

Individual odor differences and their social functions in insects

(pheromones/social behavior/halictid bees/habituation/individual differences)

EDWARD M. BARROWS* §, WILLIAM J. BELL* †, AND CHARLES D. MICHENER* ‡¶

* Department of Entomology, University of Kansas; † Department of Physiology and Cell Biology, University of Kansas; ‡ Department of Systematics and Ecology and Snow Entomological Museum, University of Kansas, Lawrence, Kans. 66045

Contributed by Charles D. Michener, April 18, 1975

ABSTRACT The evolution of individual or subgroup differences in odors of halictine bees is suggested from possibly widespread intraspecific variation in pheromones. An important result of such variation may be maintenance of genetic polymorphisms; in nesting Hymenoptera odor differences may also facilitate individual nest recognition. In *Lasioglossum zephyrum* males habituate to odors of different females and perhaps thus save time by not trying to copulate with nonreceptive individuals. Guards (females) at nest entrances distinguish their few nestmates (other females) from other conspecific individuals by odors, seemingly pheromones. Duration of the habituation in *L. zephyrum* is at least an hour (perhaps much more) for males in relation to females and 6 or 7 days for guards in relation to nestmates. Studies of pheromones should take into consideration the possibility of pheromonal polymorphism in any species and the likelihood that it may be significant from biological and practical viewpoints.

Bee behavior described below shows the existence of individual differences in odors of conspecific females and initially suggested individual recognition of the odors. A terminological framework for the studies follows: Individual recognition is learned discrimination among conspecific individuals. It is based on a complex of individual differences such that if certain attributes change, recognition nonetheless occurs. For example, as an individual grows, its recognition still is possible on the basis of other attributes, even though its size has increased. There is little evidence for individual recognition in invertebrates, although a few papers suggest it (e.g., ref. 1).

Even in invertebrates, however, all members of a population are not identical from the viewpoint of other members. Age, sex, or physiological groups often elicit different responses from conspecific individuals. Recognition of membership in these groups is commonly innate—thus males and females are often recognized without learning, and receptive females may be recognized as a group by adult males.

Groups may be divisible into subgroups of one to many individuals, each subgroup eliciting different responses from at least some other members of the population. Subgroups may be genetically determined or may result from effects of physiological, experiential, or environmental conditions operating on the individuals being grouped, or may result from experiences of the individuals that recognize the subgroups. Recognition of subgroup membership is commonly by learning, perhaps most often by habituation.

A subgroup may consist of individuals having some common biological attribute by which its members are recog-

nized. This is here called a homogeneous subgroup, because its members are alike in a feature by which they are grouped. Thus in a *Drosophila* population, females may become habituated to males of a common phenotype (subgroup) and more readily accept males of a rare one. We suppose that subgroups might be composed, however, of individuals not united by any common feature of their own but rather grouped as a result of the experience of the individual grouping them. These we call heterogeneous subgroups; their content can be different for every individual that is recognizing subgroups. Heterogeneous subgroups are likely to be like the following: (a) individuals that I have seen before versus (b) individuals that I have not seen before.

If heterogeneous subgroups exist, they imply perception of individual differences among the animals being grouped. This does not necessarily mean that there is individual recognition, for as suggested above, familiar individuals might constitute one subgroup and unfamiliar individuals, another, with no memory of the individually distinguishing features.

Through multiplication of subgroups by perception and memory of more, or more complex attributes, subgroups may become very small. If most or all of them contain only one individual, then a species may be said to show individual recognition, as the term is explained in the first paragraph above.

Our data on bees provide no means for distinguishing recognition of heterogeneous subgroups from recognition of individuals. The simpler nervous mechanism may be one which, in spite of the input of individual differences, outputs familiar individuals as a group. However, the ability of a bee to fly to individual, learned places in the environment, e.g., a nest and different food sources, using a vast complex of cues (distant and nearby landmarks, celestial objects, odors), several of which can often be removed without causing failure to reach the goal, speaks to a nervous system capable of individual recognition. There is no evidence that individual recognition would be more advantageous to the bees than subgroup recognition. In either case individual differences must be perceived.

