

# A six-arm olfactometer permitting simultaneous observation of insect attraction and odour trapping

TED C. J. TURLINGS, A. C. DAVISON† and CRISTINA TAMÒ

Laboratory of Animal Ecology and Entomology, University of Neuchâtel, Switzerland and †Institute of Mathematics, Swiss Federal Institute of Technology, Lausanne, Switzerland

**Abstract.** Behavioural assays to study insect attraction to specific odours are tedious, time consuming and often require large numbers of replications. Olfactometer and flight tunnel tests can usually only be conducted with one or two odour sources at a time. Moreover, chemical information on the odour sources has to be obtained in separate analytical studies. An olfactometer was developed in which six odours can be tested simultaneously for their relative attractiveness while during the assays, part of each test odour can be trapped for further analyses. The effectiveness of this six-arm olfactometer was tested by observing the responses of the solitary endoparasitoid *Cotesia marginiventris* (Cresson) to host-induced odours from young maize plants. For statistical analyses, we used log-linear models were adapted to account for overdispersion and possible positional biases. Female wasps responded extremely well in tests where they were offered a single odour source, as well as in tests with multiple choices. The responses of wasps released in groups were the same as those released individually and it was found that females did not attract or repel each other, but males preferred arms in which females had been released. Dose-response tests with varying numbers of plants or host larvae on plants revealed that the wasps responded in a dose-related manner, thus showing that the system is well suited to measure relative preference. The clear choices of the insects amongst six possibilities provided substantial statistical power. Gas chromatographic analyses of sampled air revealed clean and effective odour trapping, which largely facilitates the comparison of results from behavioural assays with the actual blends of volatiles that were emitted by the various odour sources. Advantages and disadvantages compared to other methods are discussed.

**Key words.** *Cotesia marginiventris*, induced plant volatiles, log-linear model, maize, olfactometer, parasitoid behaviour, *Spodoptera littoralis*, *Zea mays*.

## Introduction

Olfaction is the primary sense used by insects to detect and locate various resources (Whittaker & Feeny, 1971; Tumlinson *et al.*, 1993). Studies that aim to unravel odour-

mediated interactions among insects and other organisms usually involve behavioural as well as chemical assays. These have resulted in the identification of numerous pheromone blends that insects use in sexual interactions (Mayer & McLaughlin, 1991; Cardé & Minks, 1996; Howse *et al.*, 1998), but have also revealed the sources and identities of substances that insects use to locate food and other resources (Bell & Cardé, 1984; Cardé & Bell, 1995). The studies are often tedious because of the time consuming behavioural assays that are involved. In the laboratory,

Correspondence: Dr Ted C. J. Turlings, University of Neuchâtel, Institute of Zoology, Laboratory of Animal Ecology and Entomology, C.P.2, CH-2007 Neuchâtel, Switzerland. Tel.: +41 32 718 3158; fax: +41 32 718 3001; e-mail: ted.turlings@unine.ch

insect responses are usually observed in olfactometers or flight tunnels of varying complexity (Hare, 1998).

Olfactometers have been used for over a century. One of the earliest and best descriptions of an olfactometer comes from McIndoo (1926); see also Snapp & Swingle (1929). He tested the attractiveness of host-plant odours to beetles by releasing the insects in the base of a Y-shaped glass tube where they were exposed to odours that were introduced through the two arms of the tube. An insect that walked into one of the arms was assumed to have a preference for the odour introduced through that arm. Y-Shaped olfactometers, or comparable T-shaped linear tract olfactometers (Sakuma & Fukami, 1985), are still commonly used to test the olfactory responses of various arthropods (Sabelis & vande Baan, 1983; Steinberg *et al.*, 1992; Bartlett *et al.*, 1997; Pallini *et al.*, 1997; Bernasconi *et al.*, 1998; Sullivan *et al.*, 2000).

Pettersson (1970) developed the four-arm olfactometer, which has been modified and described in more detail by Vet *et al.* (1983). In this olfactometer, small insects are introduced into a chamber in which four distinct odour fields are created. In principle, four different odour sources can be tested, but usually it is not used for more than two types of odours. Four-arm olfactometers are well suited for direct behavioural observations. Other types of olfactometers are mainly static, without airflow, but in which odour gradients determine the orientation of responding insects (Ruther & Steidle, 2000). Directional responses to odours by walking insects can be precisely recorded and measured with walking spheres, as developed by Thiéry & Visser (1987).

For flying insects, flight tunnels can be employed (Miller & Roelofs, 1978; Baker & Linn, 1984). Because the insects will have to exhibit their full behavioural repertoire to reach an odour source, flight tunnel assays are often more sensitive to varying abiotic conditions, as well as the condition of the insects. In particular, the tendency for insects to fly towards light sources frequently prevents them from orienting towards odour sources. However, positive responses in flight tunnels provide the most convincing evidence that an odour source is truly attractive to an insect.

All these behavioural assays are very time consuming. They are usually designed for the release of one insect at a time, responding to a very limited number of odours. Moreover, the chemical identities of the substances that evoke the responses have to be determined in separate experiments. Recent developments in the odour trapping and sensitive analytical chemistry techniques have greatly facilitated such efforts (Millar & Haynes, 1998). In studies on the role of plant-provided cues in the host-location processes of parasitoids, several of these techniques have been employed (Turlings *et al.*, 1991b, 1998). However, the need to compare large numbers of odour sources for their relative attractiveness requires the development of novel assay techniques that allow for faster testing of multiple odour sources with larger numbers of insects.

