

An airflow olfactometer for measuring olfactory responses of hymenopterous parasitoids and other small insects

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ABSTRACT. A new type of airflow olfactometer is described, and results given of experiments using it to measure behavioural olfactory responses of hymenopterous parasitoids. Compared with Y-tube olfactometers it shows several advantages. In its exposure chamber four separate abutting odour fields are presented so that the test insect can readily enter and re-enter them. More than one odour (or different concentrations of one odour) can be tested at the same time, thereby providing complex preference test situations. The various behavioural measures that can be assessed in the apparatus are examined and discussed.

Key words. Olfactometer, anemotaxis, olfactory responses, parasitoid, Hymenoptera, host-habitat searching, host searching, behaviour.

Introduction

Many parasitic Hymenoptera use olfactory cues and responses to orient first towards a potential host habitat and second towards their host (Vinson, 1981; Weseloh, 1981). To be able to study these processes in the parasitoids on which we are working, we needed an olfactometer in which the animals could choose between several different odours presented simultaneously.

We wished to avoid Y- or T-tube olfactometers which have often been used in the past (e.g. by Akinlosotu, 1978; Carton, 1977; Monteith, 1955; Read *et al.*, 1970; Rotheray, 1981; Shahjahan, 1974; Wylie, 1958) as they do not create clearly distinct, contiguous odour fields that can be easily entered, left, and re-entered by walking insects. Moreover there is usually turbulence at the junctions

of Y- and T-tubes which results in mixing of the odours offered. Small parasitoids therefore have difficulty in making chemotactic responses in this situation; having entered one of the arms they are usually behaviourally trapped (e.g. by a strong phototactic response) and thus have effectively made their first and final 'choice'.

In the olfactometer system described below four distinct fields of odour can be created in a chamber in which individual parasitoids can move around freely. This freedom of movement is important as it allows the parasitoids to explore the odour fields presented, sampling freely between areas containing different 'attractants' separated by sharp boundaries.

The four-armed exposure chamber of the apparatus is a development of that originally designed for the study of sex pheromones by Pettersson (1970). Our innovations include an odour inlet system for solid or fluid odour sources, catching vials to facilitate the testing of individual insects, and a sensitive

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airflow control system which creates sharp boundaries between the test odour fields.

Materials and Methods

Description of the apparatus

Fig. 1 shows the basic form of the olfactometer system; the exposure chamber is made in two parts, of transparent perspex. The four-pointed star-shape of the exposure chamber is constructed of four perspex crescents (90° arc, radius 135 mm), glued to the sheet of 3 mm thick perspex forming the ceiling (the glue used is 'Tensol' cement no. 70, ICI; a methyl methacrylate mixture, added to a solution of benzoyl peroxide in di-methyl-phthalate). Each point of the star runs without obstruction into a 5 mm (inside diameter) stainless steel tube. This array is then held firmly down on the perspex floor sheet by four metal clamps. Air leaks are prevented by a teflon tape gasket (0.1 mm thick).

Four odour fields are created in the chamber by sucking air out through the hole in the centre of its floor (membrane vacuum pump/compressor, capacity 10 l/min). With the extractor tube disconnected, this hole is also used to introduce test insects into the chamber. Airflow is regulated by pinching off the rubber extractor tube with a screw clamp. Reducing a strong airflow is essential to stabilize the desired – lower – speed, which is measured by a flowmeter (Brooks, type R-215-A, carboloy float).

Each arm of the chamber is connected to a set of three 50-ml glass vials. That closest to the chamber serves as a trap to catch insects reaching that tube. The odour source is provided by the second vial, and the outer vial contains distilled water over which the incoming air is passed to create a high, uniform humidity. The airflow through the four arms is equalized by regulating each arm separately with a Rotaflo fine metering valve and a Brooks R-2-65-5 flowmeter. All connections are made of silicon rubber tubing.

Two fluorescent tubes are hung either side of the video camera above the chamber, providing an even illumination of about 350 lx in the chamber. The video camera (positioned centrally above the chamber) and a monitor are used to observe the beha-

viour of test insects; if necessary observations are recorded on video-tape.