Our studies on perception of individual differences were made using two types of behavior: (a) responses of males to females and female odors and (b) responses of guard bees (females) at nest entrances to their nestmates and to other conspecific bees.

MATERIALS AND METHODS

Our studies were made indoors, and except as otherwise noted concern the primitively eusocial sweat bee, *Lasioglossum zephyrum* (Halictidae). This species nests in burrows in

§ Present address: Smithsonian Tropical Research Institute, Balboa, Canal Zone.

† To whom all reprint requests should be addressed.

the soil and the midsummer population of a nest consists of several adult females. Males leave nests upon maturity and do not form part of the colony.

Mating. Responses of males to females were studied by presenting tethered females, one after another, to a group of males. The experiments were made in net flight cages, $0.3 \times 1.0 \times 1.3$ m, in a brightly lighted flight room, 20 males per cage. Records were made of the number of male contacts with each female.

Male responses to aphrodisical odor from females were tested in similar cages with black dots (3 mm in diameter, arranged eight in a horizontal row) on a white card (21×28 cm). Moist filter papers (5 cm^2) were impregnated with female odors by placing two pieces edge to edge inside of a closed plastic container (17.2 cm^3) so that a bee in the container could walk on both pieces with approximately equal frequency. Unimpregnated control papers were treated similarly except that no bee was in the container. Pounces upon dots by males were counted after a piece of impregnated or control paper was placed in the middle of a row of dots. (For details, see refs. 2 and 3.)

Each experiment was composed of a sequence of trials which were repetitions of a particular procedure. Experiments were performed on different days; all trials of a given experiment were done in immediate succession. Differences in male responses (contact or pouncing rates) were analyzed using Wilcoxon's signed rank test (WT); probabilities for independent statistical tests were combined using the method of Sokal and Rohlf (ref. 4, p. 623).

Recognition of Nestmates. To study actions of guards in admitting or rejecting other conspecific females, we used artificial nests in which bees burrowed in soil between pieces of glass (5); the entrance was the end of a plastic tube (6). Colonies were established by placing female pupae (from diverse field origins except when sisters or close relatives are specified, in which case the pupae came from carefully excavated nests) in artificial cells in the nests. After emergence, the adults in such a nest interact to form a colony, with guard, queen, forager, and others, recognizable by behavior (7).

To eliminate or reduce possible environmental components of individual odors, we made all nests with mixed and sifted soil from the same place, and fed bees in them only with *Apis* honey from the same bottle and *Typha* pollen collected from flowers, mixed within a storage jar, and frozen. Mature larvae completely void the gut contents, then shed the cuticle as well as fore- and hindgut linings at pupation, and the pupa sheds its cuticle when it becomes an adult; thus it is unlikely that field nest odors would be transferred to adults emerging in our artificial nests. We therefore speak of the odors as pheromones, although a remote possibility of environmental origin remains. In the field, environmental odors may supplement the pheromones and facilitate recognition of nestmates.

Actions of the guard in admitting or rejecting other bees were observed at the nest entrance. Guard action was tested by bringing bees (foreign or nestmates) to the entrance one at a time, each in a piece of plastic tubing 5 cm long. The bee was then prodded out of the tube and into the nest entrance with a pipe cleaner. The interactions as it met the guard were recorded, often with cinematography (8, 9).

RESULTS

Mating

Males of *L. zephyrum* patrol in flight over areas where females rest or walk, and pounce upon conspecific females as well as other insects, shadows or dark spots of similar size (2, 11). Attempted matings occur through most or all of the life of a female, whether she has mated or not, although mated females are usually not receptive. In the laboratory pouncing upon black dots was enhanced by the presence of either female bees or pieces of moist filter paper impregnated with female odors.