The present study introduces an olfactometer in which six odours can be offered to test insects at the same time, and

where part of each odour is collected on trapping filters for subsequent chemical analyses. Using naïve females of the parasitoid *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae), it is shown that this olfactometer can be highly effective in determining the relative preference for various odours. The wasps were strongly attracted to maize plants releasing an odour blend that is typical for plants under caterpillar attack. In dose-response tests, the wasps also clearly preferred sources that released more of these odours. By releasing wasps in groups, the experiments could be conducted in a relatively short time span. In separate experiments, it was shown that female wasps do not interfere with each other's behaviour, but that males are attracted to females.

## Materials and methods

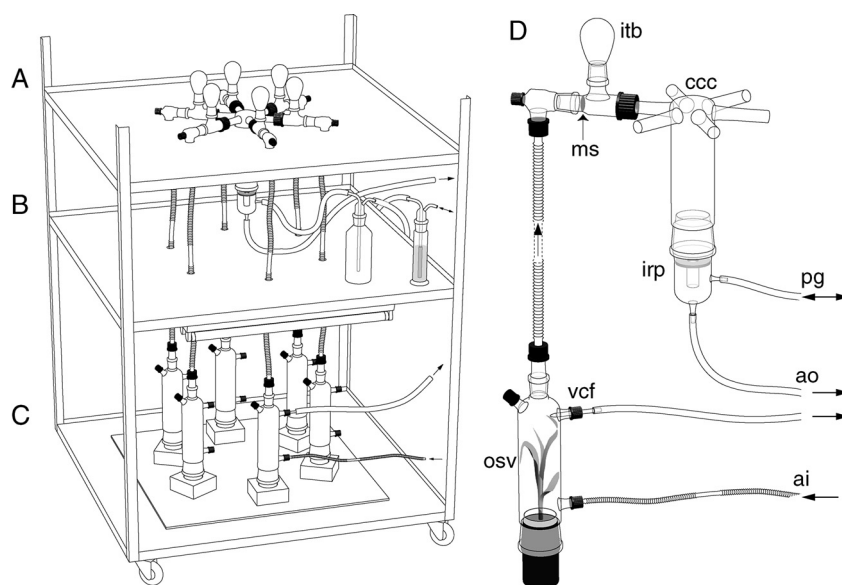
### *The insects*

For all bioassays, 2–3-day-old naïve individuals of the solitary endoparasitoid *C. marginiventris* were used. The rearing colony originated from the USDA-ARS, Biological Control and Mass Rearing Research Unit (Mississippi). For rearing, 25 *Spodoptera littoralis* (Noctuidae: Lepidoptera) caterpillars (3–4 days old) were offered to a single mated female (4–7 days old) for 3 h in a plastic-box (diameter 9.5 cm, height 5 cm). The parasitized caterpillars were kept on a wheatgerm-based artificial diet in an incubator (25 °C and LD 16 : 8 h) until cocoon formation. Cocoons were kept in Petri dishes until adult emergence. Emerging adults were sexed and kept in cages (30 × 30 × 30 cm) in the same incubator at a sex ratio of 1 : 2 (male : female), with moist cotton wool and honey as a food source.

*Spodoptera littoralis* larvae were reared from eggs provided by Syngenta (Stein, Switzerland). The eggs were kept in the above incubator and, after emergence, larvae were placed on the artificial diet at room temperature. In several experiments, second-instar larvae were directly placed on small maize plants to induce the production of volatiles. In other cases, caterpillar regurgitant was used for this induction. Regurgitant was collected from third- and fourth-instar larvae that had been fed on maize leaves, as described by Turlings *et al.* (1993). The collected material was centrifuged and the supernatant was filtered through a 0.22 µm filter to remove large particles and microorganisms, and subsequently stored at –70 °C until used for the treatment of plants.

### *The six-arm olfactometer*

Details on the olfactometer are available at <http://www.unine.ch/zool/leae/olfactometer.html>. The device was assembled on a 1 × 1 m and 1.5 m high three-tiered metal frame on small wheels, with each level consisting of a wooden



**Fig. 1.** An overview of the six-arm olfactometer. (A) Top shelf where the insects can make choice for one of six possible odours. (B) Middle shelf from where the insects are released. (C) Bottom shelf with the six odour sources (only for one of the chambers are all connecting tubes drawn). (D) Detailed depiction of the various parts and connections; ai, air inlet; osv, odour source vessel; vcf, volatile collection filter; ms, metal screen; ccc, central choice chamber; itb, insect trapping bulb; irp, insect release point; pg, pressure gauge; ao, air outlet. The corrugated tubes are made of Teflon and the exhaust tubes of Tygon (drawing by Dr Thomas Degen).

shelf (Fig. 1). Holes in the top two shelves allowed for the connection between the different levels via Teflon and glass tubing. The bottom shelf (10 cm from the floor) held the odour sources. A middle shelf (83 cm from the floor) served as a release point for the insects, whereas the top shelf (123 cm from the floor) carried the olfactometer itself. One of the main advantages of the separation of the different parts of the olfactometer is that each of them can be subjected to different light conditions. Moreover, the test insects have no visual contact with the odour sources. This separation was found to be practical and optimized the use of space. Almost all glass parts were custom made by Uni-Glas (Zurich, Switzerland) and VQT (Neuchâtel, Switzerland).