During preliminary tests it appeared that movements by the observer disturbed the parasitoids. The whole system (except for the monitor) is therefore surrounded by white paper walls, and observations are made exclusively via the video set-up. A video borderline detector can also be used to perform completely automated observations, as described by van Lenteren *et al.* (1976).

Fig. 2 indicates the nature of the view of the chamber provided by the monitor screen. The form of the chamber with its curved walls prevents the four air flows from mingling and creates sharp borderlines between adjacent fields. This pattern, however, is obtained only with a specific flow rate. To determine this and to show the form of the flow fields we sucked NH_4Cl 'smoke' (created from NH_4OH and HCl in the inlet vials) through the apparatus over a black background. Figs. 3(a) to 3(c) show the establishment of the flow fields during the first few seconds after starting the flow. At first the fields are only partially filled with smoke (Figs. 3a and 3b), but within seconds they become uniformly white with visible straight borderlines (Figs. 3c and 3d).

The sharpest boundaries, with no detectable intermingling, were produced with a flow rate of 300 ml/min through each arm. Only with deviating flow rates were turbulence and mixing of adjacent fields observed around the centre and in the corners of the fields. For experimental observations the borderlines of the odour fields were drawn on the monitor screen. We did not try to measure concentrations within the fields.

Procedure of the experiments

The parasitoids were tested individually. First the experimental odour fields were set up in the chamber (with 4×300 ml/min overall flow rate). Then the extractor tube was disconnected to allow the test animal to enter the 50 mm long teflon tube leading up to the hole in the centre of the chamber floor. After this the air flow tube was re-connected and the air flow restarted.

As most of the parasitoids showed a positive phototactic reaction under these circumstances, they walked up into the chamber.

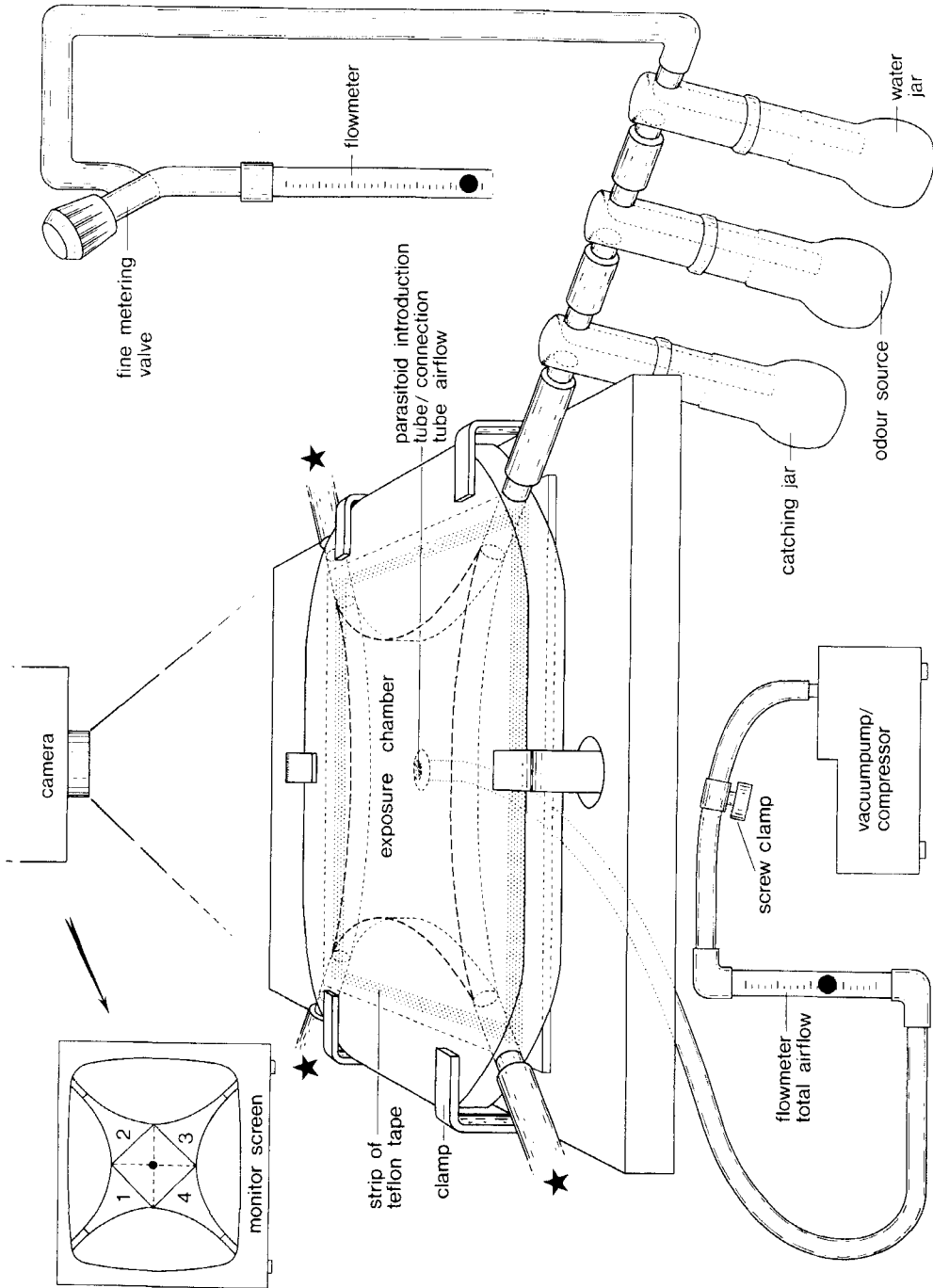
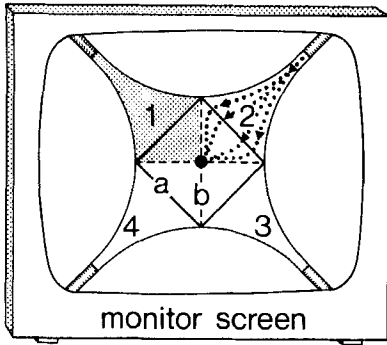


