Research Article

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Short-chain alkanes synergise responses of moth pests to their sex pheromones

Alexandre Gurba* **and Patrick M Guerin**

Abstract

BACKGROUND: The use of sex pheromones for mating disruption of moth pests of crops is increasing worldwide. Efforts are under way to augment the efficiency and reliability of this control method by adding molecules derived from host plants to the sex attractants in dispensers.

RESULTS: We show how attraction of the European grapevine moth, *Lobesia botrana* Den. & Schiff., and the codling moth, *Cydia pomonella* L., males to underdosed levels of their sex pheromones is increased by adding heptane or octane over a range of release rates. Pheromone–alkane mixtures enhance male recruitment by up to 30%, reaching levels induced by calling females, and shorten the flight time to the sex attractant by a factor of 2.

CONCLUSION: The findings show the promise of using short-chain alkanes as pheromone synergists for mating disruption of insect pests of food crops. Alkane–pheromone combinations are expected to increase the competitiveness of dispensers with females, and to reduce the amount of pheromone needed for the control of these pests.

Keywords: *Lobesia botrana*; *Cydia pomonella*; heptane; octane; pheromone synergist

1 INTRODUCTION

Most animals, including insects, rely on chemical signals to find mates and food. Since the discovery of sex pheromones in moths, efforts to control such pests using their own communication chemicals has led to the development of the mating disruption method, which does not require the use of insecticides.[1,2] The codling moth, *Cydia pomonella* L., and the European grapevine moth, *Lobesia botrana* Den. & Schiff., are two major worldwide fruit pests in orchards and vineyards. The identification of their key sex pheromone components, (*E*,*E*)-8,10-dodecadien-1-ol[3] (or codlemone) in *C. pomonella* and (*E*,*Z*)-7,9-dodecadien-1-yl acetate[4] in *L. botrana*, has permitted the development of pheromone dispensers for mating disruption. The increasing extent to which fruit and other crops are being treated with sex pheromones to control these and other moth pests underlines the success of this control method.[5]

In mating disruption, dispensers releasing pheromones act as point source attractants that outcompete females.[6] The efficiency of the method relies on the propensity of each dispenser to recruit males during their daily short flying period. Products that increase the range of action of a dispenser are therefore at a premium. The addition of secondary sex pheromone components from female sex glands to the principal pheromone component, for example, has already been shown to increase dispenser efficacy.[7]

Efficient mating disruption is in demand because the codling moth has become resistant to most conventional insecticides, especially in areas under intensive fruit production,[8] and the European grapevine moth, which has recently invaded the Americas,[9] is already showing resistance to different products.[10] Protection of high-value-added crops such as apples, pears and grapes can also be achieved by using a sex pheromone released as an aerosol in ethanol from puffers at a precise time each day.[11]

Short-chain C_2–C_{10} alkanes occur in the airspace around fruit crops colonised by both *L. botrana* and *C.* pomonella[12] as byproducts of lipid peroxidation on the waxy surface of fruits and leaves.[13] We hypothesised that these plant products could serve to enhance the attraction of males to sex pheromones, as has been demonstrated for other host plant products.[14–18] For example, pear ester, ethyl (2*E*,4*Z*)-2,4-decadienoate, has a synergistic effect on the attractiveness of codlemone to male *C.* pomonella,[19] and a mixture of the two products is used for field trapping and mating disruption.[20–22] We set out to test the effect of selected short-chain alkanes on male *L. botrana* and *C. pomonella* responses to their sex pheromones, and to show how these products serve as pheromone synergists, opening up the possibility of their use as carriers to generate pheromone aerosols with a wider active space in crops.

