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SHORT COMMUNICATION

(*Z*)-9-tricosene identified in rectal gland extracts of *Bactrocera oleae* males: first evidence of a male-produced female attractant in olive fruit fly

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Abstract It is well-known that *Bactrocera oleae* (olive fruit fly) females attract conspecific males by using 1,7-dioxaspiro[5,5]undecane (**1**) as the main component of their sex pheromone, and that **1** is produced in the female rectal gland. Although some authors have claimed that *B. oleae* males also attract females, to date no male-produced female attractants have been found in this species. In this paper, we report the first identification of a substance unique to males and able to attract females. The findings of the study include the following: (1) females responded in a bioassay to hexane extracts obtained from rectal glands of 15-day-old *B. oleae* males, (2) the presence of (*Z*)-9-tricosene (**2**) was consistently and unambiguously identified in these extracts using gas chromatography (GC) and GC-mass spectrometry methods, (3) in preliminary bioactivity tests, low doses (equivalent to a few males) of chemically and stereoisomerically pure synthetic (*Z*)-9-tricosene (**2**) attracted olive fruit fly females. Interestingly, compound **2**, commonly called *muscalure*, is also a well-known component of the house fly (*Musca domestica*) sex pheromone.

Keywords *Bactrocera oleae* · Male rectal glands · (*Z*)-9-tricosene · Behavioural bioassay · Semiochemicals · Chemical ionization mass spectrometry

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Introduction

Bactrocera oleae (Rossi) (Diptera: Tephritidae), the olive fruit fly, is a pest of great economic importance (Daane and Johnson 2010), and a better understanding of its intraspecific communication could help to enhance its control.

It is known (for key references see: Fletcher and Kitching 1995; Wicker-Thomas 2007) that in *B. oleae*: (1) females attract males, in contrast with the majority of Tephritidae; (2) the main component of the female sex pheromone is racemic 1,7-dioxaspiro[5,5]undecane (**1**), produced in the rectal gland; (3) males also produce racemic **1** in their rectal gland where it serves to aggregate conspecific males, but it does not attract females; (4) as we recently demonstrated (Canale *et al*. 2010), the male production of **1** reaches a maximum when gonad maturation is complete (5–8 days old), and then decreases to 0 by the 11th day of life: after this age, no detectable amounts of **1** have been recorded; (5) to date no male-produced female attractants have been found in this species.

While Mazomenos and Pomonis (1983) specifically claimed that male rectal gland extracts did not affect the behaviour of virgin females, De Marzo *et al*. (1978) reported that *B. oleae* males attract females pointing to a chemical

attractant. Their evidence is questionable; however, more recently Mavraganis *et al*. (2010) have proven that virgin females have been attracted by extracts of male bodies. There are no other published data on the attraction of females to males. In conclusion, although males probably attract females, there is no experimental evidence clearly demonstrating this.

Given the contradictions in these results and the presence of a known pheromone in the female rectal gland, our initial aim was to search for male-produced attractants by revisiting the contents of the male rectal gland. Here, we report the first result of this research on male rectal gland extracts: the identification of a female attractant produced by *B. oleae* males.

Materials and methods

Insect rearing

Insects used in this study were obtained from field-collected pupae in October. Twenty-four hours after emergence, flies were separated according to sex and placed in different cages; to obtain mated insects, virgin couples were isolated and observed until mating occurred.

Gland extraction

Typically, 15 days after eclosion 10 virgin males (or females) were maintained at −18°C for 5–10 min, then their rectal ampullae were dissected out and immediately immersed in hexane (140 μl) for 2 h. Three replicates were performed. Extracts of virgin males or females of other ages (1, 3, 6, 9, 11, 13, 20 days old) were similarly prepared.

