased in comparison to the other testing baits. Taken into consideration that the ammonium salt and the major pheromone component are primarily attractants of females and males respectively, the observed increase in catches level should be attributable to an attractive effect of γ-hexalactone upon both sexes. This is partially in agreement with previous observations, which addressed the role of males exerting attraction on females. Evidence of male-mediated attraction on females dates back from the decade of 1970s, when a particular odor emanating from males was reported when the mating activity of the insect peaks (Economopoulos et al. 1971). Later, it was described that "an oily substance from the glandular epithelium" resulted attractive to females in the laboratory (De Marzo et al. 1978), albeit the conclusions of this study generated some skepticism, due to some methodological weaknesses that may have led to artificial results (Mavraganis et al. 2010). Further evidence was provided by Mavraganis and coworkers, who tested the activity of male body extracts, which resulted to be attractive for mature virgin females in test cages (Mavraganis et al. 2010). In the last years, (*Z*)-9-tricosene has been isolated from the rectal gland of mature

males, and it attracts virgin females in olfactometer tests (Carpita et al. 2012). As in many other tephritid species (Baker et al. 1982a; Perkins et al. 1990; Zhang et al. 2019; Noushini et al. 2019, 2021a, b), the rectal gland is regarded as the biologically-active compounds secreting organ in *B. oleae* (Gariboldi et al. 1983; Carpita et al. 2012; Canale et al. 2013b, 2015). In addition, male urotergal gland in the olive fruit fly males has been reported to be attractive for both sexes, with females attracted to urotergal glands from old males (15 days old), and males attracted to urotergal glands from young males (5 days old) (Canale et al. 2013b). However, it is remarkable that these previous works have not found any of the lactones reported by us when analyzing the content of male glands (Carpita et al. 2012; Canale et al. 2013b) and thus, the characterization of the production and release site of both lactones deserves further attention.

Interestingly, in our case γ-hexalactone was not only attractive for females, but unexpectedly also for males. We suggest that this male-male interaction mediated by γ-hexalactone might be related to lek formation. It is well known that *B. oleae* males form leks at dusk, and within these swarms they compete for female attraction, and courtship takes place. Similar lekking behavior has been reported for most tephritid species (Iwahashi and Majima 1986; Dodson 1986; Whittier et al. 1992; Segura et al. 2009). Cues involved in lekking are not fully understood in fruit flies, although intraspecific and host chemical cues, visual and acoustic signals are suggested to be relevant (reviewed by Benelli et al. 2014). In this sense, it would be plausible that γ-hexalactone, along with other undetermined signals, may contribute to lek formation by recruiting males at first instance, and afterwards, female conspecifics may be enticed towards swarming males by the action of additional cues, with a possible role of γ-hexalactone. Nevertheless, in the light of our results it is too premature to draw such conclusions, and hence in-depth research would be needed to test whether γ-hexalactone is attractive by itself under field conditions, along with determining its possible role as a potential lek formation elicitor.

In conclusion, our findings demonstrate that the combination of ammonium bicarbonate and γ-hexalactone is highly attractive for both sexes of *B. oleae*, and this bait even improves the performance of ammonium bicarbonate and olean. Therefore, the use of γ-hexalactone should be taken into consideration for replacing olean as a complement of this kind of food lure-based baits. Remaining challenges, such as determining the stereochemistry or γ-hexalactone, the role of δ-hexalactone as a potential synergist, and the optimum release rate (Navarro-Llopis et al. 2011), may contribute to gather pivotal information for the development of novel trapping strategies against *B. oleae* populations. Furthermore, the identification of reported male-specific lactones represents a novel step towards deciphering the chemically-mediated intraspecific interactions of *B. oleae*, supporting the idea that the sexual communication of the species is not governed only by the chemical cues released from females. Future work will be aimed to address the putative role of γ-hexalactone as an aggregative cue.

Author contributions

SL, PA and CQ conceived the study. SL and CCM reared the insect colony. SL conducted the volatile collections, behavioral tests, and data analysis. PA and AGZ designed and coordinated the field assays. SL wrote the initial draft. CQ and PA led the project administration. All the authors contributed to the edition, revision and approval of the final version of the manuscript. All authors contributed to the conception, design, and research of this study. The first draft of the manuscript was written by SL and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability Additional data will be provided by the authors upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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