FIG. 1. Perspective view of the olfactometer. Air is drawn by the vacuum pump through the exposure chamber equally from four odour sources (only one shown; rest represented by stars) and flows towards the centre of the chamber and out through the central hole. Internal dimensions of chamber: height, 10 mm; narrowest width across, 1.10 mm; inside diameter of air inlet tubes, 5 mm.



- a first choice line
- b borderlines odour fields
- ... direction of flow

FIG. 2. Diagrammatic representation of video monitors' view of the olfactometer exposure chamber indicating the boundaries, and direction of airflow of the four odour fields.

In the vertical entry tube the test animal was exposed to a mixture of the four odours offered until it reached the floor of the chamber where, depending on which sector of the hole it climbed out of, it started walking in one of the four odours. Thereafter it could move at freedom over the floor or on the ceiling and either stay in the same odour field, or leave it, sample the others and select one of them.

When the parasitoid crossed one of the lines of the arbitrary 'first choice' square (Fig. 2) it was recorded as having made a first choice. This first choice was highly correlated with the sector of the entry tube via which the parasitoid first approached the chamber floor. The first choice may therefore in fact have been made upon leaving the entry tube, but it was easier to define on the monitor as the animal crossed the first choice line.

After this first choice was recorded, the parasitoid was given 10 min in the olfactometer. During this period the times when it left and entered the different odour fields were recorded. Depending on the responses to the stimuli offered, the parasitoid might make a 'final choice' by walking all the way up one of the air flows to end up in a catching vial. If it had left the exposure chamber for more than 2 min (a period after which returns to the chamber were rare), the experiment

was stopped and the remaining experimental time assigned to the odour field finally chosen. Each odour situation was tested with at least forty different animals.

Although statistical analysis indicated no directional bias in the chamber (see below), the whole apparatus was rotated 90° after every ten to fifteen insects were tested, and at that point the chamber was cleaned out with 96% ethanol. Between each different odour test, the whole system was dismantled, thoroughly washed with hot detergent and swabbed out with ethanol. Odourless control tests revealed only random distribution of the insects, showing the thoroughness of this cleansing procedure.