Chemical analyses, identifications and synthesis

Gas chromatography (GC) analyses were performed on a Dani GC 1000 with PTV injector, FID detector and two bonded FSOT columns (Alltech, 30 m×0.25 mm i.d., 0.25 μm): AT-5 (column 1) and AT-35 (column 2). Gas chromatography–mass spectrometry (GC/MS) analyses were performed with an Agilent apparatus: mass selective detector 5973 Network, 6890 N Network GC system and HP-5MS bonded column (30 m×0.25 mm i.d., 0.25 μm, column 3). Chemical ionization mass spectrometry (CI-MS) experiments were performed by a Varian apparatus (a Saturn 2000 coupled to a GC 3800) equipped with an Rxi-5MS column (Restek, 30 m×0.25 mm i.d., 0.25 μm). CI reagent was acetonitrile (ACN). Both GC and GC/MS analyses of extracts were carried out under splitless conditions, injecting 3 μL of hexane extract.

The compound in *B. oleae* male extracts was identified by comparing its mass spectra and retention times to those of commercial or synthesized (*Z*)-9-tricosene (**2**), as well as by CI-MS experiments (to determine the double bond position in the unknown male extract component by using the method developed by Moneti *et al*. 1997).

Chemical and stereoisomeric purities of commercial **2** (Sigma-Aldrich) were 95% and 93%, respectively, as shown by GC and ^{1}H or ^{13}C nuclear magnetic resonance analyses. Instead, the (*Z*)-9-tricosene (**2**) synthesized in our laboratory following exactly the procedure reported by Fisher and Tyman (1998) was 99% chemically and stereoisomerically pure. Quantifications were performed by GC, using absolute calibration curves obtained with pure **2**. The same pure compound was also used in bioassays.

Behavioural bioassays

Attractiveness of rectal extracts or synthetic **2** was evaluated in a two-choice bioassay using a plexiglas unit (15×15×1.5 cm) as a still-air arena. In the centre of this unit, there was a circular chamber (4 cm ∅, the specimen release chamber), connected to two other identical chambers by means of two linear paths (2 cm in length, 1 cm in width), forming a 90° angle. The top of the arena was covered by means of a removable glass panel. A subject was placed in the centre of the release chamber. It was judged to have chosen a given cue if it moved to the cue within 3 min after being released and if it engaged in searching behaviours on the chosen cue for at least 30 sec (i.e. walking inside the chosen chamber followed by arrestment).

Two kinds of bioassays were carried out. (1) Attractiveness of rectal gland extracts of 15-day-old virgin *B. oleae* males or females to coeval *B. oleae* virgin females: extracts of 10 rectal glands in 140 μl of hexane were prepared as described above, then 60 μl of these extracts (corresponding to 4.3 glands) were adsorbed on a filter paper (1.5×1.5 cm) and were placed in one of the two side chamber of the arena; an equal filter paper with 60 μl of hexane (control) was placed in the other side chamber and a single specimen of *B. oleae* was placed in the release chamber. (2) Attractiveness of (*Z*)-9-tricosene (**2**) to *B. oleae* females or males: 99% chemically and stereoisomerically pure **2** was diluted in hexane (124 ng/μl), then 0.5, 1.0, 5.0 or 12.5 μl of this solution (corresponding to 62, 124, 620 or 1,550 ng of **2**, respectively) were tested vs. control (corresponding volumes of pure hexane) in bioassays performed with 10-day-old *B. oleae* (a) virgin males, (b) virgin females, (c) mated males, (d) mated females. Given that we have estimated that the rectal glands of 15-day-old males contain about 42 ng/gland of **2** (standard deviation, ±2.26), the amounts of **2** used for the bioassays correspond to ca. 1.5, 3, 10 or 37 15-day-old male equivalents, respectively.

Statistical analysis

A chi-square test (with Yates's correction) was used to evaluate behavioural data from two-choice bioassays and a probability of 0.05 was considered significant (Sokal and Rohlf 1981). See the Electronic Supplementary Material (ESM) for further experimental details.

Results and discussion

A simple attractiveness test was carried out on rectal gland extracts of 15-day-old *B. oleae* males, chosen because at this age they no longer produce **1**, which might complicate the bioassay interpretation. As shown in Fig. 1a, 15-day-old females were significantly attracted by 60 μL of these male extracts, corresponding to ca. four males (χ^2=24.02, d.f. = 1, P<0.005). However, females did not respond to the same dose of 15-day-old female rectal gland extracts (Fig. 1b).