2 EXPERIMENTAL METHODS

2.1 Insects

L. botrana pupae were taken from a laboratory colony maintained in a climate chamber at 65% RH and 25 °C in the photophase (16 h) and at 85% RH and 18 °C in the scotophase (8 h). The larval stages were reared on a semi-artificial medium.[23] Pupae

* *Correspondence to: Alexandre Gurba, Institute of Biology, University of Neuchâtel, 2000 Neuchâtel, Switzerland. E-mail: alexandre.gurba@unine.ch*

Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland

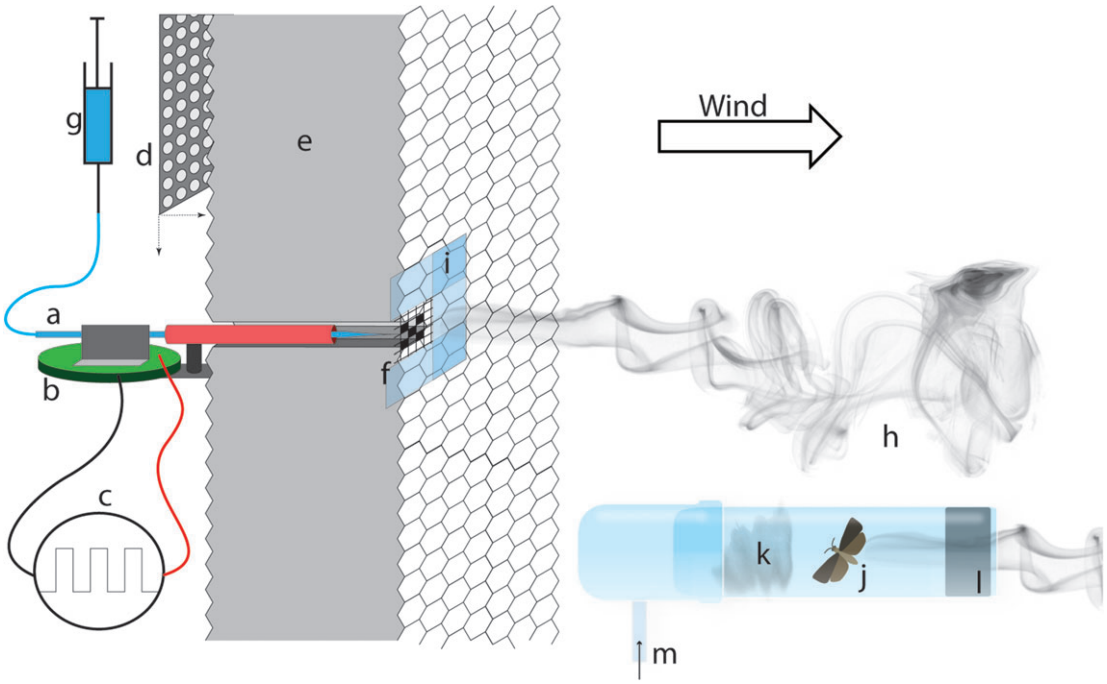

Figure 1. Chemical treatment release in the wind tunnel and laminar air flow system. The piezonebuliser included a glass capillary (a) mounted on a ceramic chip (b) that vibrated when voltage was applied to both faces using a frequency generator (c). A metal cylinder (red) protected the capillary. The nebuliser was mounted on a perforated grid (d) screwed to a perforated honeycomb structure (e), which rendered the air flow laminar. The downwind surface of the honeycomb structure was covered with mosquito netting (not shown), except for the aperture containing the capillary tip where a metal grid sat to protect the tip from the moths (f). A syringe pump (g) pushed the test solution via a Teflon tube connected to the glass capillary with two layers of heat-shrink tubing. Oscillations of the capillary tip served to nebulise the test solution within the wind tunnel (h). A frame of sticky tape (i) placed around the metal grid generated turbulence locally to increase the plume diameter to 10 cm at ca 8 cm from the source. To test the attraction of males to calling females, the females were enclosed in a glass tube (j) with cotton wool on the upwind side (k) and with a honeycomb structure on the downwind side (l). The tube was connected to a charcoal-filtered air supply (m).

of *C. pomonella* were obtained from Andermatt Biocontrol AG (Grossdietwil, Switzerland). Pupae of both species were sexed and placed on a cloth mesh over a dish filled with water for emergence. Males emerged daily into cages (BugDorm, 30 × 30 × 30 cm; MegaView Science Education Services Co., Taiwan). A 10% sucrose solution was provided to males in a glass vial stoppered with a cotton wool wick suspended from the roof of the cage. Female moths were not tested.

