Offprint from
Semiochemistry: Flavors and Pheromones
Editors: Terry E. Acree · David M. Soderlund

BEHAVIORAL ANALYSIS OF PHEROMONES

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Introduction

Insect pheromone research involves great effort and exacting techniques, not only for isolating and identifying chemicals, but also in recognizing which chemicals are behaviorally active. Well-designed bioassays to demonstrate the behavioral activity of a compound are essential to proving that a compound is used in intraspecific communication, i.e., that it is a pheromone component. Such assays can also be invaluable for deducing the communicative function (alarm, aggregation, sexual communication, etc.) of a chemical identified from an insect. They can, in addition, give information as to the mechanisms that are used by responding insects to move toward or away from the chemical source.

Any researcher must determine the objectives of the behavioral tests before choosing among the possible bioassay setups. There is no single "correct" assay, and a simple one may be just as useful in meeting the objective as a type that is very elaborate. A good assay is one that will answer the key questions quickly and efficiently, discriminating unequivocally among a variety of chemical fractions, synthetic analogs, or types of possible response.

Behaviorally discriminating assays (1) need not be expensive or technologically complex. However, their design should make

use of the mechanisms of response used by the individuals receiving the chemical message. These responses may involve direct reactions to the pheromone gradient (2), taxes as opposed to kineses (3), or the integration of these with a response to cues from another modality (4, 5). An example of the latter is found in moths flying in response to sex pheromone; the chemical blend elicits internally programmed "zigzagging" turns, which are polarized in the upwind direction due to the optomotor response to wind-induced drift (5).

General Considerations for Conducting Bioassays

1. Type of Dispenser for Chemicals. - Test chemicals or fractions can be dispensed from a variety of materials such as filter paper, rubber septa, glass rods, or metal discs. A major consideration is to ensure that the surface emits the compound at a constant rate over the course of the assay. This may be more difficult for very volatile, low molecular weight chemicals, and so the dispenser, as well as the length of time it is used 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 this rate has changed 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. Tobin et al. (6), using a tube-type bioassay modeled after one designed by Persoons (7) to assess the activity of synthetic Periplaneta americana sex pheromone, 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, presumably because the emission rate from paper was lower.

2. Quantity of Stimulus Used. - For a natural extract, the quantity of stimulus used is usually expressed in terms of insect equivalents. 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. If too high a dosage had been used, it would be possible that an incomplete set of compounds could elicit the maximum response.

Even when assaying synthetic compounds, lower dosages may be more discriminating. For instance, Baker et al. (8) found that high dosages (1-100 ug) of either of the Argyrotaenia velutinana sex pheromone components (Z)-11- and (E)-11-tetradecenyl acetate presented alone could elicit high levels of wing fanning; however, at a dosage of 2 ng of (Z), only the optimal [8% (E) added to (Z)] ratio could cause more than 80% of the males to fan their wings simultaneously.

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. Baker et al. (8) found that increasing the amount of (E)-11-tetradecenyl acetate from 0% to 100% of a constant 2 ng (Z)-11-tetradecenyl acetate caused first an increase, then a decrease of wing fanning by male A. velutinana as the proportion of (E) approached, then exceeded 8%. Blend quality was responsible for the behavior.

3. Standardization of "responsiveness". - In trying to obtain reproducible responses from insects, it should be remembered that behavior is a result of the interaction between external (stimulus) and internal (physiological state) factors. The insect's internal state may fluctuate throughout a 24-hr period due to an underlying circadian rhythm or a periodicity triggered by regularly occurring cues in the environment, such as dawn and dusk (9). Other environmental factors, such as temperature, may also have immediate modulating effects on the internal state, by modifying either periodicity (10) or the threshold of responsiveness (11). Fluctuations in responsiveness are the rule and thus the time of assay should be first optimized to the period when the insect demonstrates the most intense response to the pheromone. Among nocturnal species, particular attention should be given to the ambient light levels. When the light levels exceed those of full moonlight (ca. 0.3 lux), then the threshold for responses may be raised (12). "Red" light has been used with success in many bioassays, but its use should be based upon experiments showing that the behavioral reactions parallel those under nocturnal conditions lacking red light.