Subsequently, GC analyses of the bioactive 15-day-old male extracts showed the presence of several unknown compounds (see Fig. S1 in the ESM). Some of these were not found in 15-day-old female extracts and could potentially be part of an attractant blend; however, we focused on one of them because (1) it is the most volatile, (2) in general it was less variable than the other compounds, (3) its presence was clearly age dependent, (4) it increased significantly when males were sexually mature (8-day-old, see Fig. S2 in the ESM) and (5) it is not present in hexane extracts of male cuticle portions. This compound was unambiguously identified as (*Z*)-9-tricosene (**2**). Its electron ionization (EI) mass spectrum was almost identical to that of authentic **2**, and its retention times on three different columns (columns 1–3) were superimposable to that of authentic **2** but different to that of (*E*)-9-tricosene (which is contained in commercial **2**, see "Materials and methods" and Fig. 2a). An ACN-CI-MS was used to unequivocally establish that the position of the double bond in the hydrocarbon chain of the unknown compound was between C9 and C10. A comparison of the retention times (column 3) of the unknown compound, commercial **2** and (*E*)-9-tricosene is shown in Fig. 2a, while in Fig. 2b, the EI mass spectra of the unknown compound and authentic **2** are compared. Figure 2c reports the ACN-CI mass spectrum of the unknown compound, which exhibits two adduct ions that allow the assignment of the double

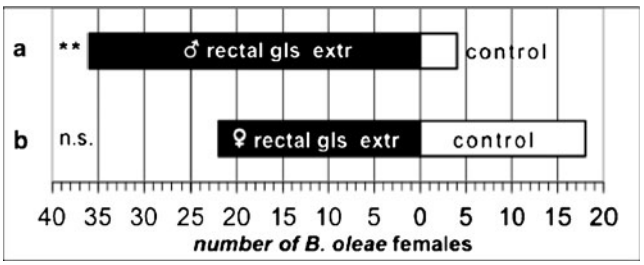

Fig. 1 Attractiveness to 15-day-old virgin females of **a** male rectal gland extract, **b** female rectal gland extract (extracts prepared with 10 rectal glands of 15-day-old virgin *B. oleae* specimens in 140 μl of hexane); both tests carried out with 60 μl of extract (equivalent to 4.3 glands); control = hexane; *asterisks* denote significantly different at 0.01% probability level, *n.s.* not significantly different (chi-square test, Yates's correction)