#### *The air delivery*

A central inhouse compressor supplied the air. Starting at the laboratory valve, air was first passed through two 16-cm long 1/2" Ø (external diameter) stainless steel tubes filled with 20–60 mesh activated charcoal (Sigma, St Louis, MO) for purification. The air then passed through a 1/4" Ø copper tube into a 1-L water bubbler that was half filled with deionized water. The pure and humid air was then pushed into a manifold with six flowmeters (Aalborg, Oranenburg, NY). In all experiments, the flow rate through each flowmeter was set at 1.2 L/min. The exit of each flowmeter was connected to a 13-cm long, 6-mm Ø glass tube filled with activated charcoal, held in place by two 325 mesh stainless steel screens (Small Parts Inc., Miami Lakes, Florida). These tubes served to filter out

any remaining impurities. The glass tubes were connected to 1/4" Ø corrugated Teflon tubes (Cole-Parmer, Vernon Hills, Illinois) of sufficient (variable) length to reach one of the six odour source vessels.

#### *The odour source vessels*

The vertically-placed cylindrical odour source vessels (Fig. 1) were designed to hold small living plants and consisted of three parts. The bottom part was an 11-cm high 5-cm internal diameter (i.d.) glass pot with a 50–55-mm male ground glass joint. This pot fitted the female ground glass joint of the 28.5-cm long, 6-cm i.d. central part of the vessel. Just above the female fitting, a horizontally connected glass port with a screw cap fitting (GL14) allowed for the connection to one of the Teflon air supply tubes through a rubber Teflon-coated 6-mm i.d. ferrule. The air was introduced via these tubes into each vessel, a few cm above the glass pot. Two identical glass ports were positioned at the other (top) end of the central part one perpendicular (horizontal) to the vessel and one under an angle pointing up. These ports served to hold trapping filters for volatile collections (Fig. 1D). Just above these ports, the glass cylinder narrowed into a 22–25-mm ground glass connector. A fitting with a glass male joint and a screw-cap fitting (GL25) on the other end, formed the connection for a 3/4" corrugated Teflon tube that transfers the air from the odour source to one of the arms of the olfactometer on the top shelf. For this purpose, holes were drilled just above each vessel in the middle and top shelf.

Six such vessels, all connected to one of the Teflon tubes from the flowmeters, were placed in a 50-cm Ø circle at equal distances from each other. For stability, the bottom pots were placed in 4.5-cm high wooden blocks with a fitting hole in the centre that were glued to a wooden base (Fig. 1). They were illuminated with 10 neon tubes (five Osram 18 W/21-810, alternated with five Sylvania Gro-Lux F18W/GRO-T8) that were connected to the bottom of the second shelf of the olfactometer. These lamps provided approximately 8300 lux at plant height.

### *The olfactometer tiers*

As shown in Fig. 1, the odour sources were connected to the olfactometer with corrugated Teflon tubing. The first connecting part was a glass elbow with a GL25 screw-cap fitting. This part ended in a 22–25-mm female ground glass connector and also had a GL14 screw-cap fitting, which was sealed during all experiments. The next glass part had a 22–25-mm male ground-glass connector and a similar upward connection for the insect-trapping bulb (50 mL) (Fig. 1D). A 325 mesh stainless metal screen (Small Parts Inc., Miami Lakes, FL) was placed just after the horizontal male connector and served to block the passage of insects that had made a choice. The opposite part was attached to one of the arms of the central choice chamber by another GL25 screw-cap fitting. This central chamber consisted of six 15-mm Ø arms attached to a 6-cm i.d., 22-cm long vertically placed cylinder into which the insects were released. The top of the cylinder was closed just above the arms and the rest was hanging through a hole in the top shelf. The base of the chamber was connected via a 50–55-mm ground-glass joint to a shorter cylinder with a glass frit into which a 15-mm Ø insect release vial was sealed (Fig. 1D). The bottom of this cylinder tapered off into a 6-mm tube connector and a similar connector was attached to the side. The first was connected with a Tygon tube to the vacuum pump via another flowmeter, whereas the second connection was with a wash bottle with water that served as a pressure gauge. Both were used to balance the incoming and outgoing air ( $6 \times 600$  mL/h).

To eliminate any visual distractions and to ensure uniform illumination, the olfactometer on the top tier was surrounded by a 56-cm Ø, 25-cm-high white cardboard cylinder (not shown in Fig. 1). A second such cylinder (23-cm Ø, 25-cm high) with holes cut out for the olfactometer arms was placed just around the central choice chamber and a milky white Plexiglas disk (28-cm Ø, 3-mm thick) was placed on top of this cylinder. This disk was illuminated with a 60 W light bulb placed 25 cm above it. Because the inner-cylinder was covered, the central choice chamber was not visible to the experimenter, but the insect trapping bulbs were positioned outside of this cylinder and could be readily checked for the presence of insects by looking into the larger cylinder.

### *Bioassays*

Mated 2–3-day-old female (in one case male) wasps were used and all test insects were naïve in that they had never encountered a host or plant as an adult. In most cases, the wasps were released in groups of six (exceptions are specified below). They were removed from their cage with an aspirator and directly placed in the vial attached to a glass frit at the bottom of the central choice chamber (the insect release point in Fig. 1D). The great majority of the wasps walked up, attracted by the light above the chamber. If attractive odours were present, most of the wasps would walk into an arm with such an odour until their path was blocked by the stainless steel screen. Eventually, they walked up in the direction of the light source into the insect trapping bulb (Fig. 1D), where they could easily be counted and removed. All insects were given 30 min to make a choice, after which they all were removed and a new group was released. Three groups of six wasps were tested on a given day. Statistical comparisons among choices are described in the results section.

On a given day, all wasps were tested with the same odour sources, which remained in the same position. This was performed because preliminary results had shown that the arms remained attractive after removal of attractive sources due to adsorption of volatiles to glass and Teflon surfaces. For this reason, all glass and Teflon parts were carefully cleaned at the end of an experiment (day), first with water and then by rinsing with acetone and pentane. After the solvents had evaporated, the glass parts were placed overnight in an oven at 250 °C.