Successive presentations of a pheromone stimulus have been shown in many species to raise the threshold for subsequent responses or even entirely eliminate responsiveness. Both effects are presumably the result of CNS habituation, rather

than transient sensory adaptation (13, 14). Responsiveness may also vary with age, particularly in species that are not reproductively mature upon eclosion. Standardization of the level of responsiveness for behavioral assays thus necessitates a single stimulus presentation per 24-hr interval, unless it is first demonstrated that more than one presentation does not alter the threshold. For most assays it is best that insects initially are used only once to simplify interpretation of results.

A series of assay treatments should be standardized so that each will have an equal opportunity to be presented to the insects during the interval of peak responsiveness. This is especially important when a large number of treatments is to be tested; there is a danger that the internal state of the insects will have changed drastically by the time the last treatments are tested. This type of variation can be accounted for and factored out of the bioassay by using a randomized, complete-block design, but in addition, it can be avoided by designing the assay so that a complete series of treatments is tested during a period of relatively shallow fluctuations of the internal state.

4. Recording the responses. - Recording equipment for bioassay responses can range from a pencil and paper to high-resolution video cameras and recorders. The recording method must be matched with the objectives of the assay. In general, the more complex the recording device, the more time-consuming it is 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.

In many assays, particularly those 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 (15). Now we know that pheromones more typically are blends of components and that in some species 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 wing fanning, is thus a legitimate concern.

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 lose.

Examples of Bioassays

1. Bioassays without airflow, displacement not monitored. - Bioassays without airflow are very useful and simple, but they generally rely on diffusion to transport the test chemical toward the receiving insects. In one category of windless bioassay, the insects are stationed such that they cannot make use of a spatial concentration gradient to move toward or away from the source, and of course they cannot steer with respect

to wind because there is none. Hence, the insect cannot use a direct response to chemicals in such cases. A second class of bioassays without wind is the type in which a sufficient concentration gradient is present and direct responses to the gradient of the test chemical can occur. These assays are often larger and more elaborate because the researcher is interested in the insect's displacement in space.

An example of the first type of windless biossay system is the one used by Baker et al. (8) in studies of the sex pheromone of the redbanded leafroller moth, Argyrotaenia velutinana. One of the objectives of the study was to see whether (E)-11 tetradecenyl acetate showed activity as a sex pheromone component along with the (Z) isomer, and if so, whether there was an optimal Z:E ratio. Small plastic boxes (12.4 x 9.0 x 7.0 cm) were used and each had a small hole through which 10 males were introduced about 1 hr before assay. The hole was then plugged with a cork, and a filter paper tab containing the chemical blend was introduced into the box through a narrow slit at the time of testing. The optimal time of bioassay had been determined by placing a standard amount (10 female equivalents) of extract on the filter paper and presenting it to a different box of 10 males every 2 hr and observing them for 1 min to count the maximum number of males simultaneously exhibiting wing fanning while walking. This behavior is performed by A. velutinana males just prior to copulation. Baker and Carde (16) later showed for another moth species, Grapholita molesta, that preflight wing fanning while walking was the behavior most highly correlated with ability to locate the pheromone source in a laboratory wind tunnel. Although these behaviors may not be correlated in other species (17), wing fanning has been a useful response for bioassays of tortricid moths. Despite its simplicity, this assay discriminated among some blends that differed by only a few percent of the E isomer, and the results from the laboratory

agreed well and with A. velutinana field trapping experiments showing 8% (E) to be the optimal blend.

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 (18, 19) 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. A dilution series of the synthetic alarm pheromone, (E)-beta-farnesene, was made in methanol. A filter paper triangle was saturated with pheromone solution by capillary action. 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, and also pointed to innate differences in type of response according to whether species are usually tended by ants or not.

Assaying putative trail pheromone components, such as those used by insects for recruiting nestmates, also involves monitoring spatial displacement, and so these assays must allow for movement in two dimensions. Again, with no moving air, the pheromone must be presented to the insect with a sufficiently steep gradient so that displacement aided by the gradient can occur. The responses of the tent caterpillar (Malocasoma sp.) to trail pheromones deposited between the

nest and foraging sites have been studied in detail by means of several innovative assays (20, 21). With one setup 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.