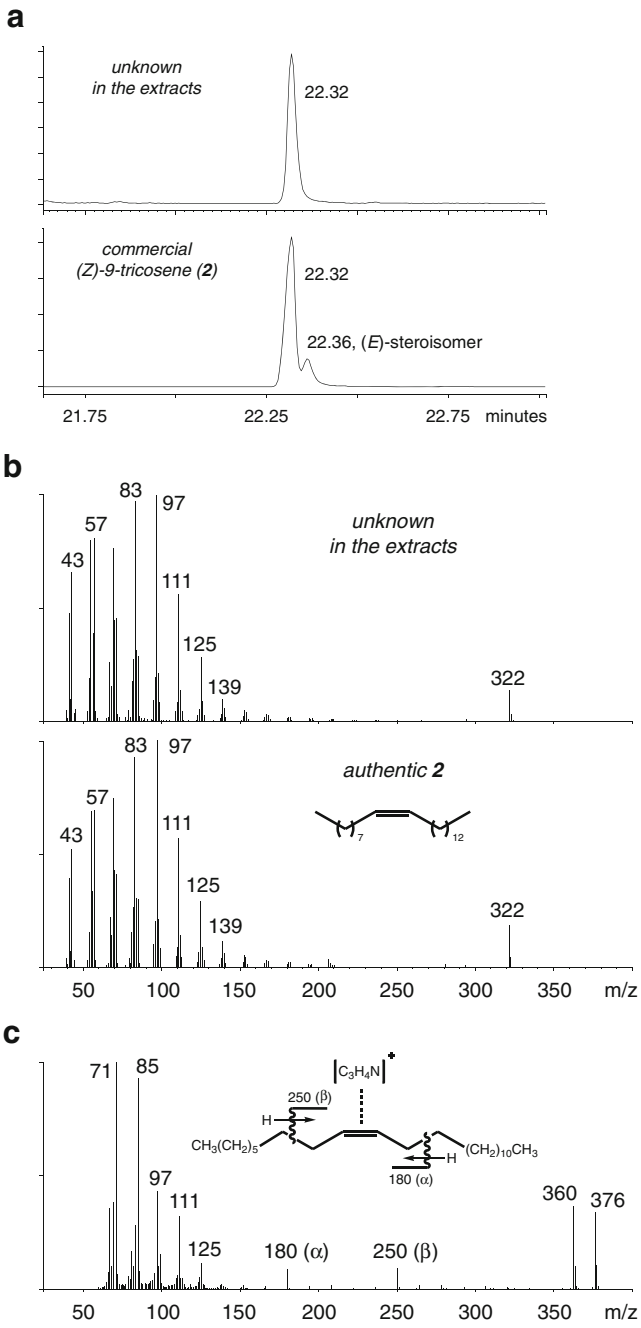

Fig. 2 Identification of unknown compound in *B. oleae* male rectal gland extracts by **a** comparison of its GC retention time (on column 3) to that of commercial (*Z*)-9-tricosene (**2**), **b** comparison of its EI mass spectrum to that of pure **2**, **c** CI mass spectrum

bond position: *m/z* 250 ($[C_3H_4N–CH–(CH)_{12}–CH_3]^+$) and 180 ($[C_3H_4N–CH–(CH)_7–CH_3]^+$).

Therefore, the new compound found in the rectal gland extracts of *B. oleae* males was unequivocally identified as (*Z*)-9-tricosene (**2**). Significant but highly variable amounts of **2** (very small in sexually immature males) were also found in the rectal gland of *B. oleae* males of other ages (see Fig. S2 in the ESM). In contrast, compound **2** was never detected in the rectal gland extracts of *B. oleae* females. Quantitative GC analyses enabled us to estimate that 15-day-old males contained 42 ng of **2** per gland (standard deviation, ±2.26).

Finally, in order to investigate the biological role of (*Z*)-9-tricosene (**2**) in *B. oleae*, preliminary laboratory tests on the attractiveness of compound **2** for *B. oleae* males or females were carried out using the 99% chemically and stereoisomerically pure **2** which we synthesized. As shown in Fig. 3, doses of **2** equivalent to ca. 1.5 or 3 15-day-old males were significantly more active than the controls for *B. oleae* females, both virgin [χ^2=5.625, d.f. = 1, P<0.025 vs. 1.5 me dose (1 me = one 15-day-old male equivalent, ca. 42 ng of **2**); χ^2=9.025, d.f. = 1, P<0.005 vs. 3 me dose)] (Fig. 3a) and mated (χ^2=5.625, d.f. = 1, P<0.025 vs. 1.5 me dose; χ^2=7.225, d.f. = 1, P<0.01 vs. 3 me dose) (Fig. 3b). Interestingly, larger amounts of **2** were no more attractive than the hexane control (Fig. 3a, b). None of the tested doses of **2** attracted *B. oleae* males (virgin, Fig. 3c, or mated, Fig. 3d).

Therefore, (Z)-9-tricosene (**2**) found in *B. oleae* males is sex specific and attracts *B. oleae* females. Mazomenos and Pomonis (1983), in the only previous analytical study on *B. oleae* male rectal gland extracts, did not report the presence of **2** and claimed that these extracts do not attract females. This is probably because: (a) they missed the higher-boiling compound **2**, due to the use of the old, non-bonded and thermally unstable, stainless-steel capillary columns for their GC analyses; (b) their bioassays were influenced by the presence, in the extracts, of compound **1**; or (c) they extracted rectal glands from very young males (containing small amounts of **2**).

The presence, in male rectal gland extracts, of other compounds not yet identified suggests that the chemical communication of the *B. oleae* male may consist of several substances acting in combination. On the other hand, in male rectal glands, we did not find any compounds unique to males and more volatile than **2** (see Fig. S1 in the ESM); however, we cannot rule out that they are produced in other parts of the male body. Further studies on *B. oleae* males and females are ongoing to obtain more supportive evidence of the biological role of **2** (for example by using field trapping experiments or other bioassays), as well as to search for other bioactive compounds and verify that males attract females in nature.