### *Odour sources*

In almost all cases, 8–9-day-old maize plants (var. Delprim) served as odour sources. They were individually grown from seed placed in plastic pots (6-cm high, 8-cm diameter) in fertilized commercial soil (Coop, Switzerland). The pots were placed in a climate chamber (23 °C, 60% RH, and LD 16:8 h, 50000 lux) and, the day before an experiment, the plants were transplanted into a glass pot with a ground-glass male connector that could be inserted into an odour source vessel. Plants were induced to emit volatiles by either placing a certain number of second-instar *Spodoptera littoralis* larvae on them on the evening before an experimental day, by scratching two leaves (2-cm<sup>2</sup>) and applying 10 µL *S. littoralis* regurgitant to the damaged sites (also on the evening before). In an experiment to test for intraspecific interference, female parasitoids were used as potential odour sources, as detailed below.

### *Odour trapping*

Volatiles emitted by the various odour sources in the above-described vessels were trapped in a similar manner

to lings *et al.* (1998). Trapping filters consisted of 7-cm glass tubes in which 25 mg of 80–100 mesh Super Q adsorbent (Altech, Deerfield, Illinois) was placed and kept in place by two fine mesh metal screens, as described by Heath & Manukian (1992). In all experiments, one filter was attached to the horizontal port at the top of each odour source vessel (Fig. 1D). The other ports were sealed with a Teflon-coated septum in the screw cap. A 6-mm i.d. Tygon tube was connected to each collection trap. These tubes were attached to a second manifold with six flowmeters (Aalborg Instruments & Controls Inc., Monsey, New York). Air was pulled through each tube at a rate of 0.6 L/min (half of the incoming flow) via additional Tygon tubing connecting the flowmeters to a vacuum pump (model ME2, Vacuubrand, Germany). The vacuum was turned on 1 min after the air was first pushed into the system.

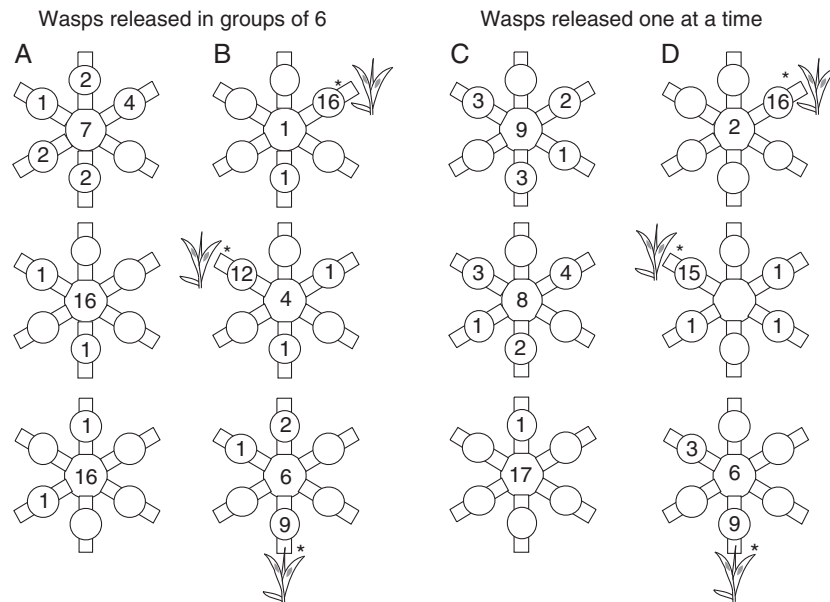
Before each experiment, the traps were rinsed five times with 200  $\mu$ L methylene chloride. Collections always lasted 3 h. The first olfactometer tests started 30 min after a collection began. After each collection, the traps were removed, extracted and analysed as described below.

#### Chemical analysis

During all experiments 50% of the air passing over the odour sources was pulled through a trapping filter for 3 h (see above). Immediately after each experiment, the volatiles collected on these filters were extracted with 150  $\mu$ L of methylene chloride and two internal standards (*n*-octane and nonyl acetate, each 200 ng in 10  $\mu$ L methylene chloride) were added to these samples. The samples were either analysed immediately or stored at  $-70^{\circ}\text{C}$  before analysis. For the analysis of each sample, an aliquot of 3  $\mu$ L was injected on column with the use of an automated injection system onto an apolar HP-1 capillary column (30 m, 0.25-mm i.d., 0.25  $\mu$ m film thickness). The column was housed in a Hewlett Packard model HP 6890 gas chromatograph equipped with a flame ionization detector. The oven was held at  $50^{\circ}\text{C}$  for 3 min and then programmed at  $8^{\circ}\text{C}/\text{min}$  to  $230^{\circ}\text{C}$ , where it was maintained for 9.5 min. The column was preceded by a deactivated retention gap (10 m, 0.25-mm i.d., Connex) and a deactivated precolumn (30-cm, 0.53-mm i.d., Connex). Helium (24 cm/s) was used as carrier gas. HP GC Chemstation software was used to quantify all major components based on the known quantity of internal standards. Initial identification of most compounds was based on comparisons of retention times from previous studies (Bernasconi *et al.*, 1998; Turlings *et al.*, 1998). Identities were confirmed with the mass spectrometry analysis of some samples, using the same column and temperature programme (Agilent 5973, transfer line  $230^{\circ}\text{C}$ , source  $230^{\circ}\text{C}$ , quadrupole  $150^{\circ}\text{C}$ , ionization potential 70 eV, scan range 0–400 amu). Total quantities of volatiles were calculated based on their peak areas compared to those of the internal standards.