3. Bioassays with airflow, displacement not monitored. - By the use of moving air in bioassays one can examine anemotaxis, or steering with respect to wind direction. This indirect response to pheromone (2) can be triggered by odor and used by the insect to move toward or away from the source (1, 4). The researcher can use this wind-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 (22) in bioassaying the sex pheromone of the light-brown apple moth, Epiphyas postvittana, and for the pheromone's identification (23).

Groups of 10 unmated male moths were placed in copper wire-mesh cylindrical cages. The cages then were placed individually into vertically standing glass cylinders into which compressor-generated air was blown from the bottom at a rate of 5 liters per minute. 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 airstream through a port in the inlet tube at the bottom of the cylinder. Pheromone and air mixed in a small chamber before entering the chamber containing the

moths, where movements were limited to a plane perpendicular to the airflow or only minimally up and down in the chamber. The observer focused only on one "key" response and scored the group of moths for the percentage of individuals moving. The overall measure, therefore, was a general "stimulation" due to pheromone without displacement with respect to the wind direction. This type of assay has also been useful for identifying sex pheromone blends of a large number of other moth species such as Heliothis virescens (24).

4. Bioassays with airflow, displacement is monitored. - When wind is parallel to the plane of displacement, it can aid both the speed of delivery of odor to the insects and the insects' movements along the windline toward or away from the source. The simplest arrangement allows movement along the windline, but restricts it perpendicular to this line.

A very useful assay using such a one-dimensional displacement was developed by Sower et al. (25) to document the reaction of Sitotroga cerealella, the Angoumois grain moth, to sex pheromone. Groups of 8-12 males were placed in Plexiglas tubes. Charcoal-filtered air entering a manifold was distributed to 15 such tubes and a system of stoppers and screens at each end prevented the moths from escaping while allowing air to move through. The tubes were lighted from below by diffuse light of 0.3 lux. Pheromone-laden air was exhausted through a fume hood. The pheromone treatment, either female extract or a synthetic analog, was deposited from solution onto a 0.5 cm glass applicator which was inserted through a stopper into the airstream through a port at the upwind end of the tube. 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 (26), 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 best treatments induced them to move away from the light to the upwind end of the tube. However, as noted earlier, the possible suppression of responsiveness by light levels above moonlight 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 (27).

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. (28), who modified Wood and Bushing's design (29).

To test the activity of synthetic Periplaneta americana sex pheromone against natural extract, Tobin et al. (6) used a low-airspeed wind tunnel. Using this system 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 desirable and often necessary to gain maximal discrimination between treatments. But the types and patterns of movements used by the insect to gain this displacement can also be monitored. Usually for this, only photographic or video records will capture the movements in enough detail to allow analysis. Of course, the amount of time needed to record and analyze these recordings (usually of individual insects) increases dramatically.

An elaborate record of walking insects' movements was obtained using the so-called servosphere apparatus. This was used by Kramer (30) to monitor the pheromone-mediated movements of Bombyx mori, and by Bell and Kramer (31) for Periplaneta americana. The apparatus consists of a Plexiglas sphere that is mounted so that it can be rotated in two different planes by two low-inertia servomotors (30). 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 later can be plotted and analyzed as a record of the insect's movements.

One attractive feature of the servosphere assay is that the insect remains in the same spot relative to the odor source, and cannot enter a new stimulus situation. In a typical two-dimensional arena or wind-tunnel test, the test concentration will increase to some degree as the insect approaches the source, and this may not always be desirable. In addition, the servosphere technique may allow for better testing between

a choice of treatments than in the previous type of assay. This is because the insect, held at the same location between two odor streams, must continually choose between them and cannot take itself out of contact with one or the other. Thus the assay will measure a more continuous discrimination by the insect, not just an initial brief judgment after which it only moves in response to one of the stimuli. Just such a choice test was performed by Kramer, who partitioned the airstream into two halves and presented pheromone of differing concentrations in each side. He found that B. mori males could discriminate between concentrations of pheromone that differed by a ratio of only 5:3.

Wind Tunnel Design

Monitoring insect movements in three dimensions in most cases pertains only to assays in which insects are allowed to fly. In-flight bioassays in wind are performed indoors using wind tunnels, or "sustained-flight" tunnels, and they have some definite advantages over outdoor flight bioassays involving field capture of insects. First and most important, the flight tunnel is a physical model of the environment, allowing the experimental manipulation of one variable at a time. Temperature, humidity, wind velocity, and chemical plume conditions can be reproduced day after day, and the experimenter does not encounter as severe a daily variation in results common to field tests that must be factored out by replication and experimental design. Cause-and-effect relationships from these variables can be gained more easily in a wind tunnel than in the field.