Analysis of experimental data and Discussion

Control tests for random distribution

First we conducted tests to determine whether there was any deviation from a random distribution over the exposure chamber. Table 1 gives the results of an experiment with female *Leptopilina clavipes* (Hartig) (Eucoilidae) in which an equal amount of decaying mushroom was put in each test vial. Females are strongly attracted to this medium in which their host, *Drosophila phalerata* Meigen, normally develops (Vet, in prep.).

Note that the mean per cent of time spent per odour field is given in all tables for ease of interpretation. However, it is relative times

TABLE 1. Response of female *Leptopilina clavipes* in the exposure chamber, with each arm producing odour of an equal amount of decaying mushroom.

Response	n	Odour field in arena				P
		1	2	3	4	
No. first choices	56	14	12	15	15	0.93*
No. final choices	53	10	16	12	15	0.63*
Mean % time spent/field	56	19.2	28.9	22.7	29.2	0.82**

*Chi-square test. **Friedman two-way analysis of variance by ranks, corrected for ties, based on relative times. n = number of females tested.

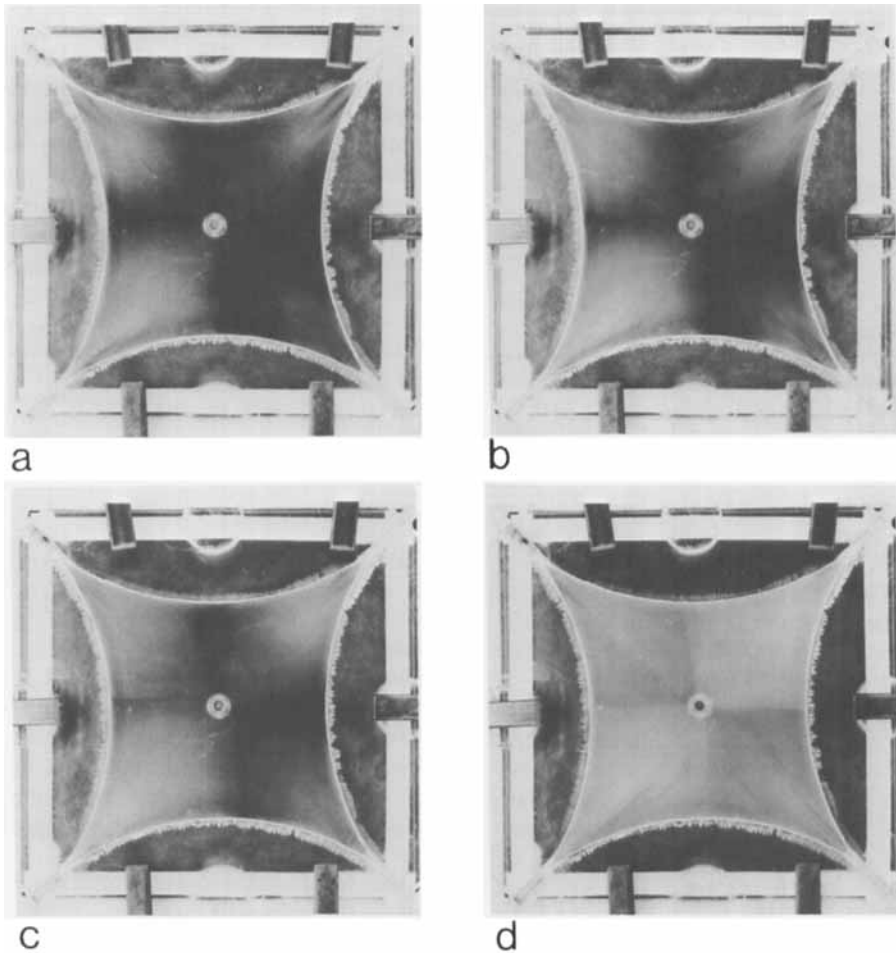


FIG. 3. Photographic representation of the four flow fields. NH_4Cl 'smoke' was sucked through the apparatus with a flow rate of 300 ml/min through each arm. (a) to (c), establishment of flow fields during the first seconds after starting the flow: (a) and (b), partially smoke-filled fields (c), sharp boundaries between fields becoming visible. (d), well-established flow fields; due to a somewhat stronger smoke concentration which was applied to two of the fields to achieve good photographic contrast, a slight extension of these fields occurs.

allocated to the different fields by each female that are used in statistical analysis throughout.