It is worth mentioning that compound **2** is also known as *muscalure* because it is a well-known component of the female house fly (*Musca domestica* L.) sex pheromone

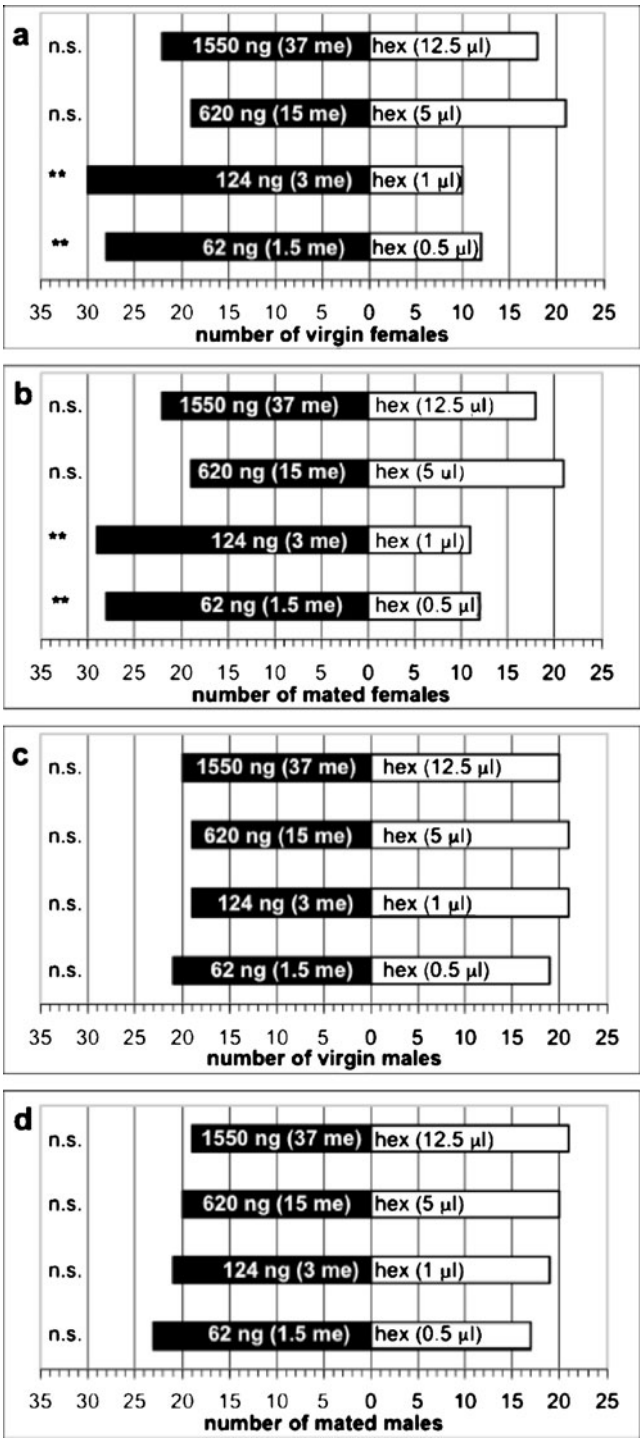

Fig. 3 Attractiveness of pure (*Z*)-9-tricosene (**2**) to *B. oleae* **a** virgin females, **b** mated females, **c** virgin males, **d** mated males; **2** was used as hexane solution (124 ng/µl); 1 me = one 15-day-old male equivalent (ca. 42 ng of **2**); *hex* hexane (control); all tested specimens were 10 days old; *double asterisk* denotes significantly different at 0.01% probability level, *n.s.* not significantly different (chi-square test, Yates's correction)

(Carlson *et al*. 1971; Rogoff *et al*. 1973; for recent literature see, for example, Butler and Mullens 2010 and references therein). Several other insect species use compound **2** in their chemical communication (see for example El-Sayed 2011).

In conclusion, we have demonstrated that rectal gland extracts of *B. oleae* males attract females and that this attractiveness can be attributed, at least in part, to the (*Z*)-9-tricosene (**2**) that we found in these glands. This is the first evidence of a male-produced female attractant in olive fruit fly.

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