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Chapter 2

Techniques for Behavioral Bioassays

T. C. Baker[1] *and R. T. Cardé*[2]

I. Introduction

Insect pheromone research involves tremendous effort and exacting technique not only for isolating and identifying chemicals, covered in earlier and lat chapters in this volume, but also in recognizing which chemicals are behavioral active. Tests to demonstrate the behavioral activity of a compound are essenti to proving that a compound is a pheromone component, i.e., that it is used intraspecific communication. In addition, behavioral tests, or bioassays, chemicals' effects on receiving individuals can identify the type of response th elicit. Therefore, well-designed bioassays can be invaluable for deducing t communicative function (alarm, aggregation, sexual communication, etc.) of chemical identified from an insect. They can also give information as to t mechanisms that are used by responding insects to move toward or away fro the chemical source.

The researcher must determine the objectives of the behavioral tests befo choosing among the possible bioassay setups. Perhaps none of the types of assa described in this chapter will be useful in meeting the objectives, in which case new type will be devised. There is no single "correct" assay, and elaborate on may be just as "incorrect" for an objective as types that are too simple. T assay must answer the key questions as quickly and efficiently as possible. Hen

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important to design assays so that they discriminate unequivocally among
ety of chemical fractions, synthetic analogs, or types of possible response.
haviorally discriminating assays (Kennedy, 1977) need not be expensive or
ological complex. However, their design should make use of the mecha-
of response used by the individuals receiving the chemical message. These
nses may involve direct or indirect reactions to concentration gradients
and Tobin, 1982), taxes as opposed to kineses (Fraenkel and Gunn, 1940),
e interplay of response with a cue from another modality (Kennedy, 1978).
rect reactions are caused by the chemical concentration gradient itself (Bell
Tobin, 1982), eliciting taxes (responses steered with respect to the gradient)
nkel and Gunn, 1940). Indirect reactions (Bell and Tobin, 1982) may in-
a heightened reactivity to another stimulus, for instance, when sex phero-
elicits steering with respect to wind direction (Kennedy, 1978). Or, they
simply involve an internal program of movements that are self-steered,
using proprioceptive feedback (idiothetically controlled), or feedback
the environment (allothetically controlled, Kennedy, 1978) or both.
actions previously labeled as kineses (ortho- and klino-) appear to fall into
ategory of indirect responses but either together or integrated with other
ct responses the movements can result in displacement in the "correct"
ion regardless of the lack of a "direct" response. In general, bioassays that
use of the entire pattern of natural responses and do not restrict the be-
r to one or two simple activities will be most discriminating. The principles
trategies involved in designing discriminating assays involving orientation
ons have been reviewed by Kennedy (1977).

General Considerations for Conducting Bioassays

e course of designing a bioassay, the experimenter must make several major
ons that influence the type of information generated. These include the
of apparatus in which to house and observe the insects, the way to obtain
ducible responses, whether to observe groups of insects or individuals, the
of substrate used to release the chemicals, the strength of the stimulus, the
in which two or more chemicals should be mixed, the types of behavioral
nses to be scored, and how best to record these responses.

Type of Dispenser for Chemicals

chemicals or fractions can be dispensed from a variety of materials such as
rods, metal discs, filter paper discs, rubber septa, etc. A major consideration
ensure that the surface emits the compound at a fairly constant rate over
course of the assay. This may be more difficult for very volatile chemicals
w molecular weight, and so the dispenser as well as the length of time it is
before reloading and using a fresh dispenser must be chosen properly.

Unfortunately, without actually measuring the emission rate of chemical from the surface, one suspects that either of these factors should be altered only when there is a change in the level of response over the course of using the same treatment. Such variation in response due to the release of chemical should be avoided.

2.2. Quantity of Stimulus Used

Another decision is the quantity of material to use. For a natural extract, this is usually expressed in terms of insect-equivalents, but for synthetics there may be a wide range of possibilities if the "natural" dosage of the synthetics is unknown. Ideally a dosage first should be found that is the minimal amount needed to produce a level of response useful for the purpose at hand. Usually, lower dosages are best for discriminating among the responses to various treatments.

For instance, in assaying natural fractions of moth sex pheromone, it is best first to find the minimum dosage from the complete set of fractions or crude extract that will elicit the maximum level of response from males. Then when separate fractions are tested alone or in various combinations at this concentration (in terms of female equivalents), the observer can be confident that a response as great as to the crude extract means that the full complement of necessary compounds is present. Likewise, if the optimal level of response is not generated by recombining the fractions, it can be assumed either that the fractionation process has left out a necessary compound, or that one or more compounds was altered by fractionation and rendered less active.

Another decision concerning the quantity of stimulus is whether to keep the total quantity of compounds, or the quantity of a single compound, constant across treatments. For instance, for a two-component sex pheromone blend, an increased response to the two components together might be explained as purely a quantitative effect—more total molecules are present—but if the total dosage had been held constant, the quality of the new blend could be responsible for the increased response. Of course there are other ways to clarify the interpretation. If adding increments of a second compound to a constant amount of a first compound causes first an increase of response, then a decrease as the increments increase, then it is the quality of the specific blend, not the total quantity of chemical, which is the cause of higher levels of response. Each researcher is faced with these interpretation problems, and must decide what is happening on the basis of the particular bioassay and method of blending the chemicals, plus the blends chosen as controls.

2.3. Standardization of "Responsiveness" (Internal State)

In trying to obtain reproducible responses from insects, it should be realized that behavior is a result of the interaction of external (stimulus) and internal (physiological state) factors. The insect's internal state may fluctuate throughout a 24-

period due to an underlying circadian rhythm or a periodicity triggered by
ularly occurring cues in the environment, such as dawn and dusk (Corbet,
6). Other environmental factors such as temperature may also have imme-
e modulating effects on the internal state, by modifying either periodicity
., Cardé et al., 1975) or the threshold of responsiveness (Cardé and Hagaman,
3). Fluctuations in responsiveness are the rule and thus the time of assay
uld be first optimized to the period when the insect demonstrate the most
nse response to the pheromone.

Successive presentations of a pheromone stimulus have been shown in many
ies to raise the threshold for subsequent responses or even entirely eliminate
onsiveness. Both effects are presumably the result of CNS habituation,
er than peripheral sensory adaptation, a rather transient phenomenon
tell and Lawrence, 1977; Kuenen and Baker, 1981). Additionally, respon-
ness may vary with age, particularly in species that are not reproductively
ure upon eclosion. Standardization of the level of responsiveness for be-
oral assays thus necessitates a single stimulus presentation per 24-hr interval,
ss it is first demonstrated that more than one presentation does not alter the
shold. Ideally insects could be used for an assay only once. Similarly, the age
he tested insects should be held constant, unless it is shown that the ages to
sed are comparable.

As noted earlier, the time of the assay should be optimized to the period
n the most intense response (or lowest threshold) is exhibited. Among noc-
al species, particular attention should be given to the ambient light levels.
n the light levels exceed those of full moonlight (ca. 0.3 lux), then the thres-
for responses may be raised (e.g., Shorey and Gaston, 1964). "Red" light
been used with success in many bioassays, but its use should be based upon
riments showing that the behavioral reactions parallel those under noctural
litions. The search for the quintessential environment for assay of a particu-
pecies may seem entirely superfluous, but development of a useful assay usu-
requires some description of the optimal set of environmental conditions.

series of assay treatments should be standardized so that each will have an
l opportunity to be presented to the insects during the interval of peak
onsiveness. This is especially important when a large number of treatments is
e tested; there is a danger that the internal state of the insects will have
ged drastically by the time the last treatments are tested. This type of varia-
can be accounted for and factored out of the bioassay by using a random-
complete-block design, but in addition, it can be avoided by designing the
so that a complete series of treatments is tested during a period of rela-
y shallow fluctuations of the internal state. These procedures will enhance
observer's ability to discriminate among responses to treatments.

Recording the Responses

ecording bioassay responses, it is important to optimize the time spent
rding data. Recording equipment can range from a pencil and paper to high-
lution video cameras and recorders. The recording method must be matched

with the objectives of the assay. Do not record more data than you need to answer the question for your assay. In general, the more complex the recording device, the more work it takes to analyze the results.

Audio- and video-taped observations take at least twice as long to analyze as the original assay takes to conduct. If a simple presence or absence of a behavior needs to be scored, it would be much quicker to record the results immediately with a pencil and paper. Video recording is not always necessary; for instance, the positions of a group of moths in a sex pheromone olfactometer tube can be incremented by marking the tube in five sections or so, and recording at set intervals the numbers of moths in each section. If these position numbers are tape recorded, transcribing the tape would more than double the time involved.