2.2 Electroantennogram recordings

The sensitivity of the antennal olfactory receptor cells of *L. botrana* males to hexane, heptane, octane, nonane, decane, dodecane and tetradecane was compared by electroantennogram (EAG) recordings. For this, an excised grapevine moth male antenna with the distal tip cut off was held between two glass electrodes filled with 0.1 M KCl in a humidified charcoal-filtered air stream (90–100% RH, 23 ± 2 °C) flowing at 1 m s^{-1} via a glass water-jacketed tube (7 mm i.d.). The tube outlet was about 0.8 cm from the antennal preparation. The EAG recording method was as described in the literature.[24] The responsiveness of the antenna was tested with a 1 s air puff from a 5 mL stimulus syringe containing 10 µg (*Z*)-3-hexenol on a filter paper strip, which served also as the reference stimulus.[25] The EAG responses of antennae to the alkanes were tested by placing 0.1, 0.5 and 1 µL of an alkane on a filter paper strip already enclosed in a transparent 5 mL stimulus syringe (Plastipak Luer; Becton Dickinson S.A., Madrid, Spain). Stimulation was made when the alkane drop had evaporated from the filter paper. Responses were normalised with respect to 10 µg of (*Z*)-3-hexen-1-ol.

2.3 Wind tunnel behavioural assay

The wind tunnel (flight section 60:60:195 cm) was made of non-reflecting glass. Air was blown by a centrifugal fan (Fischbach GmbH, Neunkirchen, Germany) through charcoal cartridges at a flow rate of 30 cm s^{-1}. The resulting air was sucked by another fan and cleaned by an additional set of charcoal filters placed at the downwind end of the wind tunnel. Overhead illumination was provided by high-frequency fluorescent tubes (36 W, >1 kHz; Philips, Zurich, Switzerland) running the length of the tunnel. Light was dispersed using a Perspex Prisma® crystal-clear plastic sheet placed under the fluorescent tubes, and intensity was regulated with a potentiometer. A sheet of brown paper was added to the wind tunnel roof to produce illumination of ca 6 lux along the floor. Below the tunnel floor, black shapes of irregular form were placed on a white sheet as optomotor cues. The wind tunnel was housed in a walk-in climate chamber (Schaller Uto AG, Bern, Switzerland), which allowed the air stream to be maintained at 18 ± 0.2 °C and 85 ± 2% RH during experiments. Male moths (3–4 days old) were placed individually in glass tubes (125 mm long, 24.2 mm o.d., 21 mm i.d.) at the beginning of the scotophase within the wind tunnel chamber, 15 min prior to experiments. A male moth was presented on a stand (25 cm high) placed in the centre of the wind tunnel and 25 cm from its downwind end. Moth behaviours recorded were (1) no activation, (2) activation, (3) take-off, (4) casting, (5) lock-on, (6) passing the midline of the wind tunnel, (7) close in within 10 cm of the source and (8) contacting the source, as well as the time to each behavioural step, by means of the OBSERVER software package (v.5.0; Noldus Information Technology, Wageningen, The Netherlands). Behavioural responses of