Although wind tunnels cannot duplicate the combination of wide plume dimensions and low concentration that occur in the field at great distances from a source, the use of low, as well as high, emission rates in a wind tunnel can shed light on the

responses that occur at both ends of the active space (32, 33). The prolonged (sustained) flight over a distance of many tens or hundreds of meters can be mimicked in the wind tunnel by rotating a visual floor pattern beneath the insects as they fly. The insects compensate for this higher velocity of optomotor stimulation by reducing their airspeed and hence can be made to fly for many minutes, even hours, at zero net up-tunnel ground speed while in the pheromone plume. This can increase discrimination among treatments (34, 35). Another key advantage of wind tunnels over field tests is that experiments can be performed throughout the year. For pheromone identifications, much progress can be made under inclement conditions in preparation for the time when field tests can ultimately be performed.

It is helpful to categorize wind tunnels by mode of air movement. The most common form of wind tunnel in pheromone research involves horizontally moving air, and displacement using anemotaxis (steering with respect to wind direction) is possible. But just as it is possible for bioassay tubes and chambers to employ moving air that does not permit anemotaxis by moving the air perpendicular to the plane in which displacement is permitted, so too can wind tunnels measure movements of flying insects that are not steered anemotactically. Such a situation is found in vertical wind tunnels, in which air moves either straight up or down and the insect's horizontal displacement in or out of pheromone has no wind-induced drift component. Also, air movement can be stopped in a horizontal tunnel to observe pheromone-mediated movements lacking an anemotactic component. Finally in at least one tunnel, horizontal air flow has been super-imposed on vertical air flow to create an elaborate assay for a pheromone's behavioral effects (36).

Kennedy and Marsh (37) used a wind tunnel for demonstrating the optomotor anemotactic response of flying Anagasta kuhniella males. Miller and Roelofs (34) demonstrated the usefulness of the wind tunnel for discriminating among several pheromone treatments in the redbanded leafroller moth. This technique has been used since then to discern differences between pheromone blends for several other moths, including G. molesta, the oriental fruit moth (16, 32), the noctuid moth, Euxoa ochrogaster (38), the gypsy moth, Lymantria dispar (17, 35), Heliothis virescens (39), and the cabbage looper moth, Trichoplusia ni (40).

Two fundamental decisions to make regarding wind tunnels are: how to get the air to move; and, how to keep the air in the room and tunnel free of contamination. Contamination must be avoided because the insects waiting to be tested can slowly become habituated, or they may respond poorly because the air in the tunnel can become tainted with an antagonistic compound. The second decision is somewhat related to the first in that if air is pulled through the tunnel rather than pushed, air-cleansing can be accomplished by having the exhaust fan pull the entire volume of the tunnel's air out of the room, and usually, out of the building. This may be the easiest way to ensure that all the pheromone, especially from a tunnel permeated with a cloud of pheromone (see below), is exhausted from the room. If an exhaust hood is present in the laboratory, usually it is easy to adapt it for air-pulling by building a box to fit the hood to the end of the tunnel or by running a tube from its duct to hook up to the end of the tunnel.

Other than for pheromone permeation tunnels, however, it is better to push the air rather than pull it, for the simple reason that access to the inside of the tunnel can be gained through doors, holes, slits, etc., without worrying about disturbing the pheromone plume's position or structure. With

pulling-type tunnels any crack, hole, pinhole leak, or especially any open tunnel door creates visible, severe perturbations of the (smoke) plume. Therefore, these tunnels must always be operated completely sealed along their length, and this makes them less adaptable for photography and for in-flight experimental manipulations of all types. This is true even during the act of releasing an insect into a plume, because the turbulence caused by opening a door at the downwind end creates uncertainty as to where the plume has moved.

In contrast, in push-type tunnels it is readily apparent using smoke plumes that even with access doors left open, actions such as sticking hands, head, arms, etc., into the tunnel do not disturb the plume, unless of course they are placed directly upwind of the smoke source. If the tunnel is housed in a small room, though, walking rapidly past the open doors can push the plume off course momentarily, and so it is always wise to operate the tunnel with the doors closed if the operator is expected to do much moving around while the insects are in flight.