In many assays, particularly those (cf. Section III below) in which displacement is not monitored, only a single behavior, often termed the "key" response, is scored. In the past this was judged to be suitable in the Lepidoptera because the pheromone was thought to consist of a single chemical and the *sequence* of behavioral response to pheromone was thought to be mediated in part by increases in pheromone concentration (Shorey, 1970). Now, we know that pheromones more typically are blends of components and that in some species (see Chapter 3 by Baker and Linn, this volume) early and late behaviors in the normal sequence of response can be elicited by different combinations of pheromone components. The possibility that a critical behavior in the normal sequence of response to pheromone will be missed by monitoring only a single reaction, such as preflight wingfanning, is thus a legitimate concern.

2.5. Number of Insects in the Assay

Another consideration in recording data is whether to use groups of insects or individuals. This decision must be made case by case, and again rapidity and discrimination among treatments can be optimized by the correct choice. Group effects may alter the results and so the experimenter must decide whether such a danger exists, and if so, whether it will increase, decrease, or not affect discrimination. Again, to optimize the speed with which bioassay information is gathered, groups of insects may be best, but this decision always must be balanced with the type of recording method to be used and how much detailed information the experimenter is willing to loose.

III. Examples of Bioassays

Bioassays may be divided into two categories, those with and those without moving air. In addition, in both of these classes displacement of the insects in space may or may not be monitored, or even allowed. In still air, there is usually an insufficiently steep gradient to allow preferential movement toward or away from the stimulus unless the insect is very close to the source. In moving air, movement toward or away from the source along the windline may be restricted, as in some apparati with vertical airflow. In the remainder of the chapter, we

examine examples of bioassay devices that have been used in pheromone arch.

Bioassays without Airflow, Displacement Not Moni'ored

ıssays without airflow are very useful and simple, but they generally rely on usion to transport the test chemical toward the receiving insects. In one gory of windless bioassay, the insects are stationed such that they cannot e use of a spatial concentration gradient to move toward or away from the ce, and of course they cannot steer with respect to wind because there is e. Hence, the insect cannot use a direct response to chemical in such cases often these bioassays do not allow much movement of the insect in space way. A second class of bioassays without wind is the type in which a ıcient concentration gradient is present, and direct responses to the test nicals' gradient can occur. These assays are often larger and more elaborate ıuse the researcher is interested in the insect's displacement in space.

An example of the first type of windless bioassay system is the one used by er et al. (1976) in studies of the sex pheromone of the red-banded leaf- r moth, *Argyrotaenia velutinana*. One of the objectives of the study was to whether (*E*)-11 tetradecenyl acetate showed activity as a sex pheromone ponent along with the (*Z*) isomer, and if so, whether there was an optimal ratio. Small plastic boxes (12.4 X 9.0 X 7.0 cm) were used and each had a ll hole through which 10 males were introduced about 1 hr before assay. hole was then plugged with a cork, and a filter paper tab containing the nical blend was introduced into the box through a narrow slit at the time esting.

The optimal time of bioassay had been determined by placing a standard ount (10 female equivalents) of extract on the filter paper and presenting it different box of 10 males every 2 hr and observing them for 1 min to count maximum number of males simultaneously exhibiting wing fanning while ing. This behavior is performed by *A. velutinana* males just prior to copu- on. Only this behavior was monitored because (a) it was easy to observe and efore the percentage of males performing it could be deduced quickly; the number of males assayed simultaneously did not allow the observer to nitor the percentages of males performing other behaviors; (c) the chambers nselves were so small that they prohibited prolonged flight and other re- ses that usually occur during response to sex pheromone; (d) it became clear moths did not accumulate regularly on the filter paper tab at the end of the n period as anticipated, and so this extra measurement was unnecessary.

Baker and Cardé (1979) later showed for another moth species, *Grapholita esta*, that preflight wing fanning while walking was the behavior most highly related with ability to locate the pheromone source in a laboratory wind nel (Fig. 1). Although these behaviors may not be correlated in other species rdé and Hagaman, 1979), wing fanning has been a useful response for bio- ays of tortricid moths.

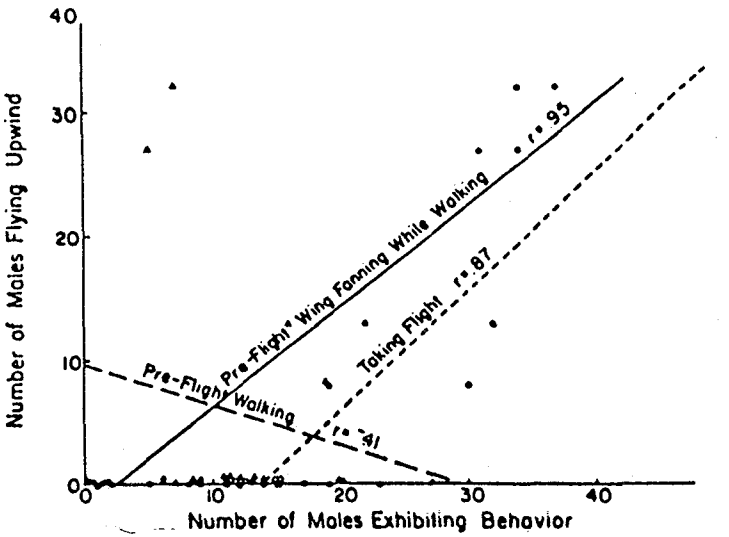


Figure 1. Correlations between pre-upwind flight behaviors and the number of *G. molesta* males flying upwind in the pheromone plume in a wind tunnel. Note the high correlation coefficient between wingfanning while walking and upwind flight (from Baker and Cardé, 1979).

Through other experiments it became apparent that the optimal response period for *A. velutinana*, normally occurring during scotophase (darkness) of the photoperiod at 24°C, could be advanced into the photophase several hours before lights-off by lowering the temperature to 16°C. Now full room illumination could be used, simplifying observation of behavior. A series of ratios of (*Z*) and (*E*)-11-tetradecenyl acetate were prepared in solution so that the same amount (2 ng) of (*Z*) was present in each. The filter paper tabs were each stored in a separate sealed vial and impregnated with the test blend only minutes before the bioassay was to begin. The vials were chosen randomly for assay, and the code number on each vial corresponding to the ratio of isomers was hidden until after all ratios had been tested. Pheromone blends were tested in a randomized complete-block design to try to factor out any unexpected time-dependent variation in responsiveness. Before each box of males was tested, it was observed for 60 sec, and the maximum number of males simultaneously fanning their wings was recorded as the "spontaneous response." Then males were observed for 60 sec after introducing the paper tabs. The maximum number of moths simultaneously fanning their wings at any time during the 60 sec was recorded. This number was corrected for spontaneous levels of wing fanning according to the formula:

$$\frac{\text{response to stimulus} - \text{spontaneous response}}{10 - \text{spontaneous response}}$$

For optimum discrimination, the importance of using a minimal dosage that elicits a maximum response became apparent here. High quantities (1-100 μg)

of (*E*) or (*Z*) alone could elicit high levels of wing fanning; however, at a dosage of 2 ng of (*Z*), only the optimal (8% *E*) ratio could cause more than 80% of the males to fan their wings simultaneously (Baker et al., 1976). This assay, despite its simplicity, could discriminate among some blends that differed by only a few percent (*E*), and the results from the laboratory agreed well with field trapping experiments showing 8% (*E*) to be the optimal blend.

Another simple bioassay without wind in which displacement was not monitored was that used by Vick et al. (1970) for sex pheromones of the dermestid beetle species of the genus *Trogoderma*. Each beetle was housed alone for 1 hr in a 3.7-ml glass vial. Then the vial's top was removed and 0.01 female-equivalents of pheromone on a 12.7-mm-diameter antibacterial assay disc was placed in the vial. The assay disc was held at the end of a glass rod, which itself was affixed to a rubber stopper. When the stopper was seated in the mouth of the vial, the assay disk was suspended 1 cm above the male. Each beetle was observed for 60 sec for evidence of running in circles beneath the disc and stretching toward it. This assay again, despite its simplicity and lack of displacement, was able to discriminate among several *Trogoderma* pheromones and demonstrate that some were species specific.