The results from first choice, final choice and time allocation do not differ significantly from random. Similar random distribution results were obtained from tests using other species and conducted in the absence of odours, but with the standard air flow.

Testing one odour

When testing an odour likely to be an attractant, or when interested only in detecting attraction (and not repellency), we used a

single-arm, one-odour test situation. This way an olfactometer with four arms has a higher ability to detect attraction than has a simple Y-tube, since only 25% of *non*-responding animals will end up in the odour field by chance, as opposed to 50% doing so in an olfactometer with two arms. Thus, either fewer animals need to be used to obtain the same statistical power (i.e. the probability to reject the null hypothesis) or a higher statistical power is achievable for a given number of animals.

For example, if forty animals are tested in the olfactometer with four arms, and if

TABLE 2. Response of female *Leptopilina clavipes* to the odour of a living yeast suspension, offered in one arm; the three other arms produced humidified air only. Details as for Table 1.

Response	n	Odour field		P
		1 yeast	2+3+4 air	
No. first choices	40	18	22	0.003*
No. final choices	21	10	11	0.017*
Mean % time spent/field	40	49.7	50.3	0.006†

† Distribution-free multiple comparisons, calculated with Friedman rank sums, showed that the significant difference was caused by a significantly higher sum of ranks for the yeast field (Hollander & Wolfe, 1973).

the fraction of non-responding animals varies from 0.8 to 0.5, then more than twice as many animals (varying from 107 to 88) is needed when using a Y-tube in order to obtain the same statistical power. In this example the power varies from 0.56 to 0.998.

If repellency is being measured, the same reduction of the number of animals needed (relative to Y-tubes) is achieved when the odour is offered through three arms simultaneously and one arm is left blank. If the characteristics of an odour are totally unknown, or one is interested in detecting attraction as well as repellency, the test situation is two-sided statistically. In this case, the test odour should be offered through two arms, and clear air through the other two arms.

Results of sample attractant experiments are given in Tables 2 and 3. *Leptopilina clavipes* females tested with the odour of living

TABLE 3. Response of female *Leptopilina fimbriata* to the odour of uninfested host food (decaying beet leaves), offered in one arm; the three other arms produced humidified air only. Details as in Tables 1 and 2.

Response	n	Odour field		P
		1 leaves	2+3+4 air	
No. first choices	50	38	12	$< 10^{-10}$ *
No. final choices	44	39	5	$< 10^{-10}$ *
Mean % time spent/field	50	83.3	16.7	$< 10^{-10}$ **

yeast (the micro habitat of several potential host species) in one arm against air in the other three, a highly significant choice was made for the yeast arm (Table 2). An identical response level with forty females tested in a Y-tube, with the non-responding females entering both arms at random would have failed to demonstrate significant attraction. Using *Leptopilina fimbriata* (Kieffer) females (endoparasitoids of larvae of *Scaptomyza pallida* (Zetterstedt) in decaying plant materials), much stronger attraction was revealed (Table 3), and experiments with several other species showed that a time allocation of 90% to one field is not exceptional.

Testing two odours

Table 4 gives the results of an experiment on *Asobara tabida* Nees (Braconidae, Alysiinae) (a solitary endoparasitoid of frugivorous drosophilid larvae) in which we tested two different odours through adjacent arms. It shows not only that females were attracted to the odour of a living yeast suspension (i.e. field 2 v. 3+4), but also that they distinguished between the odour of host-infested and uninfested yeast (i.e. field 1 v. 2), being significantly more attracted to the former.

The females also spent significantly more time in the infested yeast odour field than in any other. Many spent all their time in this field. Further, on the assumption that at least one of the fields 2, 3 or 4 were visited, the multiple comparison test shows that time spent in the yeast only field (2) is significantly longer than in fields 3 or 4 ($n=22$). The walking track of one of these females is shown in Fig. 5 (see below).

Table 5 shows the results of an experiment to test *A. tabida* females for their response to odours from a different micro habitat (decaying plant materials) and for their ability to discriminate between *S. pallida* infested and uninfested material, *S. pallida* being an alternative host. Females were reared from and had oviposited in *D. melanogaster* in yeast but were naïve to *S. pallida* and to its food.