Series I. In four experiments, 33 tethered females of *L. zephyrum* from the field were presented to a group of males. A female was presented for 5 min (first presentation), removed from the cage for 1 min, then returned for a second 5 min (second presentation). After 1 min the second female was similarly presented for two 5-min periods, then the third, etc., sequentially, up to eight or nine for each experiment. The frequency of approaches toward females by males did not differ significantly between first and second presentations; this probably indicates continued visual responses. However, males contacted females more during first than second presentations ($P < 0.001$); in fact, of the 33 females, only three were contacted more by males during the second presentation than the first. Very few matings occurred; females were generally not receptive. These data are interpreted to mean that males habituated to females, i.e., after approaching or contacting a female, they became habituated to her and the number of contacts diminished. But it increased again when a different female was presented. Thus discrimination between females by males in most trials was established. (Fig. 1, based on other experiments, see below, may nonetheless help to clarify the method and results.)

The same experiment repeated twice (a total of 16 females) with another halictid bee, *Augochlora pura*, gave similar results ($0.005 > P > 0.001$). Moreover, males of *Bombus pratorum*, a bumblebee, habituate toward certain gynes that refuse to mate. (10).

Series II. Because living females, when bothered by males, might reduce the liberation of sex pheromones or produce repellents, or males might mark females with substances repellent to other males, we employed impregnated filter papers to assess male response to the odors alone of different females of *L. zephyrum*. Thirty-five females of diverse origins were used in four experiments and 26 other females from five colonies, segregated as to colony and therefore within each experiment probably sisters, were used in five other experiments. The method was similar to that of Series I except that instead of sequential presentation of tethered females to males, moist papers impregnated by odors of females were presented, along with a row of ink dots (see *Materials and Methods*). In each of the four to 13 trials of each experiment, the first paper was presented to the males for 5 min, after which it was replaced by the second paper from the same female's container. Until that moment the second paper had been with the female, so that the second paper should have been at least as odoriferous as the first. The process was continued through all the trials of an experiment so that the presentation of papers was in this order: first paper impregnated with odor of female 1; second

¹¹ E. M. Barrows, manuscript in preparation. "Mating behavior in halictine bees. III. Copulation and olfactory communication."

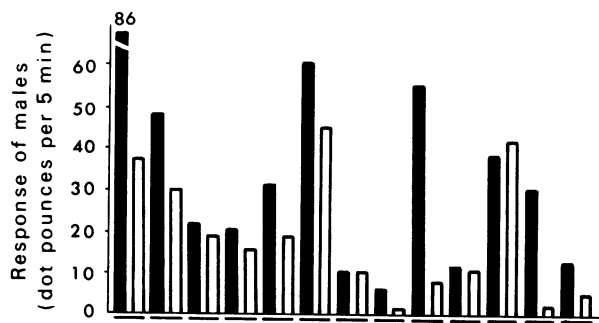


FIG. 1. Responses of males to female odors. Black, first papers; white, second papers. Each short line below links results from two papers impregnated by one female.

paper, female 1; first paper, female 2; second paper, female 2; etc. Possible repellent marking by males is unlikely, since males pounced upon the dots, rarely contacting the papers. Moreover, the same dot card was used throughout each experiment; had the dots become repellent the later part of each experiment could not have given results like the early part.

Impregnated papers produced higher rates of pouncing on the dots by males than control papers ($0.005 > P > 0.001$, $n = 20$) (3). The impregnated papers were attractive to males as well as aphrodisiacal, demonstrating the presence on them of one or more female odor components. In all nine experiments males pounced on dots significantly less during second than first presentations of odor of individual females ($P < 0.001$). In only six out of 61 trials was pouncing during the second presentation equal to or greater than that during the first. Thus males habituated toward odor of individual females as they did toward tethered females themselves. Moreover, males pounced on dots significantly more during first presentations of female odors than during immediately previous second presentations of already familiar odors ($P < 0.005$). Fig. 1 shows a sample of these results.