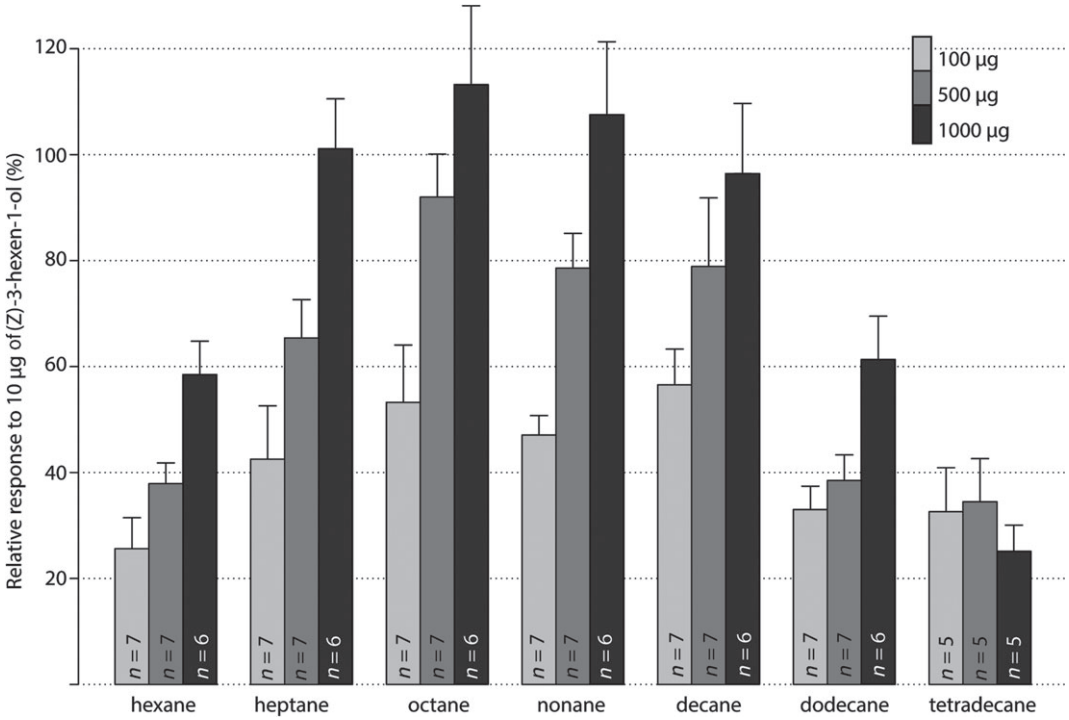

Figure 2. Electroantennogram responses of *L. botrana* males to alkanes. Mean ± SEM of the antennal responses at 100, 500 and 1000 µg source doses of alkane. Responses are normalised with respect to a source dose of 10 g of (*Z*)-3-hexen-1-ol as reference (100%); *n* is the number of antennae tested.

males were recorded for 2 min. Recording was terminated when the male was not activated within 1 min or when it landed on a wall of the wind tunnel for a minimum of 5 s. Moths that were not able to fly properly were discarded (less than 3% of the tested insects).

2.4 Odour release and laminar air flow

All chemical treatments were released using a piezonebuliser modified from El-Sayed *et al.*[26] (Fig. 1). The ultrasound nebuliser was connected to a syringe pump (Model KDS-200-CE; kdScientific, Holliston, MA), which pushed the test solution at a rate of 10 µL min^{-1} from a 5 mL gas-tight syringe (Hamilton type 81527; Milian SA, Meyrin, Switzerland) into PTFE microtubing (1.5 m long, 1.02 mm o.d., 0.56 mm i.d., Hamilton type 90674) connected via a PTFE microtubing connector (2 cm long, 1.57 mm o.d., 0.97 mm i.d., Hamilton type 20919) to a borosilicate glass capillary (100 mm long, 1 mm o.d., type GC100-10; Clark Electromedical Instruments, Pangbourne, UK) with a drawn-out tip (10–20 mm tip length, 30–40 µm i.d. tip opening). The PTFE connector was sealed in two layers of heat-shrink tubing. A frequency generator (Wavetek FG- 5000A; Willtek Communications GmbH, Ismaning, Germany) producing a square-wave signal of ca 92 kHz at an amplitude of 40 V was connected to a piezoceramic disc (25 mm diameter, Philips PXE5 25/2.0), which held the glass capillary mounted on one face. This caused the capillary tip to oscillate and produce an aerosol of the test solution. The oscillating glass capillary was installed in an aperture at the centre of the upwind aluminium honeycomb structure. The capillary tip was protected from the approaching moths by a small metal grid (see below), which was cleaned after exposure to each treatment (Fig. 1).

The attraction of males to calling *L. botrana* females was tested by placing four virgin females in a 110 mm long, 26 mm i.d. glass tube closed with an aluminium honeycomb (12 mm thick, 5 mm cell diameter) on its downwind end and with cotton wool (30 mm thick) at its upwind end. This tube was placed at 30 cm from the floor and 10 cm downwind from the aperture for the glass capillary in the honeycomb. It was connected to an external charcoal-filtered air supply flowing at 6 L min^{-1} through a 6 mm i.d. Teflon® tube providing a flow of 30 cm s^{-1} over the females in the glass tube.