It is clear from the first choice differences for fields 1+2 v. 3+4 that most females were repelled by the odour of the decaying

TABLE 4. Response of female *Asobara tabida* to odours of infested and uninfested host food. One arm produced odour of a living yeast suspension containing *D.melanogaster* larvae; an adjacent arm produced odour of yeast without hosts; the two other arms produced humidified air only.

Response	n	Odour field			P	
		1 yeast + host	2 yeast	3+4 air	1 v. 2	2 v. (3+4)
No. first choices	50	31	11	8	0.002*	0.023*
No. final choices	41	31	8	2	0.0002*	0.003§
Mean % time spent/field	50	69.9	20.0	10.1	<0.0005†	<0.01†

§ Binomial test. † Testing relative times for all four fields: $\chi^2_{(3)} = 62.33$, $P \leq 10^{-10}$, R_1 is significantly larger than R_j , $j = 2, 3, 4$. Test 2 v. (3+4) based on condition that time spent in 2+3+4 $\neq 0$, then R_2 significantly larger than R_3 as well as R_4 (see text). Other details as for Tables 1 and 2.

beet leaves. Rather few females made any final choice in the absence of an obvious attractant (cf. Tables 1–4), so repellency cannot be deduced from the differences in final choices. Comparison of the response to the odour of infested and uninfested leaves (field 1 v. 2) implies, however, that females, having entered the beet leaf odour, were then attracted to the odour of the larvae.

These results illustrate another important advantage of this olfactometer over Y-tubes; repellency of an odour is shown by a preference for the two blank fields and is not interpreted as an attraction to one of the odours, as will occur in Y-maze comparisons

of two odours, where initially each odour must be tested against a blank to demonstrate apparent repellency before two odours can be tested simultaneously.

Testing three odours

Asobara tabida attacks early second instar drosophilid larvae with more success than it attacks third-instars, and its risk of fatal encapsulation is substantially lower in the younger larvae (van Alphen & Drijver, 1982). We therefore investigated females olfactory responses to infested host media of different ages, containing hosts in different developmental stages.

TABLE 5. Response of female *Asobara tabida*, reared from *D.melanogaster* in yeast, to odour from a different host species and food. One arm produced the odour of decaying beet leaves infested with *S.pallida* larvae; an adjacent arm produced the odour of uninfested leaves; the two other arms produced humidified air only.

Response	n	Odour field			P	
		1 leaves + larvae	2 leaves	3 + 4 air	1 v. 2	(1 + 2) v. (3 + 4)
No. first choices	50	11	3	36	0.03*	0.002*
No. final choices	27	10	1	16	0.007*	0.33*
Mean % time spent/field	50	21.6	6.3	72.1	0.0046**	0.0007**

**Friedman two-way analysis of variance, 1 v. 2 tested on condition that time spent in 1 + 2 $\neq 0$. Other details as for Table 1.

TABLE 6. Response of female *Asobara tabida* to odours of three infested host media of different ages, containing *D. melanogaster* in different stages. Arm 1, 1-day-old medium + first and second instars; arm 2, 3-day-old medium + third instars; arm 3, 6-day-old medium + pupae; arm 4, humidified air only.

Responses	n	Odour field				P
		1	2	3	4	
No. first choices	80	42	21	12	5	<10 ^{-9*}
No. final choices	58	40	14	4	0	<10 ^{-9*}
Mean % time spent field	80	52.0	31.0	14.2	2.8	<10 ^{-9**}

* Test used: trend-test (Meelis unpubl.); under H_0 of no trend, numbers of choices follow a multinomial distribution with parameters n , and p_i , with $i = 1, 2, 3, 4$. The test-statistic $(-3X_1 - X_2 + X_3 + 3X_4)/(5n)^{-1/2}$ follows approximately $N(0, 1)$ distribution for large n . ** A distribution-free test for ordered alternatives, based on Friedman rank sums showed significant decrease in time spent with increasing age medium (Page test, Hollander & Wolfe, 1973).