3.2. Bioassays Without Airflow, Displacement Is Monitored

Some bioassays without wind have been used successfully to test the displacement of insects in response to various chemicals. In such cases, the apparatus itself has been designed to permit movement along a chemical gradient.

A good example is the method of assaying for alarm pheromone activity in aphids. Montgomery and Nault (1977a,b) allowed groups of 20-30 aphids, all 7-9 days old, to develop on plants by removing adult females immediately after they had deposited about 30 young. Standard conditions of 21°C, 55-75% relative humidity, and light intensity of 2900 lux were used for all assays. A dilution series of the synthetic, purported alarm pheromone, (*E*)-β-farnesene was made in methanol. Then a filter paper triangle, 8 X 8 X 2 mm, was touched to a solution and allowed to saturate by capillarity. The paper was held 0.5 cm from the center of a cluster and the proportion responding by falling from the plant or walking away from the paper was recorded. The gradient was steep enough for the aphids to move away from the source, and also the intensity of the response (those falling rather than walking) decreased according to the distance from the source. This assay revealed sharp differences in sensitivity to this alarm pheromone among species and tribes of aphids (Fig. 2), and also pointed to innate differences in type of response according to whether species are usually tended by ants or not.

An example of another assay in two dimensions without wind is the type used by Hawkins (1978) and Bell and Tobin (1981) to test the activity of the natural sex pheromone extract of the American cockroach, *Periplaneta americana*. The males' movements were monitored in a 2.5-m-diameter circular arena

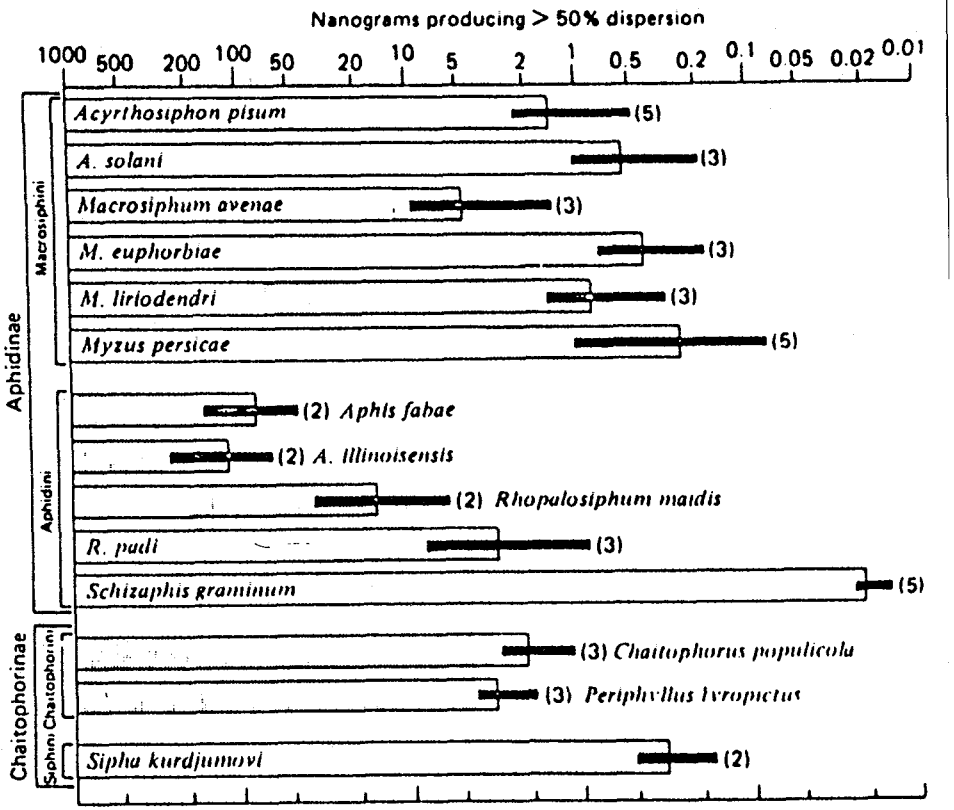


Figure 2. Responses of different species of aphids to the alarm pheromone component (*E*)-β-farnesene using a simple bioassay without wind (from Montgomery and Nault, 1977a).

with the test stimulus located in the center. In Bell and Tobin's procedure, males were kept isolated from females for at least 2 weeks in groups of 10 on a 12:12 light:dark photoperiod regime. For the photography used in the assay, a flat triangular 9 X 9 X 9-mm tab that reflected UV light was glued to each male's pronotum. The arena was painted flat black and had 20-cm-high walls which were coated with petroleum jelly to prevent the roaches from leaving the recording area. Illumination was provided by a UV light (365 nm). The males' movements were photographed from above the arena in time-lapse fashion by means of a 35-mm camera with its shutter held in the open position and a "stroboscopic" slit rotating at 60 rev/sec providing on a single frame successive shots of the roach's position 0.08 sec apart.

A single roach was placed in the arena and allowed to adjust for 15 min before its movements were photographed with only a solvent control present. Then a 5.5-cm-diameter filter paper disc loaded with extract equivalent in activity to 10^{-5} μg of synthetic periplanone B was introduced into the center of

the arena and the males' movements were photographed for 10 min. The pheromone increased the velocity of movement even at the farthest distances from the source (indirect response), and direct response to the gradient apparently was more often employed when the roaches moved to within ca 40 cm from the source (Fig. 3). Here, turns were usually toward the source (Fig. 4), indicating that the males were sampling the concentration gradient either simultaneously

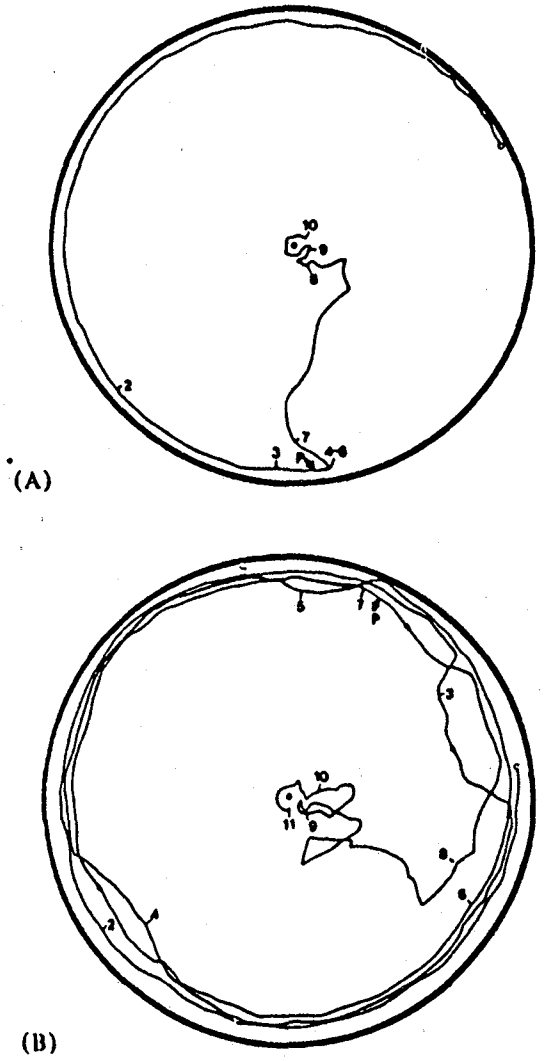


Figure 3. Typical tracks of male *P. americana* in a 2.5-m-diameter arena in response to sex pheromone located in the center (black dot). (A) Male with two antennae; (B) male with one antenna. Pheromone was introduced into the center when the males were at point P, whereupon they no longer walked around the periphery, but rather headed toward the center, of the arena.