The laminar air flow in the wind tunnel was generated using an aluminium honeycomb structure (9.525 mm cell diameter, 5.7 cm thick × 60 cm × 60 cm) covered with a perforated steel screen (1 mm thick, 3 mm round holes, 51% of air passage; Schäfer, Neunkirchen, Germany) on its upwind surface and with white mosquito netting (1 mm mesh) on its downwind side to prevent moths from flying through the honeycomb structure. A 1 cm diameter aperture was perforated at the centre of the structure to accommodate the glass capillary tip. A metal grid (15 × 15 mm, mesh 2 mm) was placed on the plume exit from the aperture. It has been demonstrated that a large turbulent plume induced more source contacts by moths than a continuous ribbon plume.[27] To increase the plume size, a frame of transparent sticky tape (19 mm wide) bordered the metallic grid. This generated turbulence locally around the plume source to increase its diameter to ca 8 cm at 10 cm from the release point (Fig. 1). The resulting plume reached the downwind end of the wind tunnel at its centre with a diameter of ca 15 cm.

2.5 Chemicals

The *L. botrana* sex pheromone components used were (*E*,*Z*)-7,9-dodecadienyl acetate (*E*7*Z*9–12:Ac, >97% pure), (*E*,*Z*)-7,9-dodecadien-1-ol (*E*7*Z*9–12:OH, >94%) and (*Z*)-9-dodecenyl acetate (*Z*9–12:Ac, 99.9%) from Plant Research International (Wageningen, The Netherlands). The *C. pomonella* sex pheromone used was (*E*,*E*)-8,10-dodecadien-1-ol (*E*8*E*10–12:OH, >99.8%)