#### Statistical analysis

Except for the first experiment, the results for each test day were used as a replication (a minimum of six replicates). An initial experiment designed to test for a directional bias of the wasps found none but, nonetheless, bias was tested in each subsequent experiment; none was found. Only those insects that made a choice were included in the analyses of the results; these represented over 75% of the wasps in experiments where an attraction to an odour source was expected. Thus the responses consist of counts ( $n_1, \dots, n_6$ ), where  $n_i$  denotes the number of wasps observed to choose arm  $i$ . Simple data analyses would suppose that the counts ( $n_1, \dots, n_6$ ) for a single replicate follow a multinomial distribution with probabilities ( $P_1, \dots, P_6$ ) and denominator  $m$  the total number of wasps making a choice; here  $P_i$  represents the probability that an individual wasp chooses arm  $i$ . Such a model accounts automatically for the dependence between the numbers of wasps choosing the different arms. Its underlying assumptions are that individual wasps act entirely independently of one another and that the  $P_i$  are constant across all replicates with the same experimental conditions. If these assumptions are true, it is possible to model how the probabilities  $P_1, \dots, P_6$  depend on the experimental treatments. For example, in the dose–response experiments, a log-linear form  $\log P_i = b_0 + b_1 x_i - c$  was used, whereby the log probability of choice of arm  $i$  is taken to depend linearly on a covariate  $x_i$ , which measures the relative attractiveness of the dose for that arm; Model I. The constant  $c$  ensures that  $P_1 + \dots + P_6 = 1$ ; it depends on the unknown parameters  $b_0$  and  $b_1$ , which must be estimated from the data. The strength of the attraction is measured by  $b_1$ ; if this is close to zero, then the attraction is weak, whereas a large positive value indicates strong attraction. If such a model is supported by the data, a test of attraction may be based on the estimate of  $b_1$ . Log-linear models such as this are widely used in applied science and their statistical properties are well-understood (Bishop *et al.* 1975; Dobson, 1989; Davison, 2003). They are readily adapted to more complex experimental setups. The interspecific interference experiments described below use a  $2 \times 2$  factorial design, for example, with arms 1, 3, 5 given no treatment, and the other three treated with plant odour (e.g. arm 2), 10 female insects (e.g. arm 4) and both plant odour and 10 female insects (e.g. arm 6). The corresponding log-linear model (Model II) sets  $\log P_1 = \log P_3 = \log P_5 = -c$ ,  $\log P_2 = g_1 - c$ ,  $\log P_4 = g_2 - c$ , and  $\log P_6 = g_1 + g_2 + g_3 - c$ , where  $c$  ensures that the total probability is one,  $g_1$  represents the relative attraction of plant odour,  $g_2$  the relative attractiveness of female wasps, and  $g_3$  is an interaction between these. Significantly positive estimates of  $g_1$  or  $g_2$  would indicate significant attraction of the corresponding treatments for the wasps, and the sizes of these estimates measure the relative strength of attraction. Such models can be fitted by maximum likelihood estimation in software packages such as *R* (<http://stat.ethz.ch/CRAN/>), and their relative adequacy can be assessed through likelihood ratio statistics and examination of



**Fig. 2.** Results for the experiments with no (A and C) or a single (B and D) odour source. Each drawing represents a single replicate during which 18 female wasps were released. The wasps were released either singly or in groups of six. Each replicate was tested separately for differences in the number of choices among the arms. Arms with an asterisk were chosen significantly more often than the other five arms.

residuals. More details for the data are provided in the results section.

A complication for the use of both standard tests and modelling is overdispersion, the statistical term for situations in which data vary by more than would be expected under a theoretical model such as the multinomial. This often arises in applications because the assumptions that underlie simple statistical models are rarely entirely valid. Overdispersion is present in the data, perhaps owing to the slight variation in experimental conditions between and within replicates. Fortunately, its effects are well studied and there are standard techniques for assessing the degree of overdispersion and obtaining reliable inferences from overdispersed data (Firth, 1991; McCullagh & Nelder, 1989; Davison, 2003). Modified  $G$ -statistics were used for comparison of log-linear models fitted to overdispersed data, and modified standard errors for parameter estimates.

## Results

### *Blank tests and single choice experiments*

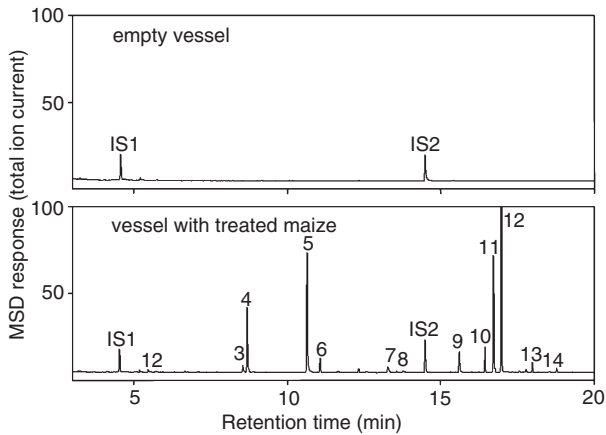
In a first experiment, the responses of naïve *Cotesia marginiventris* females were tested with no odour sources placed in any of the chambers. This experiment was conducted to test for directional biases of the insects and to determine if the wasps would enter the arms in the absence of an odour. Female wasps (2–4 days old) were released either individually or in groups of six and left in the olfactometer for 30 min or until they had made a choice. In both cases, the majority of the wasps remained in the central chamber of

the olfactometer. The few wasps that did make a choice showed no significant preference for any of the arms (Figs 2A,C) ( $P > 0.05$  in both cases). There was evidence of different behaviour in the three different replicates of the experiment ( $P < 0.01$  in both cases), though this is based on the small numbers of wasps that made choices. However, such variation between replicates performed under supposedly identical conditions may account for the overdispersion mentioned in the section on statistical treatment of the data, and for which allowance is made below.