Arm 1 of the olfactometer produced the odour of 1-day-old host medium (sugar, yeast and agar) containing first and second instar larvae. Arm 2 produced the odour of 3-day-old medium containing late third instar larvae. Arm 3 produced the odour of 6-day-old medium containing pupae only. Each arm contained equal weights of medium and hosts. The fourth arm was blank.

Table 6 summarizes the females' responses. They 'preferred' the odour of the youngest medium, which contained hosts of the most

suitable stage for parasitization (differences between all results from arm 1 and arm 2 are significant). This, and many of our other three- and four-odour tests, imply the presence of gradients of attractiveness between the fields, and the results can thus be analysed with trend tests. For example, for Table 6 we tested the hypothesis that attraction decreased with increasing age of the medium. First choices, final choices and time allocation show a significant negative trend (see Table 6 legend).

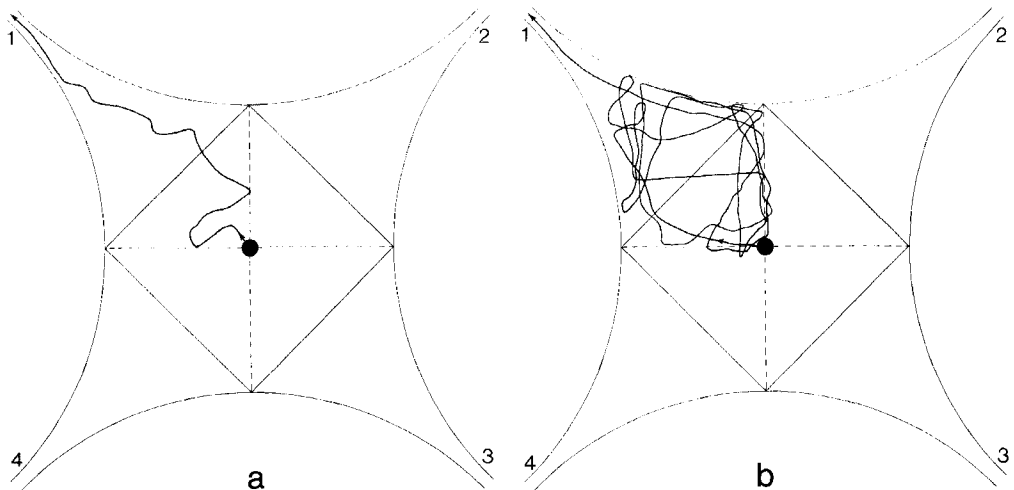


FIG. 4. Example of walking tracks of two female *Asobara tabida* in the exposure chamber during two tests with arm 1 producing the odour of a living yeast suspension; and arms 2, 3 and 4 producing humidified air only. Stops are not indicated in the tracks. Time first choice (crossing square) to final choice (leaving chamber): female a, 21 s; female b, 159 s.

Examples of walking tracks

The video records allow more detailed analysis of the insects' responses to the odour. Not only can measurement be made of the time spent in the different fields (as shown in the tables), and hence one facet of the basis of 'attraction', 'repulsion' and arrestment, but in addition, walking speed (orthokinesis) and turning (klinokinesis) can be measured, as can the nature of the insects' behaviour when they reach the borderlines between fields (e.g. whether klinokinetic or klinotactic responses). Figs. 4–6 present walking tracks of female *A. tabida* to illustrate some of these aspects.

Fig. 4 shows the walking tracks of two different females during tests with only one odour. Female *a* performed two apparently klinotactic turns at the boundaries of the odour field, followed by a presumably odour-regulated anemotactic walk (Kennedy, 1978). Female *b* spent more time searching the surface of the odour field, making repeated similar klinotactic turns. Encounters with the borders between 'attractant' odour and clean air elicited klinotactic responses which turned the females back into the odour field.

Also, in the middle of the field, females frequently stopped (not indicated in the figures), and sometimes turned 360° before continuing.

When more than one odour was presented, many females displayed tracks similar to those in Fig. 4, apparently making their first choice after encountering odour field borders, but without actually crossing them. However, especially when competing 'attractants' were tested, many parasitoids showed walking patterns of the kind illustrated in Fig. 5. Here a first choice was made for the uninfested yeast field, but 113 s after this first choice the female encountered and crossed into the field of host-infested yeast. Repeated encounters with this border elicited apparent klinotactic responses which directed the female back into the host-infested yeast field until a final choice was made 21 s after entering this field.