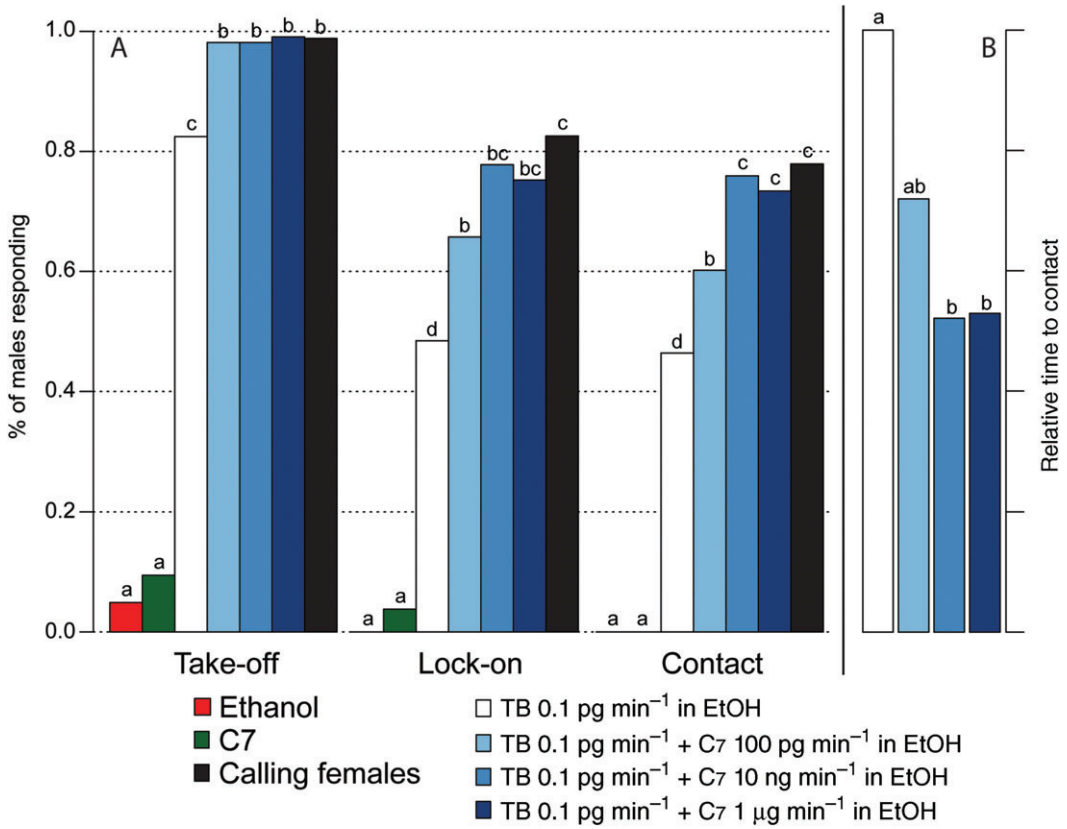

Figure 3. Attraction of *L. botrana* males to their sex pheromone, to heptane and to sex pheromone plus heptane combinations. Proportions of grapevine moth males making the behavioural steps of take-off, lock-on and source contact in a wind tunnel (A) and the relative time they needed to contact the source of semiochemicals (B) in response to underdosed pheromone (0.1 pg min^{-1}) in ethanol ($n = 97$), to heptane released at 100 pg min^{-1}, 10 ng min^{-1} and 1 pg min^{-1} with underdosed pheromone diluted in ethanol ($n = 108–109$), to heptane alone ($n = 53$), to ethanol alone ($n = 29$) and to 1–4 calling females ($n = 86$). All solutions were released at 10 µL min^{-1}. Letters assigned within a behavioural element indicate statistically significant differences [GLM (logit), $P < 0.05$ in (A); Cox proportional hazard regressions, $P < 0.05$ in (B)]. TB is the ternary sex pheromone blend (see text).

from Siegfried Ltd (Zofingen, Switzerland). Other compounds tested were *n*-hexane, *n*-heptane, *n*-octane, *n*-nonane, *n*-decane, *n*-dodecane and *n*-tetradecane (all >99.0% pure) from Fluka (Buchs, Switzerland). Ethanol was used as solvent (analysis grade, >99.8% pure; Merck AG, Dietikon, Switzerland).

2.6 Treatments tested

For *L. botrana,* the ternary sex pheromone blend of *E*7*Z*9–12:Ac, *E*7*Z*9–12:OH and *Z*9–12:Ac at a ratio of 100:20:5 was presented to male *L. botrana* at release rates of 0.1, 1 and 10 pg min^{-1} of the major component to determine its optimal concentration.[28] Secondly, an underdosed level of pheromone of 0.1 pg min^{-1} was added to dilutions of heptane and octane released at 100 pg min^{-1}, 10 ng min^{-1} and 1 pg min^{-1} in ethanol, and to the pheromone diluted in the alkanes. Pure heptane, octane, ethanol, and heptane and octane released at 10 ng min^{-1} in ethanol were tested as controls. Codlemone was tested at 1 and 100 pg min^{-1} to estimate an underdosed level to which test products were added to test for *C. pomonella* attraction. Heptane was released at 10 ng min^{-1} or on its own in combination with underdosed codlemone at 1 pg min^{-1}, and heptane and ethanol were tested as controls. Each treatment was tested on 30–113 males.

2.7 Statistical analysis

Comparisons between the responses of *L. botrana* males to treatments were made within behavioural elements by fitting general linear models (GLMs) with a logit link function. Analysis of deviance based on an asymptotic distribution was used to test whether behavioural responses were dependent on treatments. Multiple comparisons were made by applying Tukey contrasts when GLMs were significant ($P < 0.05$). When treatments affected all moths or no moths within a behavioural element, an individual was either subtracted or added, respectively, to allow comparisons. Time–event analyses were made by applying Cox proportional hazard regressions to compare the times to contact. All the data were analysed with the statistical package R 2.15.1.[29]

3 RESULTS

EAG recordings from *L. botrana* males indicate the sensitivity of the antennal olfactory cells to short-chain alkanes (Fig. 2). Antennae responded to all the tested alkanes in a dose-dependent manner, except to tetradecane, which is only moderately volatile at ambient temperature. The sensitivity of antennae was highest to heptane, octane, nonane and decane, evoking EAG responses similar to the response of the positive control at the highest dose tested.