Thus females are shown to have individually distinctive odors and males distinguish between sequentially presented odors of different females. The individually distinctive odors perceived by the males may be either pheromones secreted by the females or odors derived from the soils or foods before the bees were captured for the experiments. When all the bees in an experiment came from a single nest, both genetically controlled and environmental sources of variation should be reduced. There is, however, no evidence of this; responses of males showed the same pattern whether the females tested were presumed sisters or were of diverse origins.

Series III. To learn whether male habituation to the odor of a given female persists over time, we made two additional experiments in which first papers were presented in random order, one after another. An hour later, second papers as well as papers impregnated by odors of other females (i.e., impregnated control papers, in effect additional first papers) were also presented in random order. Because they were intermingled with control papers, second papers were actually presented 60–135 min after corresponding first papers. Male response (dot pounces per minute) in the presence of second papers was lower than that in the presence of first papers in 11 of 13 individual comparisons ($P < 0.005$). Moreover, there was no significant difference between response to dots in the presence of first papers and that in the presence of the impregnated controls. Thus males remembered odors of

given females although odors of other females had been presented in the meantime.

Recognition of nestmates

The principal defense against conspecific intruders by *L. zephyrum* is by an individual behaviorally specialized as an entrance guard (11). Adults less than 48 hr old are generally accepted if introduced into a nest entrance, suggesting that they have not yet developed distinctive attributes. Nonresident older adults are generally rejected if introduced into the nest of an established colony (8). Even if the guard is removed, the nonresident is usually rejected by other colony members within 24 hr.

Thus guards regularly distinguish nestmates, which they admit to the nest, from conspecific nonresidents, which they reject. Of 228 nonresident bees introduced into other nests, 50% elicited aggression by guards before contact with the guards and 92%, on contact. Resident bees, similarly treated and then reintroduced into their own colonies, elicited no aggression in 50 trials (12).

Tactile, auditory, and visual stimuli appear unrelated to the distinction between resident and non-resident bees (12). Of 20 residents killed by freezing and placed in nest entrances, only 10% elicited aggressive responses on contact; in contrast, 97% of 32 dead nonresident bees did so. Nonresident bees introduced into nests in a room illuminated only by far red light (590–680 nm), which presumably simulated darkness to the bees, were also usually rejected. Thus contact chemoreception or olfaction must be the modalities of recognition.

To learn whether familial relationship is important in the recognition of nestmates, we used pupae from two field nests, A and B, to establish four artificial colonies of three bees each, colonies a_1 and a_2 with pupae from A, b_1 , and b_2 with pupae from B. Those in artificial nests a_1 and a_2 were presumably sisters or close relatives, as were those in b_1 and b_2 . After colonial behavior was established in each artificial nest, 30 introductions of individuals were attempted; in each a bee from one artificial nest (e.g., a_1) was introduced into a nest containing unrelated bees (b_1), and for comparison, a bee from the same source (a_1) was introduced into the colony of its relatives (a_2). The bees were accepted by related bees, usually rejected by unrelated bees ($P < 0.01$), suggesting that odors of closely related bees are similar; they may sometimes constitute a homogeneous subgroup as defined in the *Introduction*.

In most of the experiments, however, colonies consisted of bees from diverse sources. Yet the guards regularly accepted residents and rejected nonresidents ($P < 0.001$, $n = 1175$) (8). To do this the guard must recognize the odor of each nestmate, either as an individual or as one of her familiar heterogeneous subgroup, assuming that we have successfully eliminated differential environmental odor cues. Such an ability should be important in nature, since genetic diversity and therefore probably odor diversity must often occur even among sisters.

A few (14% of 1175 introductions) mature nonresident bees are readily accepted into colonies (8). If individual odors are genetically determined by a limited number of genes, one would expect a certain number of odor duplicates; guards would not distinguish between bees with similar or identical odor mixtures.