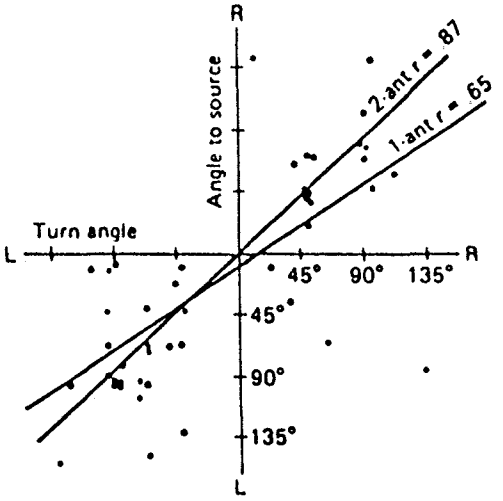
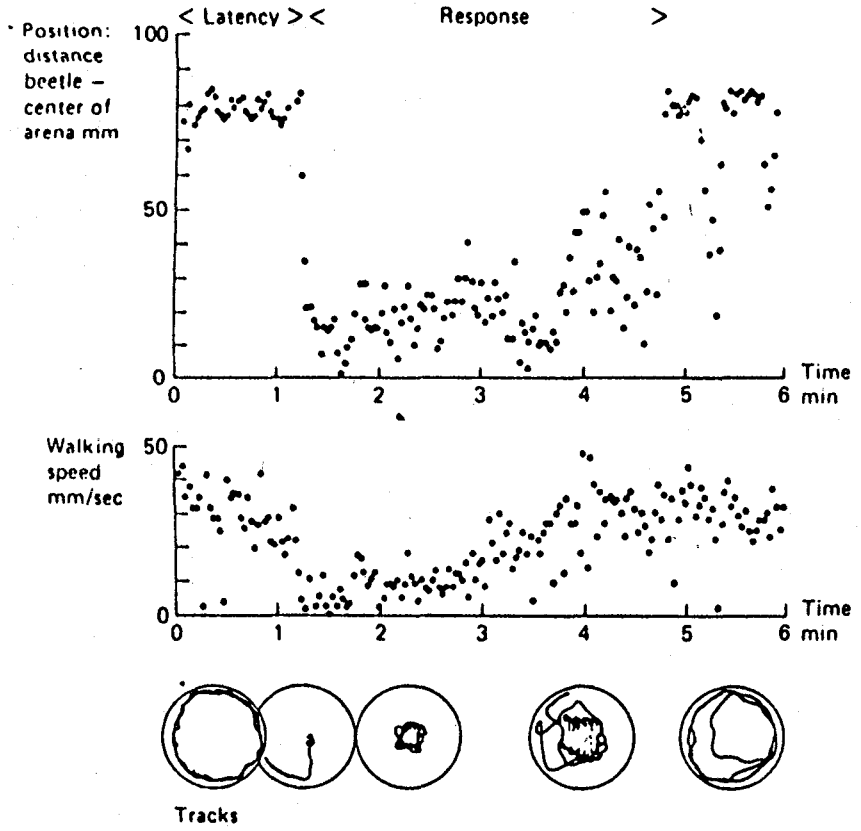


Figure 4. Correlation between the angle the roaches turned and the actual "correct" angle toward the source when males were <40 cm from the source in the arena shown in Fig. 3. The high coefficients of correlation especially for two antennae-males indicates a direct response to the concentration gradient (from Bell and Tobin, 1981).

(using their long antennae) or sequentially, and the gradient was sufficiently steep to allow them to move accurately toward the higher concentration using the direct orientation mechanisms, tropotaxis or klinotaxis.

A similar type of assay was used by Von Keyserlingk (1982) to test the activity of the aggregation pheromones of *Scolytus scolytus* as well as their responses to host odor and water. Although the "arena" was much smaller, a 5-cm-diameter petri dish, a similar type of direct response to pheromone was recorded when the beetles walked away from the walls toward the arena's center where the source was located (Fig. 5). Here the responses were recorded simultaneously with two high resolution video cameras, one stationary for track recording and a second, mobile camera with a macro lense for magnified viewing of behavioral events. Both video signals were blended onto the same videotape and later played back and analyzed with a microcomputer. The slow motion and single frame facilities of the video recorder and the use of the computer as both track analyzer and 30-channel event recorder made uninterrupted analyses of behavioral details over extended periods of time comparatively fast and easy. The computer plotted the insects' tracks and printed out a statistical analysis after each run. Again, care had to be taken to standardize the time of assay and physiological state of the beetles.

Assaying putative trail pheromone components, such as those used by ants for recruiting nestmates, also involves monitoring spatial displacement, and so these assays must allow for movement in two dimensions as in the roach assay described above. Again, with no moving air, the pheromone must be presented



gure 5. Tracks of *Scolytus scolytus* beetle responding to aggregation phero-one in the center of the petri dish arena (bottom). Computer-assisted analysis cludes simultaneous readout of the beetle's velocity and distance from the nter, and these values can be correlated with event recordings of the behavior leo-recorded on a second camera equipped with a macro-lens (from Von yserlingk, 1982).

the insect with a sufficiently steep gradient so that displacement aided by the adient can occur. The trail pheromone bioassay used by Van Vorhis Key and ıker (1981) for the Argentine ant, *Iridomyrmex humilis*, utilized a circular position of test chemical on a large piece of filter paper. The test compounds ere pipetted onto the paper as it revolved on a phonograph turntable. This lowed the chemicals to be deposited in a narrow line and with an even concen-ation along the circle; both of these deposition characteristics were important achieving reproducible responses.

Another factor crucial to reproducibility of responses was standardizing the ıternal state of the ants. Workers that had been fed only water for a few days ere given sugar water. They returned to the colony to recruit other workers, nd it was these recruits that were diverted individually through a movable

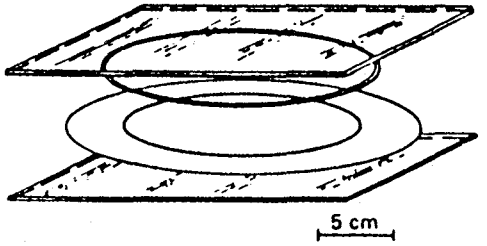


Figure 6. The assay disc (wide, lower circle) containing the circular test trail (dark circle) was housed between two glass plates separated by a Teflon spacer ring (upper circle). Thus there was no wind, and ants were scored for the durations of continuous contact with the trail (from Van Vorhis Key and Baker, 1981).

plastic (Teflon) tube onto the treated paper. Recruited workers were most likely to respond to the pheromone with continuous locomotion around the circular trail. Wind was negligible because a glass plate was placed over the trail and held above the paper by a circular Teflon spacer ring (Fig. 6). Ants were scored as trail-following when they were moving inside of two lines drawn in pencil 0.5 cm to either side of the deposited trail.

The average duration of each bout of continuous trail-following was calculated by dividing the total time spent in the trail vicinity (within the lines) by the number of times the ant crossed out of the trail's scoring area. This assay discriminated among several positional and geometrical isomers of a trail pheromone component of the Argentine ant (Van Vorhis Key and Baker, 1981, 1982).

A choice bioassay was also created to test preference of the ant for its trail pheromone extract compared to the single synthetic component. Two circular trails were deposited by using the phonograph apparatus so that the trails intersected in two places (Fig. 7). An ant following one trail encountered several choice points where it had a chance to begin following the alternate trail. The

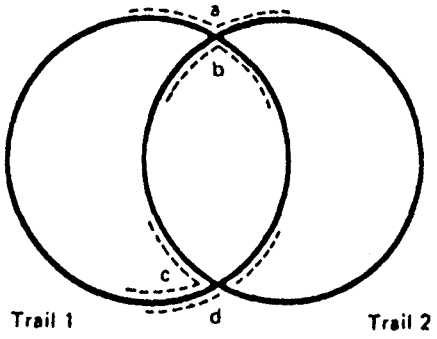


Figure 7. A choice test arrangement of circular trails deposited so that they overlapped and ants periodically had to choose to either remain on the same trail or switch to a new one (from Van Vorhis Key and Baker, 1982).

synthetic trail pheromone component (*Z*)-9-hexadecenal was found to be preferred by ants over gaster extract if the concentration of synthetic compound was high enough. At lower concentrations the ants switched equally as well between synthetic and natural extract trails. In these assays, the gradient was sufficient to cause a direct response because the ants were never more than a few millimeters from the applied trail, where the gradient was steepest. Of course indirect responses involving speed of locomotion (orthokinesis) were also integrated into the response to result in displacement along the trail.

The tent caterpillars' (*Malacosoma* sp.) responses to trail pheromones deposited between the nest and foraging sites has been studied in detail by means of several innovative assays (Fitzgerald and Gallagher, 1976; Fitzgerald and Edgerly, 1979). With one setup (Fig. 8), the responses to new trails were shown to be higher than those to old trails, and pheromone extracted from silk deposited by walking larvae was shown to be active in eliciting trail-following apart from the effect of silk alone.