To measure the effect of alkanes on the attraction of male *L. botrana* and *C. pomonella* to their sex pheromones, a range of pheromone release rates (0.1, 1 and 10 pg min^{-1}) were tested to set an underdosed level to which to add alkanes. At 1 pg min^{-1}, the sex pheromone activated 100% of L. botrana males and 63%

Table 1. Behavioural responses of *L. botrana* and *C. pomonella* males in a wind tunnel to their sex pheromones nebulised in ethanol with and without heptane (C_7) and octane (C_8, grapevine moth only). Responses of *L. botrana* to its sex pheromone with C_7 released at 100 pg min^{-1}, 10 ng min^{-1} and 1 μg min^{-1} and to C_7 alone are presented in Fig. 3

		Proportions (%)			
	n	Take-off[a]	Lock-on[a]	Contact[a]	Relative time to source contact[b]
L. botrana					
Ph. 0.1 pg min^{-1} in EtOH	97	82.4 f	48.5 bf	46.4 b	1
Ph. 1 pg min^{-1} in EtOH	94	96.8 de	65.9 cd	62.8 c	0.62*
Ph. 10 pg min^{-1} in EtOH	95	90.5 df	54.7 cf	47.4 b	0.99
Calling females	86	98.8 ce	82.6 e	77.9 d	NC
Ph. 0.1 pg min^{-1} in C_7	96	94.8 de	61.5 bcd	44.8 b	1.08
C_7 10 ng min^{-1} in EtOH	29	44.8 g	13.8 g	3.4 ae	19.27
C_8 alone	61	29.5 gi	4.9 ag	0 ae	NA
Ph. 0.1 pg min^{-1} + C_8 100 pg min^{-1} in EtOH	113	95.6 de	68.1 d	62.8 c	0.72
Ph. 0.1 pg min^{-1} + C_8 10 ng min^{-1} in EtOH	104	99 e	82.7 e	78.5 d	0.47*
Ph. 0.1 pg min^{-1} + C_8 1 μg min^{-1} in EtOH	104	99 e	72.1 de	67.3 cd	0.64*
Ph. 0.1 pg min^{-1} in C_8	104	85.6 bf	46.2 f	9.6 e	6.54
C_8 10 ng min^{-1} in EtOH	30	20 hi	6.7 ag	0 a	NA
C. pomonella					
EtOH	61	13.1 C	0 D	0 D	NA
Ph. 1 pg min^{-1} in EtOH	58	96.6 A	46.6 A	44.8 A	1
Ph. 100 pg min^{-1} in EtOH	60	100 A	85 B	85 B	0.31*
C_7	60	41.7 B	0 D	0 D	NA
Ph. 1 pg min^{-1} + C_7 10 ng min^{-1} in EtOH	78	96.2 A	73.1 BC	69.2 C	0.54*
Ph. 1 pg min^{-1} in C_7	62	95.2 A	62.9 C	40.3 A	1.16

[a] Letters assigned to a behavioural element indicate statistically significant differences [GLM (logit), $P < 0.05$].
[b] Asterisks indicate significantly reduced time to reach the source compared with the underdosed pheromone (shaded rows; Cox proportional hazard regressions, $P < 0.05$).

contacted the source, whereas the lower dose of 0.1 pg min^{-1} only activated 85.6% and attracted 46.4% of the males to the source. Therefore, the latter dose was selected for assessing any synergistic effect of alkanes.

Heptane significantly increased attraction to the sex pheromone in both species. The addition of heptane to the underdosed pheromone enhanced *L. botrana* attraction at release rates ranging from 100 pg min^{-1} to 1 μg min^{-1} (Fig. 3A) and induced source contact levels (76%) comparable with calling females (79%), in contrast to significantly lower source contacts (46%) recorded in response to the pheromone alone [GLM (logit), $P < 0.05$]. Critically, adding heptane rendered the pheromone more efficient in recruiting significantly more males to lock on to the pheromone plume (Fig. 3A), and such males practically all arrived at the pheromone source once they had locked on [GLM (logit), $P > 0.05$] (Fig. 3). Furthermore, males reached the pheromone source faster when heptane was added, requiring just over two-thirds of the time with heptane released at 100 pg min^{-1} compared with the underdosed pheromone, and only half the time with heptane released at 10 ng min^{-1} and 1 μg min^{-1} (Cox hazard regressions, $P < 0.05$) (Fig. 3B). We repeated the tests with underdosed codlemone on *C. pomonella* using the most effective release rate of heptane for *L. botrana* (Table 1). Compared with codlemone alone, where the proportion of males recruited and making source contact was, respectively, 47 and 45%, heptane released at 10 ng min^{-1} with underdosed codlemone increased recruitment to 73% and source contact to 69% [GLM (logit), $P < 0.05$] (Table 1). The latter is a level of attraction comparable with that recorded for *C. pomonella* males to calling females in the same wind tunnel.[17] *C. pomonella* males also reached the source of pheromone twice as quickly in the presence of heptane compared with the pheromone alone (Cox hazard regressions, $P < 0.05$) (Table 1).