Several experiments involving transferring bees between nests, blowing air continuously from one nest to another, transferring soil from one nest to another, and using of for-

eign odors (peppermint) failed to show that bees could acquire distinctive odors from their environment that would influence their acceptance or rejection. These findings, along with the precautions against differential environmental odors (see *Materials and Methods*), indicate that distinctive volatile or contact pheromones or both are sufficient for nestmate recognition, although odors of environmental origin may also be involved in nature.

If guards remember odors, the duration of such memory is important. When reintroduced into their own nests, bees isolated in clean tubes for 1–72 hr became progressively more likely to encounter guard aggressiveness (12). In another set of experiments** all bees were removed from nests, marked, and kept isolated in clean tubes with honey and *Typha* pollen. After various periods guards were re-established in their nests, and other bees introduced at the entrances. After up to 7 days of isolation a guard accepted her nestmates and rejected others ($n = 78$); at 8 days there were mixed results and after 12 days of isolation all reintroduced nestmates ($n = 11$) were vigorously rejected whether or not the guard was in its own nest or had been transferred to another nest burrow. The most obvious explanation is that guards respond to but later forget individual or subgroup odors; the long period before forgetting is noteworthy, but in *Apis* memory of odors also lasts for days (13). The ability of guards to accept nestmates even when in a foreign nest suggests that habituation to nest odors does not influence recognition of nestmates and supports the idea of individually distinctive secreted odors.

DISCUSSION

The following paragraphs outline a possible evolutionary sequence from indiscriminate mating to a high level of individual or subgroup mate recognition, and suggest a model for the use of such abilities in other contexts in nest making insects and in social insects. Perhaps in the primitive stage any conspecific male and female mate if they meet while in the proper physiological states.

Homogeneous subgroups

Assortative mating occurs in a wide variety of animals and indicates a relation between mate selection and genetic make-up.

A substantial series of papers shows that in various *Drosophila* species tested in small arenas, females associated with males of two strains mate preferentially with the less common male type (ref. 14). Such frequency-dependent mating success has been observed when the males' strains differed at a single locus, in chromosomal arrangement, or in geographic origin; similar discrimination has been noted between flies of the same strain reared at different temperatures. Reproductive depression in inbred lines is largely attributable to failure to mate by individuals of very similar genotypes (15).

Discrimination among genotypes is mediated in *Drosophila* at least in part by volatile pheromones (15–18). Presumably heterosis leads to selection for genetic factors that produce distinctive substances (or behavior) perceptible by members of the opposite sex. Individuals probably become habituated to (or possibly inhibited by) their own products or those of their close relatives or those produced by the ma-

jority of individuals in the population. On the other hand sexual responses are stimulated (or permitted) by products of unrelated or rare type individuals and such products promote mating within inbred lines where mating otherwise is a rare event.

Rare-male advantage could be important in maintaining genetic polymorphisms in populations. Social phenomena thus may be responsible for much of the unexpectedly high allozymic polymorphism recognized since 1966 in a wide variety of animals (15, 19, 20).

Heterogeneous subgroups or individual recognition

Mate Recognition. Evidence that female sweat bees have individually different odors and are recognized by males either as individuals or as heterogeneous subgroups is presented above. It seems likely that these bees have elaborated upon the possibly widespread insect ability to discriminate homogeneous subgroups. Attempts to mate with nonreceptive females waste time; recognition by a male of familiar females, individually or as a group, could prevent such waste. Also, if all females had the same odor, males might become habituated to it and stray away from the mating area; diversity of female odors may prevent this.