There are many ways to generate wind to push air down a tunnel, but perhaps the simplest way is with a simple, rotary-blade fan. Whether the tunnel is round or rectangular, air can be easily conducted from a fan to the tunnel by means of a duct constructed from thick, flexible plastic sheeting, such as plastic bag material, taped together and sealed to form a tube. Usually the duct must expand from the smaller fan's size to the larger tunnel's opening.

At the tunnel's opening, a mixing chamber is needed to dampen the turbulence created by the fan's blades and to balance the wind velocities throughout the tunnel's middle, sides, top and bottom. The chamber, usually a box shaped like the tunnel, creates resistance by means of several layers of narrow-mesh

evaluation of the mixture's activity is needed before initiating a large, expensive field test.

Hill and Roelofs (41) identified three chemical components from the salt marsh caterpillar moth, Estigmene acrea, using Miller and Roelofs' horizontal flight tunnel to make sure complete activity was present in the recombined fractions and synthetic components (Z,Z)-9,12-octadecadienal, (Z,Z,Z)-9,12,15-octadecatrienal, and (Z,Z)-3,6-cis-9,10-epoxyheneicosadiene. Males fanned their wings and began walking to the epoxide alone, but successful upwind flight to the source 1.5 m away occurred only to binary or tertiary combinations, not to any component presented alone. At the filter paper source, males hovered for long periods, and extended their claspers while wing-fanning on the paper. More examples of pheromone identifications which utilized horizontal wind tunnels are Hill et al. (42, 43) and Roelofs et al. (44).

2. Behavioral roles of pheromone components and blends - The sex pheromone of the gypsy moth, Lymantria dispar, is (+)-(Z)-7,8-epoxy-2-methyl octadecane (45). Although the (+)-enantiomer is produced by females and is very active, addition of the (-)-enantiomer significantly reduced captures of males in traps in the field (46, 47). The reduction in trap capture could not be explained by wing-fanning levels of males exposed to (+)- and (±)-enantiomers (48). Using their horizontal wind tunnel for choice tests between (+) and (±) disparlure, Miller and Roelofs (35) found no significant differences in the number of in-flight orientations to either source. It was only in paired, flight duration tests in which sustained flights in one location were induced by moving the floor pattern beneath the males that significant differences in the behavior began to unfold. Males flew for considerably shorter periods of time to the (+)-enantiomer when exposed first to the (-)-enantiomer. Under optimal conditions, males

exhibited continuous flight for 30 min to the (+)-enantiomer alone, and significantly shorter durations were recorded for flights to the (±) mixture.

Choice tests similar to those of Miller and Roelofs for the gypsy moth (35) were performed by Linn and Gaston (40) on the cabbage looper moth, Trichoplusia ni, to determine the interaction of the two pheromone components (49). Two copper disc pheromone emitters were placed upwind and separated by either 12 or 8 cm. Smoke plume visualization showed that pheromone component plumes merged 85 or 35 cm downwind, respectively. Males flew upwind in the merged, two-component plume, and never flew in the 12:Ac plume when they reached the "choice" point. They did fly in the Z7-12:Ac plume after that point, but not as close to the source as when both components were present on one of the discs as the choice. Therefore, 12:Ac could not be called a "close-range" component, because it did not elicit upwind flight by itself at close range. Rather, it was clear that the two component blend was a good close-range stimulus compared to Z7-12:Ac, which alone evoked optimal levels of upwind flight from long-range, with or without the addition of 12:Ac.

3. Response profiles to blends and concentrations. - In studies by Baker and Carde (16), Baker et al. (32), and Linn and Roelofs (33, 50) observed the flight behavior of individual male G. molesta in a horizontal wind tunnel. In some of the studies, the ratio of components and the dosage were varied around the natural levels emitted by females (51). Males were found to be very sensitive to changes in blend and concentration, with optimal attraction to the source occurring to a narrow range of concentrations of the natural 6% E8-12:Ac blend containing 3 to 10% of Z8-12:OH. The ability of the experimenter to discriminate among the treatments was clearly increased when moths were required to fly close to the source (50, 32). Males became activated and took flight in nearly