Second-instar larvae were allowed to construct a silk nest on an inverted tripod, and they foraged on a small cherry tree connected to the nest by a bridge (Fig. 8) (Fitzgerald and Gallagher, 1976). As often as four times a day, the larvae moved from the nest to the cherry tree to feed, and if observations needed to be performed during the dark phase of the 15:9 light:dark photoperiod, a subdued red light was used. The key to the assay was a removable section of the bridge that could be treated experimentally. For example, when an established trail on a glass rod was extracted with methylene chloride and replaced the larvae would not cross it. When the extract was added back to the silk on the glass rod, trail-following resumed.

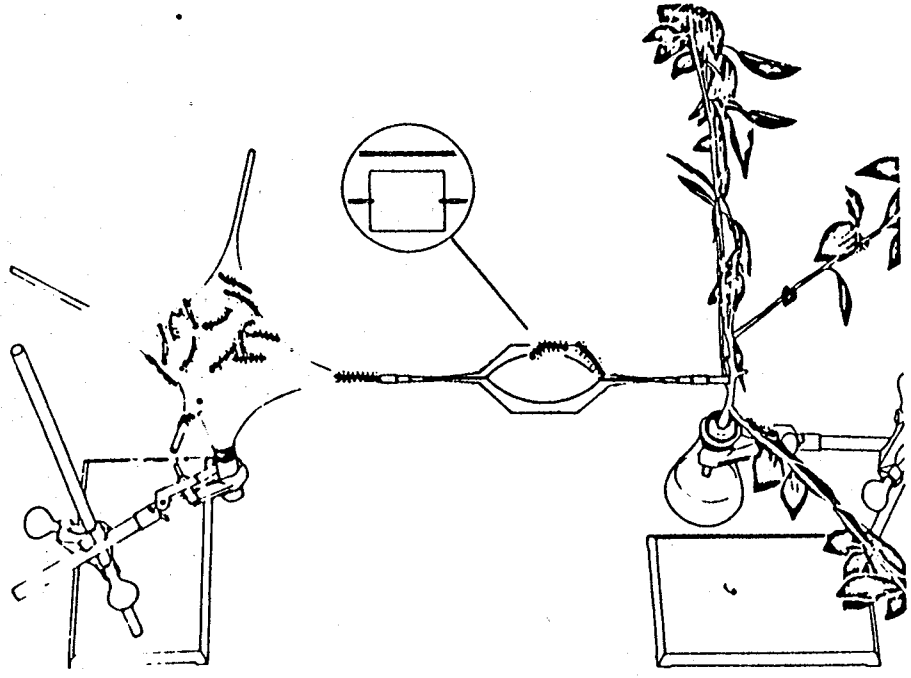


Figure 8. An assay system for the trail pheromone of *Malacosoma* larvae. The setup allows larvae to forage in cherry foliage, but to do so they must walk across a removable section of the bridge that can be replaced by an experimentally altered section (from Fitzgerald and Gallagher, 1976).

In another experiment, a plate, rather than a rod was used to test in choice fashion the response of larvae to extract-alone trails compared to washed silk alone. The larvae usually chose the extract trail and deposited new silk trails on it. They even could be induced to follow elaborate, curved trails that had been drawn with methylene chloride extract on larger plates that were inserted into the bridge. A Y-maze, choice section was also inserted into the bridge to test old versus new trails.

In another type of choice test whole silk trails were deposited on filter paper and cut up to form a "Y" (Fig. 9) (Fitzgerald and Edgerly, 1979). Larvae crawl-

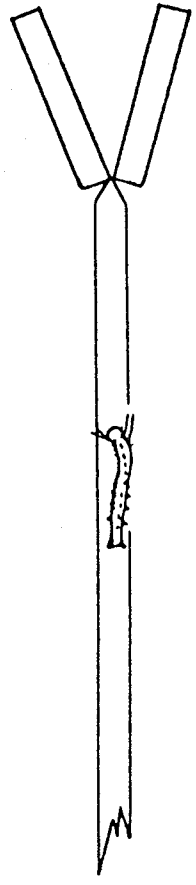
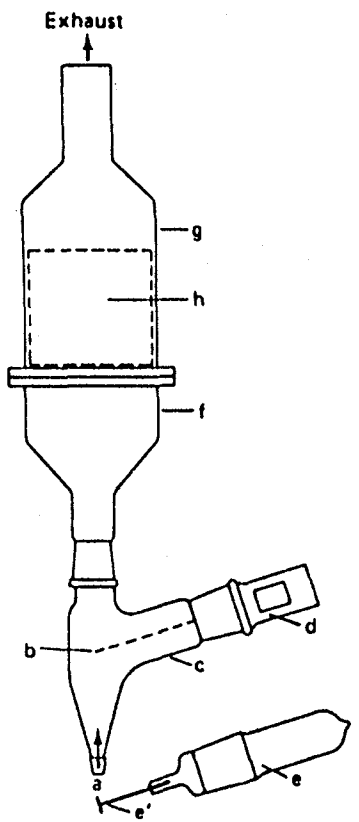


Figure 9. A choice test for the trail pheromones of larvae *Malacosoma sp.* and *Archips cerasivoranus* (from Fitzgerald and Edgerly, 1979).

ıg up the main trail had to choose a trail at the branch point. This assay was ble to show that *Malocasoma distria* and *americana* readily followed each ther's trails, but trails of a tortricid species, *Archips cerasivoranus*, were not ollowed by these two tent caterpillars.

.3. Bioassays with Airflow, Displacement Not Monitored

y the use of moving air in bioassays one can examine anemotaxis, or steering ith respect to wind direction. This indirect response to pheromone (Bell and obin, 1982) can be triggered by odor and used by the insect to move toward or vay from the source (Kennedy, 1977, 1978). The researcher can use this wind-



igure 10. A stimulation-type bioassay chamber with airflow but not allowing ıe insects to displace. Air enters the apparatus at (a) and flows over the phero- ıone-impregnated brass disc (e') attached to a ground-glass stopper (e) which is ormally inserted at (c) (the disc rests in the airstream at point (b)). The phero- ıone-laden air enters the glass cylinder (f, g) in which a cage of males (h) is oused. The observer scores the males for the "key" response of activation rom Bartell and Shorey, 1969).

steered displacement as yet another way to discriminate among treatments. There is no need for a steep gradient emanating outward from the source, and the wind delivers the odor quickly to the test insects.

However, airflow can be utilized without displacement, either by restricting the insects' movements in all directions, or more importantly, by placing the wind direction perpendicular to the only plane in which the insect is allowed to move. This technique was used by Bartell and Shorey (1969) in bioassaying the sex pheromone of the light-brown apple moth, *Epiphyas postvittana*.

Groups of 10 unmated male moths were placed in copper wire-mesh cylindrical cages, 7.6 cm diameter X 7.0 cm high. The cages then were placed individually into vertically standing glass cylinders (Figs. 10, 11), into which compressor-generated air was blown from the bottom at a rate of 5 liters/min. All assays were conducted at ca. 2 hr after lights-off on a 14:10 photoperiod regime with transitional dawn and dusk light intensities. The sex pheromone extract was impregnated onto a brass disc which was introduced into the air-stream through a port in the inlet tube at the bottom of the cylinder (Fig. 10). Pheromone and air mixed in a small chamber before entering the chamber con-

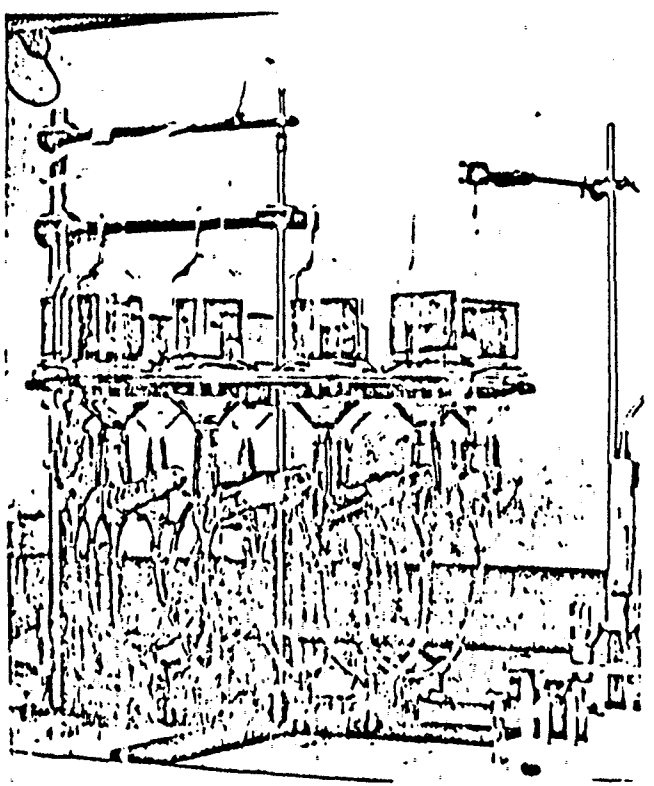


Figure 11. A group of the assay chambers depicted in Fig. 10, containing males ready for assaying (from Bartell and Shorey, 1969).