Nest Recognition. Nest-making insects may take advantage of individual odors to help in the recognition of their own nests. Many authors have marveled at the ability of nonsocial wasps and bees to return each to her own burrow even when many similar burrows are near, and especially when small landmarks and the burrow entrances have been destroyed by trampling. Return to the general locality is facilitated by visual cues but olfactory cues are likely aids in selecting the correct spot at which to dig. Shinn (21) remarked that nests of a solitary bee, *Calliopsis*, may have different odors. He exchanged tumuli of loose soil at burrow entrances between pairs of burrows a few centimeters apart while the bees were both away foraging. The lemon-like odor of this bee can be perceived by man in tumuli, showing that the female does leave some of her secretions there. On returning, eight of 14 bees went first to their own tumuli at the artificial locations while six went to the wrong tumuli at the correct locations. Presumably some bees recognized their own or their nests' odors in contrast to the neighbors' odors, recognized individually or as a heterogeneous subgroup.

Differences in nest odors could be due to different soils penetrated by the burrows or to different foods used by the bees. However, the *Calliopsis* nests were close together, about the same depth, and the bees at that site appeared to be visiting only *Trifolium* flowers for pollen. It is therefore at least possible that nest recognition was based on secreted odors.

Maternal Recognition. Young nymphs of the cockroach *Byrsotria* congregate under their own mother in preference to another female having young of the same size and age (22). Aggregation and presumably discrimination among mothers is mediated by a contact pheromone in the feces.

Individual Differences in the Context of a Eusocial Colony. In solitary bees and wasps there is no need to discriminate among other nest inhabitants; any living thing in the burrow is ordinarily ejected by the owner. In colonies, however, recognition of nestmates as different from conspecific intruders or robbers is important. It is not surprising, therefore, to find that in *L. zephyrum*, as detailed above, guard bees block nest entrances to foreign intruders but allow nestmates to enter.

** W. J. Bell, E. M. Barrows, A. Sobti, and J. Hackney, manuscript in preparation. "Further studies on intraspecific nest defense in a primitively eusocial halictine bee."

The occurrence of social hierarchies, for example in small colonies of *Polistes* wasps (23), suggests individual recognition but may well be based on responses to single physiological attributes of the colony members rather than upon memory of them as individuals; subgroups may be homogeneous rather than heterogeneous.

In large colonies of ants, bumblebees, and highly social bees, recognition of individual workers may be possible but probably is not selected for; in spite of division of labor among workers, there are many with the same activities and the important thing would be recognition of the homogeneous subgroups (refs. 24 and 25). In these forms, nonetheless, colony members are commonly accepted and non-residents attacked. A major factor making this distinction possible in *Bombus* and *Apis* is odor from the nest, stored food, and the like, adsorbed onto bodies of bees (10, 26, 27). Yet some authors have been convinced of a genetic basis for odors, and it will probably turn out that there are both genetic and environmental components. Thus Butler (28) indicates that the distinctive odor of an *Apis* colony, and of each of its members, is made up of a hereditary component (recognized when bees of different strains interact) and components of environmental origin.

Can One Pheromone Explain Individual Differences?

A reasonable assumption is that individually distinctive pheromones are made up of mixtures of compounds, the relative abundances of which provide the distinctive features. Pheromones consisting of complex mixtures are known. The sex attractant of a moth (*Archips*, ref. 29), the mandibular gland pheromones of three bee species (genus *Trigona*, refs. 30 and 31), and Dufour's gland pheromones of two ants (*Pogonomyrmex*, ref. 32), all contain about a dozen probably active substances each. Such mixtures, if the components vary in relative abundance and if the insect has the necessary sensory equipment, could provide a basis for individual or subgroup recognition. If a contact pheromone contains 10 components and if four relative concentrations of each can be distinguished, 4^{10} distinguishable combinations exist. This is enormously more than are necessary to explain our observations. Moreover, more than one pheromone source may be involved. Work with pheromones should be undertaken with the realization that polymorphisms may occur and that they may be significant from both biological and practical viewpoints.

This study was possible thanks to National Science Foundation Grant GB 38502. We wish to acknowledge the able assistance of Anita Sobti and John M. Hackney. Profs. P. W. Hedrick, R. Jander, and O. R. Taylor read the manuscript and provided useful suggestions.

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