The track in Fig. 6 is of a three odour test. Females in such tests often sampled only one or two fields before making a final choice. Fig. 6, however, shows a track from one female that explored three fields before making a final choice different from its first. It crossed

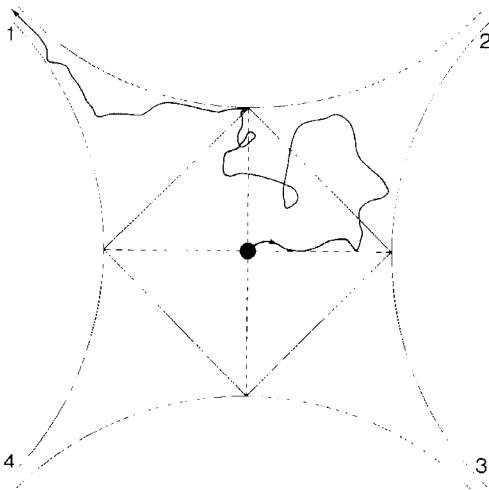


FIG. 5. Example of a walking track of a female *Asobara tabida* exploring two odour fields in the exposure chamber during a test with the arms producing the following odours: 1, host-infested yeast; 2, uninfested yeast; 3 and 4, humidified air only. The first choice was for uninfested yeast, the final choice for infested yeast. Time spent in field 2, 113 s; final choice 21 s after entering odour field 1. Same experiment as in Table 4.

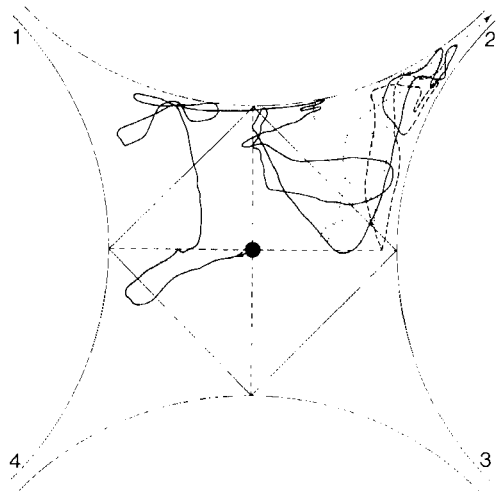


FIG. 6. Example of a walking track of a female *Asobara tabida* exploring three odour fields in the exposure chamber during a test with the arms producing the following odour: 1, 3-day-old host medium with late third instar host larvae; 2, 1-day old medium with first and second instar host larvae; 3, humidified air; 4, 6-day-old medium with host pupae. The track is broken into different lines for clarity. Time spent in fields: 4, 88 s; 1, 20 s; 2, 458 s. Same experiment as in Table 6.

two borders without hesitation from less attractive to more attractive fields, until it was retained klinotactically in the most attractive. The advantage apparent over Y-mazes is that the 'final' choice is not normally based on a single decision.

Orthokinetic responses are not quantified in these examples, but an increase in walking speed was often observed after initiation of the upwind (presumed anemotactic) walk towards the odour source.

Conclusions

Because of the klinotactic responses at the borders between attractive odour fields and blank fields, two odours can be discriminated best when offered as adjacent odour fields, leaving the other half of the chamber blank. Test animals can then freely cross the double odour boundary without having to traverse the arena through odourless air. For similar reasons, with three or four odours presented simultaneously, their sources are best changed around in the apparatus during a test in order to obtain all possible combinations of double odour boundaries.

A disadvantage of the system is the confined space in which the animals are exposed, though it is better in this regard than most Y-tubes. Since flight is virtually impossible except for very small insects, the apparatus is inappropriate for those insects which orient to odours only after initiation of flight, as seems to be the case with, for example, the braconid fruit fly parasitoid *Biosteres (Opus) longicaudatus* Ashmead (Greany et al., 1977). This drawback was of no noticeable importance, however, in tests on Alysiinae and Eucolilidae (Vet, in prep.).

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