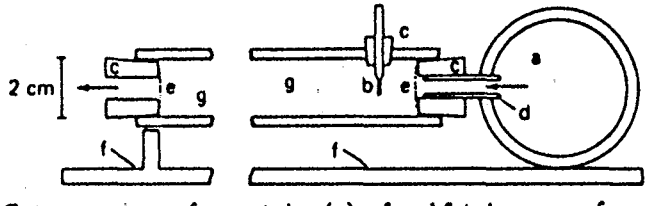
ing the moths, where movements were limited to a plane perpendicular to airflow or only minimally up and down in the chamber. The observer sed only on one "key" response and scored the group of moths for the entage of individuals moving. The overall measure, therefore, was a general mulation" due to pheromone without displacement with respect to the wind ction.

his type of assay has been used for identifying sex pheromone blends of r moth species such as *Heliothis virescens* (Roelofs et al., 1974). Other ys using wind but not permitting displacement along it have been successful dentifying sex pheromones from a large number of species. The use of ivation" bioassay chambers connected directly to a split gas chromatogra- outlet is described in Chapter 8.

Bioassays with Airflow, Displacement Is Monitored

n wind is parallel to the plane of displacement, it can aid both the speed of very of odor to the insects and the insects' movements along the windline ard or away from the source. The simplest arrangement allows movement ig the windline, but restricts it perpendicular to this line.

A very useful assay using such a one-dimensional displacement was developed Sower et al. (1973) to document the reaction of *Sitotroga cerealella*, the goumois grain moth to sex pheromone. Groups of 8–12 males were placed in -cm-inside diameter X 44-cm-long Plexiglas tubes. Charcoal-filtered air ering a manifold was distributed to 15 such tubes (Fig. 12), and a system of ppers and screens at each end prevented the moths from escaping while allow- air to move through. The tubes were lighted from below by diffuse light of lux. Pheromone-laden air was exhausted through a fume hood. The phero- ne treatment, either female extract or a synthetic analog, was deposited from ition onto a 0.5-cm glass applicator which was inserted through a stopper



ure 12. Cutaway view of one tube (g) of a 15-tube array for assaying dis- cement of male moths in one dimension in response to wind plus pheromone. manifold (a) feeds air into the 15 tubes, and it enters each tube through a e (d) covered by a screen (e). Pheromone evaporates into the airstream from a ss rod (b). A screen (e) at the downwind end prevents males from leaving that l of the tube. Response is measured as the net displacement of males upwind the tube (from Sower and Vick, 1973). Structure (f) is a supporting base.

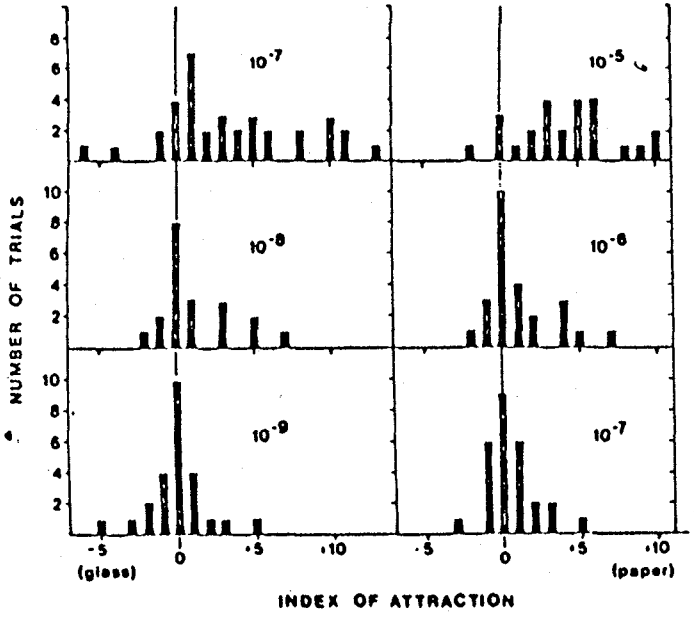
into the airstream through a port at the upwind end of the tube (Fig. 12). The number of males that had moved to within 4 cm of the source was counted after 15 and 30 sec, and from this number was subtracted the number within 4 cm of the source before introduction of the treated rod. This method of scoring allowed quick counts of the responses to be taken by hand, and the comparison of moth positions before and after pheromone introduction measured the activity of the treatment.

An advantage to measuring net movement up and down a tube is that effects of concentration can be observed. Males may accumulate farther down the tube when concentrations are too high. Daterman (1972), working with the European pine shoot moth, *Rhyacionia buoliana*, further increased discrimination among treatments by pitting positive phototaxis against the response to pheromone. He placed the downwind end of the tube in a box illuminated with dim light. The moths tended to accumulate at the downwind end of the tube before testing, and only the most attractive treatments induced them to move away from the light to the upwind end of the tube. However, the possible suppression of responsiveness by light levels above moonlight, noted earlier in the chapter, must be considered in this system. The orientation tube bioassay was used in identifying the sex pheromone components of the oak leafroller moth, *Archips semiferanus* (Miller et al., 1976).

Tobin et al. (1981) used a tube-type bioassay, modeled after one designed by Persoons (1977), to assess the activity of synthetic *Periplaneta americana* sex pheromone. A kind of choice test was performed in this assay against a blank control. Two parallel plastic tubes, 3.8 cm diameter X 30 cm, were connected to a 5.0-liter container holding 20 males. Air was drawn through the tubes at 50 cm/sec by a vacuum pump attached to the container of males. Pheromone was introduced into one of the tubes by means of a disposable pipet inserted in a stopper, and the other tube contained only a clean pipet and stopper. After 6 min, the numbers of cockroaches in the test and control tubes were counted, and for analysis the latter were subtracted from the former.

Tobin et al. (1981) were able to discriminate among several concentrations of pheromone using this assay. They also showed the importance of the release surface in influencing "threshold" values. Greater quantities of pheromone needed to be loaded onto filter paper compared to glass in order to evoke equivalent levels of response (Fig. 13).

The next increase in discrimination in moving air is gained from assays in two dimensions, usually for walking insects on a flat surface. Here, the lateral movements of the insect can take it out of contact with the pheromone, adding to the power of the assay. Under natural conditions, for instance, a male insect not only has to advance toward a sex pheromone source but also has to maintain lateral contact with it. Such a two-dimensional bioassay in wind has been utilized in studies of bark beetle aggregation pheromone by Payne et al. (1976), who modified Wood and Bushing's design (1963).



ure 13. A demonstration of the importance of the pheromone release sub-
.te. Ca. 100 times more pheromone needs to be loaded onto filter paper com-
ed to glass to elicit similar levels of *P. americana* male attraction (from Tobin
il., 1981).

The apparatus of Payne et al. consisted of a flat arena (Fig. 14), 22.5 X 28
covered with a sheet of paper that could be discarded after each assay. The
na was housed in a specially constructed 183 X 122 X 214 cm controlled-
/ironment room to optimize assay conditions for the beetles. The room was
intained at 20°C to keep the beetles from flying from the arena and relative
midity was kept at ca. 80%. The room also had adequate exhaust to remove
:romone from the room, plus an air diffuser to minimize turbulence on the
na surface. Activated charcoal-filtered airflow was provided at 1.5-2 liters/
n from a compressor, and pheromone was introduced at a constant rate from
notor-driven syringe loaded with pentane solutions of the pheromone treat-
nts to be tested. Air velocity was recorded by an anemometer at the far end
the arena. Groups of 10 beetles were placed on the arena at a starting point
:m downwind of the pheromone source and positive responses were recorded
r those walking to within 1 cm of the source. Beetles leaving the pheromone
eam were collected and rereleased for a second try. If they did not respond
this try, they were recorded as negative responses.