In a subsequent experiment, a mechanically damaged maize plant treated with regurgitant (see above) served as an odour source in one of the vessels. All other odour source vessels were empty. Again, wasps were either released individually or in groups of six. The position of the plant was different for each of the three replicates (Figs 2B,D). Of the 18 females that were released per replicate, the great majority chose the arm with the maize odour (Figs 2B,D). These arms were chosen significantly more often than arms with no odour ( $P < 0.001$  in both cases).

### *Collected odour emissions*

During all assays, the volatiles were trapped by pulling 50% of the airflow through a filter that was attached to the top of each odour source vessel. Figure 3 shows two typical chromatograms; one of volatiles collected from an empty vessel and one of volatiles collected from a maize plant treated with caterpillar regurgitant. The blank collection reveals very small amounts of a few impurities in the system, whereas the collection of the plant odour



**Fig. 3.** Typical chromatograms of collected volatiles from either an empty odour source vessel (blank) or a maize plant that was treated with the regurgitant of *Spodoptera* larvae (treated plant). The labelled peaks represent: (1) (*E*)-2-hexenal; (2) (*Z*)-3-hexenol; (3)  $\beta$ -myrcene; (4) (*Z*)-3-hexen-1-yl acetate; (5) linalool; (6) (3*E*)-4,8-dimethyl-1,3,7-nonatriene; (7) phenethyl acetate; (8) indole; (9) geranyl acetate; (10)  $\beta$ -caryophyllene; (11) (*E*)- $\alpha$ -bergamotene; (12) *E*- $\beta$ -farnesene; (13) unknown sesquiterpene; and (14) (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

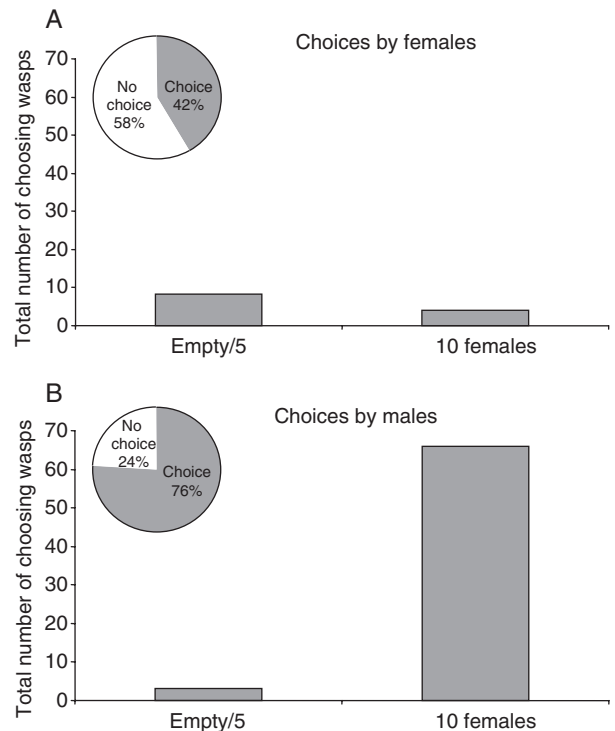
demonstrates that all typical induced maize volatiles (Turlings *et al.*, 1998) were emitted and readily collected (Fig. 3).

#### Intraspecific interference

To test the possibility that the wasps affect each other's choices by either attracting or repelling one another, two experiments were conducted with females already present in one or two of the arms. In the first experiment, 10 females were placed in one of the arms for 1 h when plugs of cotton were used to prevent them from either walking up into the trapping bulb or out of the arm into the choice chamber. By this method, any odour emitted by the females could adsorb onto the glass in the arm. After 1 h, the cotton plugs were removed and the normal experimental airflows were passed through all arms. The wasps were left in the arm and were given 30 min to settle in the trapping bulb before starting the actual assays. The five other arms were left empty.

On each test day, three groups of six females and three groups of six males were released in the choice chamber and their choices were recorded. This was repeated for 6 days, each time with the 10 females in a different arm. Most females did not enter an arm and those that did distributed themselves evenly over all six arms (Fig. 4A). On the other hand, the males entered the arm with females significantly more than the control arms (Fig. 4B) ( $P < 0.001$ ).

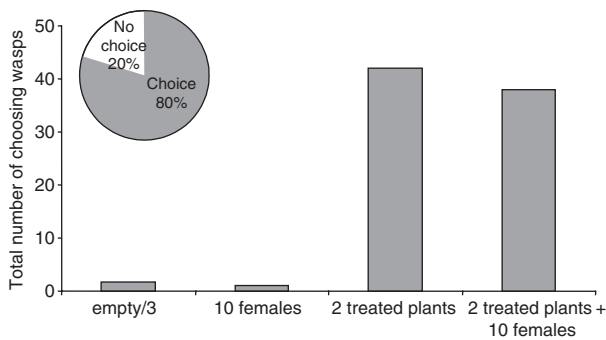
In the second experiment, three odours were introduced that were alternated with arms with clean air only. For two of the odours, two treated maize plants were placed in the bottom vessel. In one case, this was combined with 10



**Fig. 4.** Responses to the odour of female conspecifics, which was offered through only one of the six arms. The pie chart shows the percentage of wasps that entered an arm. Choices for the control arms were summed and divided by 5. (A) Choices made by females. (B) Choices made by males.

females that were placed in the arm as described above. Again, after 1 h, the cotton plugs that kept them from moving out were removed and the normal experimental airflows were passed through all arms. The third odour arm received 10 females in the same way, but did not have the plant odour.