equal numbers to a wide range of blends and dosages, but only a narrow range of treatments elicited high levels of completed flights to the source. Increasing the proportion of (E) isomer from 6 to 10% (E) caused significant arrestment of upwind flight within the plume apparently due to increased turning and decreased linear velocity. This suggested that the (E) isomer may function as a "turning" component, which may explain what happens when the "arrestment threshold" is reached (52) either with too much (E) in the blend or concentrations of the optimal blend that are too high. In the latter case, we now know that arrestment is not only a function of lower flight velocity and higher turning frequency, but also the single most important change involved in within-plume arrestment is that the males steer more obliquely across the wind, allowing more lateral drift (53).

In a subsequent study, Linn and Roelofs (50) varied all three components. Using hierarchical clustering techniques, they were able to define an area of optimal response around the natural blend ratio and emission rate, partitioned by "threshold" regions affecting specific behaviors in the sequence.

4. Habituation, sensory adaptation, disruption. - Habituation and sensory adaptation to sex pheromone are phenomena that have long interested researchers, but the traditional ways of measuring them have been by means of observing "key" responses (22). In two instances, habituation has now been measured in horizontal wind tunnels.

Kuenen and Baker (14) found that a pulsed pheromonal preexposure of cabbage looper males did not reduce their wing-fanning reaction in a small chamber over the course of an hour. However, this same regime reduced the percentage of males that flew all the way to the pheromone source in horizontal wind tunnels. Continual preexposure of males in

the chamber did not result in as severe a reduction of flight to the source in the tunnel, but nevertheless the decrease was significant compared to those receiving no preexposure whatsoever. Because electroantennogram responses of males preexposed to both pulsed and continual pheromone recovered within minutes after the exposure, habituation rather than sensory adaptation was proposed as the mechanism for reducing flights in the tunnel. This corresponded well to bioassay studies and predictions by Bartell and Lawrence (13), but contradicted the results of Farkas et al. (54) who thought pulsed exposure did not affect subsequent responses. Responses were measured by Farkas et al. in small bioassay chambers.

Linn and Roelofs (33) preexposed male G. molesta to E8-12:Ac and were able to demonstrate a prolonged effect on the males' pheromone quality perception. After such preexposure, males readily flew all the way to sources containing high percentages of the (E) isomer, something they would not do without being habituated to (E). A combination of (E)-isomer preexposure duration and dosage directly determined the subsequent tendency to fly all the way to sources emitting as high as 20% (E).

Rather than preexposing moths to pheromone, simultaneously presenting pheromone point sources in the midst of other pheromone sources has resulted in a greater understanding of the mechanisms or disruption of communication in air permeated with pheromone. Sanders (55) created a grid-array of nine synthetic spruce budworm (Choristoneura fumiferana) pheromone point sources at the upwind end of his horizontal wind tunnel and placed a cage of calling females at various points within that grid. He also created a cloud of uniformly permeated air by the tape-grid-turbulence method of Kennedy et al. (56, 57), against which he placed the cage of calling females allowed to create a discrete plume. He found that the uniform cloud was

less effective in preventing location of the females than the grid of discrete point sources of comparable overall emission concentration. He hypothesized that two different mechanisms were responsible for reduced location of females under the two regimes. The cloud produced habituation or adaptation which could be overcome by the higher-concentration bursts of pheromone in the filamentous plume from the females, whereas the discrete point sources often elicited upwind flight of males, who switched over from the female's plume. The fact that the discrete plumes caused a greater reduction of orientation to females could also be due to a higher overall effective concentration of preexposure caused by the peak concentrations within filaments and the condensed nature of the narrow plumes compared to diffuse clouds.

An elaborate wind tunnel utilizing horizontal and vertical wind was used by Phelan and Miller (36) to try to disrupt landing on host plants or host plant models by aphids by introducing clouds of the aphids' alarm pheromone over the plants. The part of the tunnel with vertically moving air was modeled after that of Kennedy (58), in which the velocity of downward-moving air could be increased quickly by the experimenter to counteract the aphid's upward flight toward a light in the tunnel's center, placed there to elicit such flight. After prolonged flight, the aphids became more responsive to vegetative stimuli (plants), and they would now tend to descend toward a green plate placed below them in the tunnel. Phelan and Miller's tunnel enabled them to try to thwart this landing behavior by introducing over the plate a horizontally moving layer of air permeated with (E)-beta-farnesene, the aphid's alarm pheromone, and to observe the possible repellent or deterrent effects of this pheromone on landing and feeding. Although they found no reduction in landing to the alarm pheromone, behavioral reductions were observed when certain fatty acids were presented. This unusual tunnel demonstrates that two columns of air moving in

different directions can be superimposed for pheromone studies of flying insects.