The internal state of the beetles was kept as constant as possible through
variety of procedures. They were collected daily from an emergence chamber,
amined for presence of all appendages (antennae, etc.), and held individually
No. 10 (ca. 1-cm-long) gelatin capsules to prevent injury. Also, daily fluctua-
ons in internal state were monitored first before performing assays on test

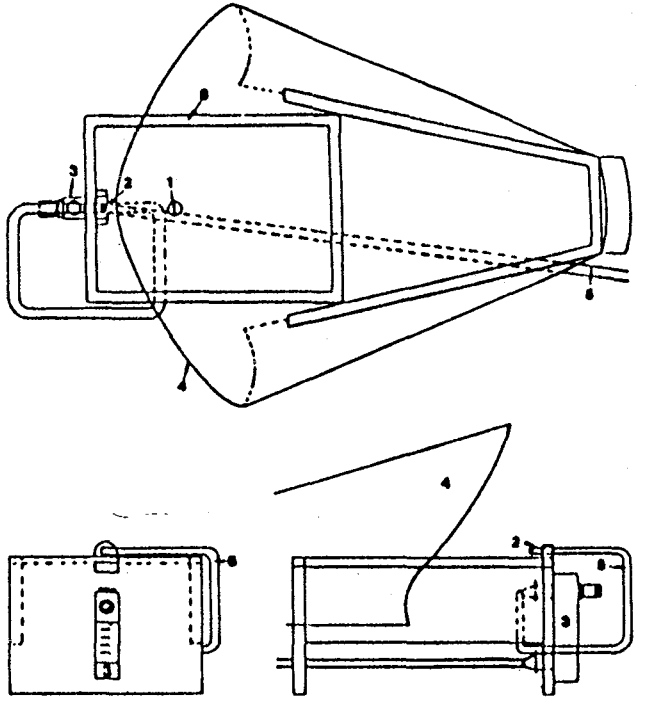


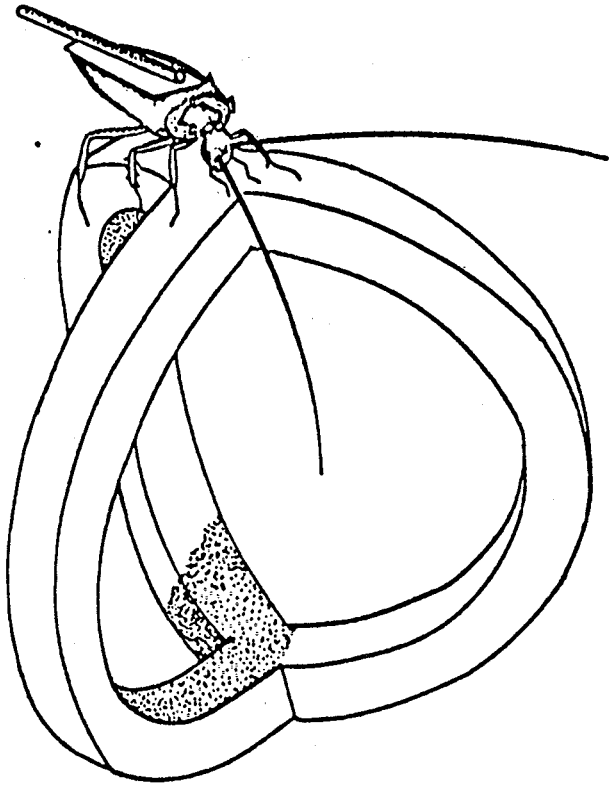
Figure 14. Top view of a bioassay arena (6) for bark beetles walking in a pheromone-laden stream. The beetles are placed on the rectangular arena at (1), from downwind of the source of the airstream (2). Beetles walking to within 1 cm of the source are scored as positive responders (from Payne et al., 1976). Bottom right is sideview. 4 is exhaust hood; 5 is a connecting tube; and 3 is an adjustable mixing chamber for pheromone and air.

materials. Groups of 10 males and 10 females were tested for their response to solvent alone and to a standard pheromone stimulus. If the positive response level to solvent was greater than 20% or if the response to the standard treatment was less than 50%, assays on test chemicals were not performed that day.

Tobin et al. (1981) used a low-airspeed, 2.4 X 1.2 X 0.6 m wind tunnel to test the activity of synthetic *Periplaneta americana* sex pheromone against natural extract. Airspeed was 22 cm/sec, and males were held individually in a wire cage for 20 min prior to assay at the downwind end of the tunnel to allow them to acclimate to the tunnel conditions. The cage was then opened and the pheromone sample introduced on filter paper suspended 2 cm from the floor. The percentage of males locating the filter paper was scored. Using this assay plus a variety of other assays and trapping experiments, they concluded that the synthetic pheromone, periplanone B, elicited the complete range of sexual behaviors in males, from long-distance orientation to close-range courtship behaviors, such as wing-raising. These were the same behaviors evoked by natural extract at comparable concentrations.

With such two-dimensional assays, measuring spatial displacement becomes :sirable and often necessary to gain maximal discrimination between treat-ents. But the types and patterns of movements used by the insect to gain this :splacement can also be monitored. Usually for this, only photographic or deo records will capture the movements in enough detail to allow analysis. f course, the amount of time needed to record and analyze these recordings isually of individual insects) increases dramatically.

A very simple and effective type of assay was used by Rust et al. (1976) to onitor turns in response to pheromone by a walking insect, the American ckroach. Individual male roaches were tethered to a 10-cm wooden applica-r stick and labeled with a mark to the back of the pronotum. The sticks were en held in a clamp on a ring stand and the cockroaches given a styrofoam Y-aze globe to "hold" (Fig. 15). Responses to sex pheromone were tested in the rk. The rate of locomotion was measured by the amount of time taken for 20 volutions of the globe to occur, and the turning tendency was measured by cording the number of left or right 60° turns taken by the males.



igure 15. Y-Maze globe used to measure the turns and locomotory rate of merican cockroach males in response to pheromone in still air and in air cur-ents (from Rust et al., 1976).

Observations were recorded on audio tape and later transcribed for analysis. Head movements of the cockroaches were filmed against a background grid (10 mm^2) using a 16-mm movie camera. From these assays, Rust et al. (1976) found that cockroaches tend to turn away from air currents without pheromone and upwind in air containing pheromone. Furthermore, by presenting the pheromone in still air to one side or the other, they found that the males could orient accurately toward the source by a simultaneous sampling of the gradient by the antennae (chemotropotaxis). If one antenna was removed, the males adapted within a few days and started sampling the gradient sequentially (chemoklinotaxis) by waving their lone antenna from side to side before executing a turn toward the pheromone.

A more elaborate record of walking insects' movements was obtained using the so-called servosphere apparatus. This was used by Kramer (1975) to monitor the pheromone-mediated movements of *Bombyx mori*, and by Bell and Kramer (1980) for *Periplaneta americana*. The apparatus consists of a Plexiglas sphere, 50 cm diameter, that is mounted so that it can be rotated in two different planes by two low-inertia servomotors (Fig. 16) (Kramer, 1975). The insect, placed on top of the sphere with a disc of reflective material attached to its dorsum, is kept in the field of an infrared light beam by the corrections of the motors on the sphere. The sphere's counter-movements required to keep the running insect in the beam are recorded and can be plotted as a record of the insect's movements.

One nice feature of the servosphere assay, and the previous one by Rust et al.

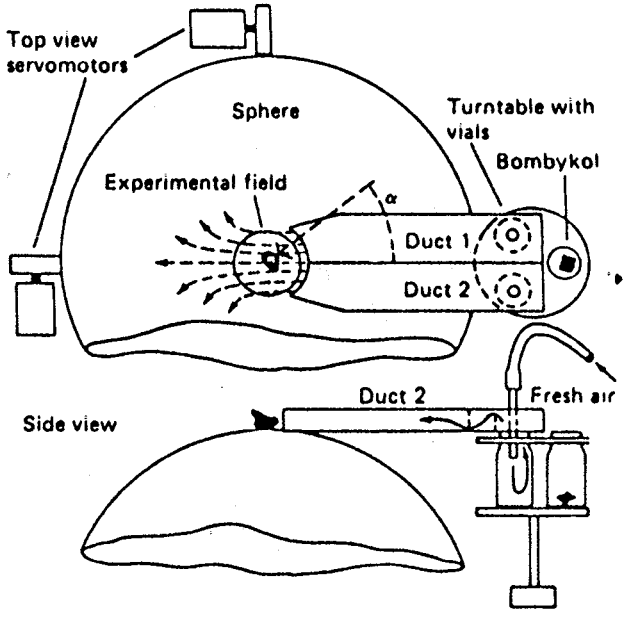


Figure 16. Servosphere apparatus for measuring movements of insects walking in response to sex pheromone plus moving air (from Kramer, 1975).