Again on each test day, three groups of six females were released in the choice chamber and their choices were recorded. This time no males were tested. This was repeated for 6 days, with the different odour being introduced through different arms. The great majority of females chose arms with plant odour and did not distinguish between arms that already contained other wasps and those that did not (Fig. 5). A fit of Model II to these data by maximum likelihood estimation with allowance for overdispersion led to estimates (standard errors) for  $g_1$  (attraction of plant),  $g_2$  (attraction of females) and  $g_3$  (interaction between plant and females) of 3.05 (0.58),  $-8.20$  (34.7) and 8.10 (34.7), with respective two-sided significance levels of  $10^{-5}$ , 0.8 and 0.8; without allowance for overdispersion the significance level for  $g_1$  would have been  $10^{-12}$ , thus showing how failure to account for overdispersion might give misleading conclusions. This confirms the visual impression from Fig. 5. The attraction of plants was highly significant and the presence of other females did not affect this attraction.

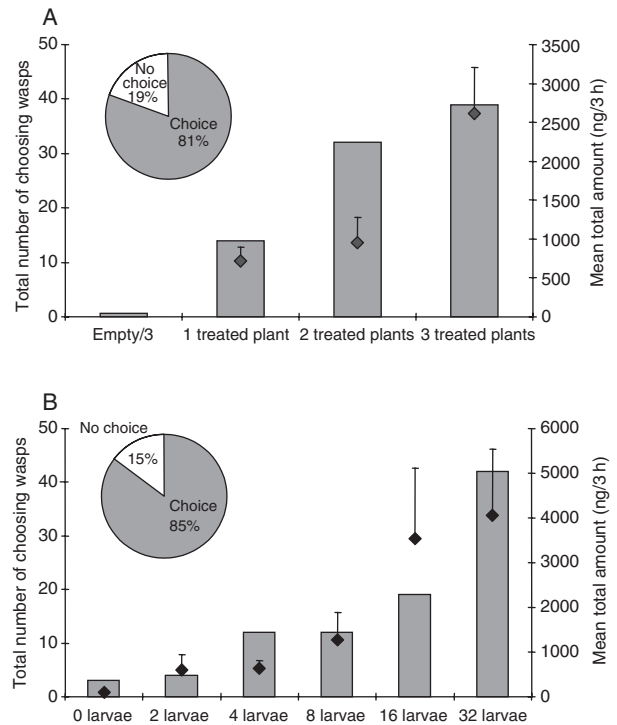


**Fig. 5.** Responses to the odour of female conspecifics in the presence or absence of induced maize odour. The pie chart shows the percentage of wasps that entered an arm. Choices for the three control arms were summed and divided by 3.

#### Dose–response tests

Two dose–response tests were conducted to test if the olfactometer was suited to measure relative attractiveness of multiple odours. In a first experiment, three arms with only clean air were alternated with arms with the odour of either one, two or three maize plants that were damaged and treated with regurgitant as described above. Three times, six naïve wasps were released per replicate (six replicates) and their choices were determined 30 min after release. For each replicate, the odour sources were placed in a different position, but always with one empty chamber between two chambers that contained at least one plant. The wasps responded in a dose-related manner (Fig. 6A), with most of the wasps choosing the arm with three plants. The analyses of the volatiles that were collected during the bioassays confirmed that the total amount emitted was positively correlated with the number of plants per odour sources, although fits of Model I showed that the numbers of wasps choosing an arm was more highly correlated with the number of plants than with the amount emitted (significance levels of 0.00005 and 0.004, respectively). There is very strong evidence that the number of wasps choosing an arm increases both with the number of plants and the volatiles emitted.

For the second dose–response experiment, two maize plants were placed in each of the six chambers, but they received different numbers (0, 2, 4, 8, 16 or 32) of second-instar *Spodoptera littoralis* larvae. The larvae were placed on the plants at 17.00 h the day before the assays. The next morning, three groups of six naïve female wasps were released in the olfactometer. Again, the choices for the arms were dose-related, with the majority ending up in the arm that carried the odour of plants damaged by 32 larvae (Fig. 6B). The more larvae that were on the two plants, the more odour was emitted (Fig. 6B). To obtain a numerical measure of the strength of the attraction of the odour from damaged plants, Model I was fitted, with  $x_i$  taken as the base 10 logarithm of total amount of collected volatiles (in ng), rescaled so that the lowest value of  $x_i$  for each experiment was zero; thus  $x_i$  represents the relative strength of the odour for



**Fig. 6.** Dose–response tests. The pie charts show the percentage of wasps that entered an arm. The bars indicate the choices made for the different arms, whereas black diamond symbols indicate the total amounts of volatiles emitted by an odour source. (A) Arms with one, two or three induced maize plants were alternated with empty arms. Choices for the three empty arms were summed and divided by 3. (B) Odour sources were two maize plants with 0, 2, 4, 8, 16 or 32 *Spodoptera* larvae.

each experiment. The fit gave an estimate (standard error) for  $b_1$  of 1.27 (0.29), with a significance level of around 0.0001. This shows a strong correlation between odour intensity and attraction. The results of both experiments show that the wasps are able to distinguish among odours in the olfactometer and that they make their choices accordingly.

## Discussion

Females of the larval endoparasitoid *Cotesia marginiventris* respond exceptionally well in the six-arm olfactometer. In previous olfactometric studies, female wasps required conditioning with an oviposition experience in the presence of a particular odour source in order for them to respond to odours in a four-arm olfactometer or a flight tunnel (Turlings *et al.*, 1989, 1991a). Here, a great majority of unconditioned (naïve) females make a choice for an arm if the odour introduced through that arm is from a treated maize plant. If no such odour is offered, most females remain in the central release chamber during the 30-min test period (Fig. 2). Similar results are being obtained with several other parasitoid species.