When heptane was used as solvent for the pheromone, its capacity to attract males to the source was comparable with the pheromone diluted in ethanol for both moth species, but heptane was more efficient in male recruitment, as more moths locked on to the plume (Table 1 and Fig. 3A). Likewise, heptane on its own induced more male take-offs than ethanol, even though males of neither species locked on to the alkane plume: heptane released alone at 10 ng min^{-1} induced take-off by 45% of *L. botrana* males and just over 40% of *C. pomonella* males, 3 times more than the response to ethanol by either species (Table 1). Octane was also tested on *L. botrana* and was as effective as heptane as a pheromone synergist at the lower release rates (up to 10 ng min^{-1}) (Table 1). However, when octane was used as a solvent for the pheromone, successfully recruited males (46.2%) avoided contacting the source (9.6%).

4 DISCUSSION

These results provide the first evidence of biological activity for short-chain alkanes on the behaviour of two major insect crop pests, and indeed for any phytophagous insect. Alkanes are released in small amounts by most fruit trees[13] where shorter alkanes such as ethane and pentane are produced, among others, through lipid peroxidation on leaves.[30] The antennal receptor cells of *L. botrana* responded to alkanes from C_6 up to C_{12}, with higher electroantennogram responses generated in the C_6–C_{10} range (Fig. 2). Other host plant volatiles act as synergists with the sex pheromones of both species studied here. Terpene hydrocarbons

such as (*E*)-*β*-caryophyllene induce only low levels of attraction in *L. botrana* males when released at 100 pg min^{-1},[31] but attraction to the pheromone increased by up to 1.7-fold in the presence of (*E*)-*β*-caryophyllene,[16] an effect equal to that of *R*(+)-limonene and pear ester in combination with codlemone for codling moth males.[17] Alkanes may also act as either molecular mimics or sensitisers. Heptane, for example, activates CO_2 receptor cells in the yellow fever mosquito *Aedes aegypti*,[32] and dichloromethane, a widely used solvent, has been suggested to act as a surrogate for CO_2 in attracting *Diabrotica* larvae.[33] Carbon dioxide has been documented as a sensitiser, enhancing responses of mosquitoes to attractants.[34] Short-chain alkanes may serve to complete an appropriate olfactory repertoire, just as host plant terpene hydrocarbons do in these two species.[16,17]

According to the competitive attraction equations of Miller *et al.*,[6] the efficiency of mating disruption dispensers in space is a function of their findability, their ability to retain males at point sources and their density compared with the density of females. We demonstrate here how short-chain alkanes are effective in recruiting males of moth pests of fruit crops to their sex pheromones. Admittedly, the levels of alkanes used in the wind tunnel experiments described here are high relative to the levels released by plants; nevertheless, short-chain hydrocarbons are very pertinent to pheromone aerosols used for mating disruption through their effects on increasing the findability of sex pheromone dispensers as measured by the proportion of males locking on to them, to the detriment of females. Our findings indicate that the use of alkanes would allow a reduction in the dose of pheromone required by at least tenfold, yet still match the attraction of optimally dosed pheromone. Furthermore, lipophilic alkanes would facilitate transport of pheromones onto the waxy outer layer of vegetation, thus extending pheromone retention on the crop.[35] Taking these factors into account, pheromone amounts used on a crop could be economised to reduce costs yet maintain competitiveness with females.

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