976) is that the insect remains in the same spot relative to the odor source, nd cannot enter a new stimulus situation as in the previous type of two-mensional test for bark beetles. In the latter, test concentration will increase some degree as the insect approaches the source, and this may not always be sirable. In addition, the servosphere technique may allow for better testing tween a choice of treatments than in the previous type of assay. This is be-use the insect, held at the same location between two odor streams, must con-ually choose between them and cannot take itself out of contact with one or e other. Thus the assay will measure a more *continuous* discrimination by the sect, not just an initial brief judgment after which it only moves in response one of the stimuli. Just such a choice test was performed by Kramer, who rtitioned the airstream into two halves and presented pheromone of differing ncentrations in each side. He found that *B. mori* males could discriminate tween concentrations of pheromone that differed by a ratio of only 5:3.

Monitoring insect movements in three dimensions really pertains only to cases which insects are allowed to fly or swim. In this case, wind is created in a large amber, usually 1 or more m long, called wind tunnels, or sustained-flight nnels. Kennedy and Marsh (1974) used such a tunnel for demonstrating the otomotor anemotactic response of flying *Anaghasta kuhniella* males. Miller and oelofs (1978a) demonstrated the usefulness of the wind tunnel for discrimi-ting among several pheromone treatments in the red-banded leafroller moth. is technique has been used since then to discern differences between phero-one blends for several other moths, including *G. molesta*, the oriental fruit oth (Baker and Cardé, 1979; Baker et al., 1981), and the noctuid moth, *Euxoa hirogaster* (Palaniswamy et al., 1983), the gypsy moth, *Lymantria dispar* iller and Roelofs, 1978b; Cardé and Hagaman, 1979), *Heliothis virescens* 'etter and Baker, 1982), and the cabbage looper moth, *Trichoplusia ni* (Linn d Gaston, 1981).

There are two major advantages of wind tunnels for assaying pheromone ends. First, the insect must perform a series of movements similar to those ed in the field to locate a pheromone source. Second, apart from the degree of atial displacement to be monitored by the experimenter, the *duration* of the sponse can be measured by making use of the moths' optomotor response to e ground pattern. The duration of sustained in-plume flight at a fixed point 'er a moving floor can discriminate among treatments that, using only flight to e source as a measure, would otherwise not have appeared to be different.

Miller and Roelofs' tunnel was a clear, somewhat flattened cylinder formed om the inverted "U" of two bowed sheets of Plexiglas joined at the middle, d included a treadmill of canvas, painted with a striped pattern (Fig. 17). ind was supplied by a small window fan connected to the Plexiglas by a exible plastic tube. Several layers of muslin were stretched across the tunnel's ntrance to smooth the airflow and make it essentially laminar. Lights were ounted overhead and could be varied in intensity from bright levels for day-ying insects (fluorescent lights) to moonlight intensities using a bank of

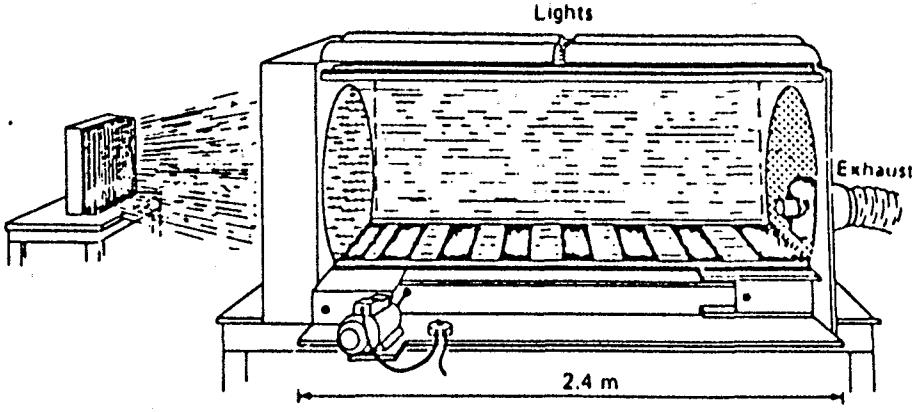


Figure 17. A typical wind tunnel for assaying pheromone compounds (from Miller and Roelofs, 1978a).

voltage-regulated incandescent bulbs covered with red cellophane. The floor pattern on the canvas treadmill was alternating 15-cm-wide red and black stripes, and the treadmill could be rotated in the direction of the wind at speeds ranging from 2 to 60 cm/sec by means of a joy stick connected to a sliding-gear variable transmission and electric motor.

Moths were introduced into the downwind end of the tunnel through a hole in the metal screening that provided a barrier to escape for moths but allowed the passage of air. Groups or individual moths were caged in screen cylinders that fit into the hole; the screen lid to the cylinder was removed just before placing it through the hole. Four different types of measurements were used to demonstrate the location and diameter of the pheromone plume at the moths' release cage in the hole. There was remarkable correspondence among titanium tetrachloride-generated smoke, a plume of hydrochloric acid on indicator paper, apple odor sensed by the human nose, and an electroantennogram as it scanned through a plume of (±) disparlure; all showed the time-averaged plume to have horizontal and vertical diameters of ca. 15 and 18 cm, respectively. In addition to the discrimination provided by sustained flight caused by the rotating floor (which showed differences between racemic and (+) disparlure, Miller and Roelofs, 1978b) many measurements could be performed with the floor station-ary that may be useful in discriminating among treatments (Table 1). These parameters are easily recorded with a pencil, paper and stopwatch, but tape-recording or use of multichannel event recorders are also very useful for record-ing wind tunnel behaviors (Baker et al., 1981). An exhaust tube (Fig. 17) scavenged the pheromone plume from the room while allowing the rest of the air to recirculate into the tunnel.

Choice tests can also be performed on insects flying in a wind tunnel. Linn and Gaston (1981) were able to show the importance of a two-component *Trichoplusia ni* pheromone blend at close range compared to a single com-

ible 1. Types of responses that can be monitored in a flight tunnel[1]

Type of behavior	$\bar{x}$ ± SD	Range
me from source introduction until first visible response (sec)	6.9 ± 2.1	3–9
me walking or running before flight (sec)	8.3 ± 4.4	3–15
me wing-fanning before flight (sec)	4.2 ± 3.3	0–9
me from source introduction until flight initiation (sec)	27.3 ± 26.8	12–85
me hovering in plume before making upwind progress (sec)	5.1 ± 3.3	1–11
ght speed (ground) over a 70-cm sector n the downwind half of the tunnel cm/sec)	17.1 ± 7.1	10.8–31.8
ght speed (ground) over a 70-cm sector n the upwind half of the tunnel (cm/sec)	14.4 ± 3.8	9.0–21.3
mber of approaches to within 2–3 cm of the source before landing	1.9 ± 0.9	1–3
mber of times dropping back more than 0 cm and reorienting before landing	0.3 ± 0.8	0–2
ot where insect lands:		
Directly on chemical source	71%	
On structures supporting source	29%	
ne landing after first coming within 5 cm of source (sec)	10.9 ± 5.3	4–19
psed time from flight initiation until anding on source (sec)	24.3 ± 5.7	15–32
ne walking or running on source (sec)	8.0 ± 9.3	3–29
ne wing-fanning on source (sec)	8.2 ± 9.7	3–30
ne genital claspers extended (sec)	8.2 ± 9.7	3–30
mber of copulatory attempts	0.1 ± 0.4	0–1
ne quiescent on source (sec)	0.1 ± 0.2	0–0.5
tal time on source	9.9 ± 14.2	3–42

ata are taken from experiments with redbanded leafroller males (from Miller and Roelofs, 78a).

nent, Z7-12:OAc, by creating two plumes that intersected at ca. 70 cm wnwind of the sources. *T. ni* males were released at their optimal period of sponsiveness into the blended, single plume. As they flew up-tunnel, they ntually had to choose to fly in one plume or the other. If one plume was the o-component blend containing both 12:OAc and Z7-12:OAc, they continued fly along it all the way to the source. If the two plumes were each compo-nt alone, the males would fly only as far as the limits of the blended plume, or haps a little farther along the Z7-12:OAc plume alone, but not all the way to e source. Z7-12:OAc alone did not elicit continued advancement toward the urce.

The use of wind tunnels in studies of orientation to sex pheromone has blos-med in the past few years. Their many uses will be explored in more detail in e following chapter by Baker and Linn.

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