The results confirm previous findings that *C. marginiventris* is attracted to herbivore-damaged maize plants (Turlings *et al.*, 1990; Turlings *et al.*, 1995). This is also clearly the case for naïve insects that have not had contact with a host and never experienced the odour of the plant before. Nearly 90% of the females choose an arm with the odour of a treated plant when it is offered next to five arms that did not carry an odour (Fig. 2).

#### *Experimental design and interpretation of the results*

Ramirez *et al.* (2000) expressed various concerns about the design of published olfactometer and wind tunnel studies. Pseudo-replication and a lack of independence are very common flaws of many studies. The design of the current experiments varies because of differences in the purpose of each test. In the first series of experiments, the principal goal was to determine whether the olfactometer can be used to test the attractiveness of an odour. For this reason, a single odour source (damaged maize plants treated with caterpillar regurgitant) is used that is known to be attractive to the wasps (Turlings *et al.*, 1990). Given that a great majority of the females enter the arm carrying the odour of the plant (Fig. 2), the olfactometer is suited to test the attractiveness of individual odours. By placing the plant in a different chamber for different replicates, the possibility that the results are due to a positional effect is ruled out. This is also confirmed with the experiments in which none of the chambers contain a plant; most females stay in the central chamber and those that do walk into an arm do not show a significant preference for a particular arm.

This olfactometer was specifically designed for 'high-throughput' studies, whereby insects are released in groups. Therefore, a second objective of the first experiments was to test if groups of females can be released at the same time without influencing each other's choices. This is clearly the case because the results for single releases are almost identical to those for group releases (Fig. 2). It is also shown that females are not attracted or repelled by each other (Fig. 4) and *C. marginiventris* females released in groups can be assumed to make independent choices. This should be determined anew for each insect species that is tested in the olfactometer. Interestingly, male *C. marginiventris* are attracted to arms with females, probably due to the presence of a sex pheromone.

The aim of the dose-response experiments was to determine if the olfactometer can be used to test the relative attractiveness of simultaneously offered odours. For such experiments, an appropriate replication is of the utmost importance. The unit of replication in the first place is the odour source and, in the second place, the insect. Ideally, each insect is exposed to a new combination of odours. This is entirely impractical, but to avoid false conclusions due to pseudo-replication, the experiments need to be replicated sufficiently with an entirely new set of odour sources. Here, each experiment with multiple odour sources is replicated six times and the position of the odour sources is rand-

omized (for the experiment with different number of larvae) or covers all possible positions of the odours (for the experiment with different numbers of plants).

The results for the dose-response tests (Fig. 5) illustrate that the insects make clear choices. Arms carrying the odour of just one treated plant are chosen less often than arms with two or three plants. Similarly, the wasps respond in a dose-related manner to plants that are fed upon by different numbers of *Spodoptera littoralis* larvae, with the highest number of wasps choosing the plants with the most (32) larvae. In both dose-response tests, the amount of volatiles collected during the bioassays corresponds with the preferences the females exhibited.

#### *Statistics*

The choice of appropriate statistics for the evaluation of the results of olfactometer studies is not always straightforward. In cases where there are only two choices, several simple tests are available but, for multiple choices, various tests have been employed. Standard analysis of variance is not appropriate because the responses are categorical rather than numerical measures. Standard log-linear models for categorical data can be employed, but it is important to adjust their output to account for the overdispersion commonly present with such data. Failure to make such adjustment could result in erroneously small *P*-values and standard errors, resulting in statements that certain effects are present, when in fact the data do not justify them. If present, overdispersion also invalidates standard approaches such as the use of tests of homogeneity based on G-statistics (Scherrer, 1984; Sokal & Rohlf, 1995).

#### *Advantages and disadvantages*

With the use of this six-arm olfactometer, multiple odours can be tested at the same time and insects can be released in groups, which constitutes a major advantage, saving experimental and rearing time. Moreover, when only one or few odours are tested, the six-arm olfactometer offers more statistical power than other olfactometric devices with fewer choices. This power of statistics stems from the fact that the likelihood of an insect selecting an arm with the test odour by chance is much smaller than in a two-choice situation.

Another important advantage is the simultaneous trapping of volatiles during the behavioural assays. The subsequent analysis of the volatiles allows for a direct comparison of the assay results with the actual quantity and quality of the odour that the insects responds to. Variation in odour emissions among similar treatment can therefore be accounted for. The volatile collections contain surprisingly few impurities despite the fact that the plants are standing in regular potting soil. All major induced maize volatiles (Turlings *et al.*, 1998) are readily detected in the analyses.

The separation of odour sources and insects by using different shelves ensures that the insects have no visual cues to orientate by. Moreover, it is possible to use different illumination for insects and plants. A central moderate light source above the olfactometer is used to initially attract the wasps up into the choice chamber, whereas the plants that serve as an odour source are exposed to considerably more light to ensure good odour emissions. The release of induced odour by maize plants requires light and increases with increased light intensity (Gouinguene & Turlings, 2002).

Disadvantages of this device are the relatively high costs for construction and the vulnerability of its many glass parts to damage. Most inconvenient is the need to clean the entire device between each replicate. Preliminary tests had shown that adsorption of the attractive odours on glass and/or Teflon parts rendered arms attractive even after an odour source is removed. This is the reason why the odour sources are only tested in one particular position on a given day. Despite these disadvantages, the six-arm olfactometer is highly efficient and practical, and promises to be a useful tool for many types of olfactometric studies.

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