5. Orientation studies. - Entomologists constructed the first wind tunnels in order to determine the mechanisms of orientation used by insects flying in wind with or without odor. Kennedy (59) developed several models for steering with respect to wind after constructing a horizontal wind tunnel and observing the odor-free flight of yellow fever mosquitoes in wind and in still air, plus visually imposed "drift" through ground pattern movement. Kellogg and Wright (60) developed a horizontal wind tunnel to study the in-flight maneuvers of insects flying to various odors. These experiments marked the beginning of quantitative, detailed studies of olfactory-mediated flight.

Farkas and Shorey (61) used a horizontal tunnel to create a pheromone plume in wind, induce males to fly within a pheromone plume, and then stop the wind in order to prove, so they believed, that only chemotaxis was used for pheromone source location in wind. Kennedy and Marsh (37) used their tunnel to negate neatly part of this hypothesis, that anemotaxis was not used, by demonstrating the optomotor-anemotactic response to a moving floor pattern. Support for an integrated system of orientation, chemotaxis plus anemotaxis, came from wind tunnel studies by Baker and Kuenen (62) and Kuenen and Baker (63) in which they repeated and extended Farkas and Shorey's experiment of stopping the wind and observing whether males could locate the source. The definitive experiment, in which the pheromone source was removed and the wind then was stopped to create a truncated plume without wind, demonstrated a chemically-modulated behavior program of zigzagging apart from anemotaxis. Males zigzagging along the plume in still air changed the amplitude, frequency, and angle of their zigzags upon entering clean air

as they flew out the end of the plume, showing that their movements were in fact pheromone-mediated.

Later it was found that in-flight experience with wind-induced drift tended to polarize the zigzags of males in the toward-source direction, even when wind was later stopped when males were part way to the source. Males released into a stationary plume in zero wind performed their zigzag flight movements, but the zigzags meandered with no consistent directional component, in contrast to the cases in which the wind was stopped after males had launched themselves in the plume (64).

Horizontal wind tunnels provided even more support for an endogenous program of zigzagging. Using a tunnel uniformly permeated with a cloud of pheromone or with only a side corridor-cloud, Kennedy et al. (56, 57) showed that upon encountering the cloud, male Adoxophyes orana decreased the width and increased the frequency of their zigzagging. However, after several seconds of this tonic stimulation, the reversals became wider and the surge of upwind progress that had accompanied the narrow zigzags now ceased. Willis and Baker (65) obtained similar results with G. molesta, supporting the findings of Kennedy et al. (56, 57).

The mechanisms of counterturning in walking insects that may be analogous to zigzagging in flying insects have also been studied in horizontal wind tunnels (6, 66). In these studies, a plume of periplanone-B was positioned 2 cm off the ground at the upwind end of a 2.4 x 1.2 x 0.6 m tunnel and the tracks of male Periplaneta americana were recorded from above. The males walked upwind in the plume by means of a combination of anemotaxis, endogenously triggered counterturns, and counterturns back into the plume triggered by a decrease in concentration at the boundary of the time-averaged plume.

Thus again an integrated system of chemically mediated movements and anemotaxis was implicated, this time for a walking insect.

New advances in the understanding of the orientation of insects flying in response to pheromone will be gained through the use of wind tunnels in which flight tracks are recorded and analyzed in three dimensions. Kellogg, Wright and their colleagues in the late 1950's and early 1960's realized the importance of three-dimensional analysis (67, 60), but until recently technical and data-handling constraints have limited this approach to descriptive studies.

It is certain that the use of wind tunnels in pheromone research will continue to expand and, as this is done, further understanding of odor perception and orientation will be gained. Meanwhile, wind tunnels will remain among the most useful tools with which to bioassay fractionated natural extract, blends of synthetic components, or potential field formulations for use as lures in traps.

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