Mate finding via a trail sex pheromone by a parasitoid wasp

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Low population density poses a problem for sexually reproducing species because of the difficulties females and males face in locating each other (1). Decreased growth of low-density populations [i.e., the Allee effect (2)] may cause extinction of small populations (3) or prevent the founding of new populations (4). Even in haplodiploid Hymenoptera, where unmated females can produce male progeny from unfertilized eggs (arrhenotokous parthenogenesis), low population density may lead to an overabundance of male progeny and low population growth.

Among the Hymenoptera, parasitic wasps (parasitoids) may suffer Allee effects because hosts are often rare or widely dispersed. Allee effects may be especially acute for wasps released into new environments for the biological control of insect pests (4). Some aspects of the life histories and mating systems of parasitoids can increase the probability of mating at low density (5, 6). First, some species lay many eggs in each host so that wasp larvae develop gregariously. In such species, males often emerge before females and mate them as they emerge (7-10). Second, volatile sex attractants are commonly found in parasitoids. Females emit sex pheromones that attract males at long distances (6, 11-13) and other pheromones that elicit courtship and mating at short distances (14-16). Third, some parasitoid mating systems are based upon male aggregation and landmarks that females visit to mate (17, 18).

Here, we report a mate-finding mechanism in a solitary parasitoid (a single offspring per host). Aphelinus asychis Walker (Hymenoptera: Aphelinidae) is an endoparasitoid of aphids; one host is the Russian wheat aphid, Diuraphis noxia (Mordvilko) (Homoptera: Aphididae). A. asychis is over-dispersed among D. noxia colonies in wheat and barley fields. In cereal fields in the Montpellier region of France in 1991 and 1992, where infested plants harbored aphid colonies of up to several hundred individuals, 55% of the D. noxia colonies (281 of 515) had only one aphid parasitized by A. asychis, and 91% of the colonies (470 of 515) had fewer than five (K. Chen and K.R.H., unpublished data). Even at peak aphid densities, only six A. asychis per m² (median) were found in quadrats that contained at least one wasp (K. Chen and K.R.H., unpublished data). Since A. asychis are small (~2-mm adult body length) and usually move by walking or jumping from plant to plant, the dispersed distribution of emerging adults could reduce the mating success of both males and females.

In a series of laboratory experiments and field observations, we demonstrate the existence and function of a trail sex pheromone that A. asychis males use to find females among the leaves of wheat and barley plants. To our knowledge, this is the first report of a trail sex pheromone used for mate finding in any insect species.

MATERIALS AND METHODS

General Methods. We tested individuals from a mass culture of A. asychis started in August 1992 from 150 parasitized D. noxia nymphs collected from two wheat fields near Montpellier. Parasitoids were reared on D. noxia on wheat (variety Tam 107) or barley (variety Dominique) for more than nine generations with a 16-h daily photoperiod (2500 lux provided by fluorescent tubes) at 20-22°C and 50-70% relative humidity. Adults emerged from aphid mummies (dead aphids, each containing one parasitoid nymph) isolated in 1.5-ml gelatin capsules and were fed with honey for 1-2 days before testing. To avoid the possibility of learning and a lack of independence between trials, individuals were tested only once. Experiments were carried out in the laboratory under the same abiotic conditions and on the same plant varieties used for rearing. Data were analyzed with the SAS statistical package (19).

Volatile Pheromone. We used a four-field airflow olfactometer in the laboratory to test the olfactory response of males to virgin females (for a detailed description of the apparatus and its use with parasitoids, see refs. 20-24). This type of olfactometer exposes insects to four odors simultaneously, one in each of four contiguous fields. Wasps are released at the center of the chamber and are free to walk from field to field. Because A. asychis is smaller than the species studied by Vet et al. (20), we used a slower airflow (~5 liters/h). A virgin female was placed in a glass tube 10 cm upwind from the exposure chamber so that one field had undiluted female odor and the three others had filtered pure air. In each replicate, a male was released at the center and observed for 10 min after its
departure from the center. The female was replaced every five replicates, and a total of 30 males and 6 females were tested over a 2-day period. Male response was measured by the first field visited (first choice) and the time spent in each field. The frequency of first choices for each field was compared to a uniform distribution with a \( \chi^2 \) goodness-of-fit test, and the time spent in each field was analyzed with Friedman’s test (25). To test for bias resulting from the orientation of the olfactometer in the laboratory, we carried out an initial experiment testing males in four pure-air fields. In this test, male locomotion was unaffected by airflow, and males spent equal time in each field (\( n = 30, F_{3,116} = 1.25, P = 0.29 \)).

**Trail Pheromone.** We carried out a series of laboratory experiments to determine whether males respond to cues left on barley leaves previously visited by conspecifics. The tested leaves were exposed to virgin females, mated females, virgin males, or neither sex. In this experiment, adults were kept without aphids for 24 h after emergence in 40-ml plastic vials at a density of 10 wasps per vial. Wasps were either separated by sex to provide virgin individuals or mixed to provide mated females. Thirty minutes before testing, a 5-cm barley leaf was cut and placed in a glass tube (0.4 cm inside diameter, 6 cm long) with an adult parasitoid of one type or without parasitoids for controls. The leaf was then placed at the center of a white filter paper (20-cm-diameter Whatman no. 5), and a male was immediately released on the middle of the leaf. We measured the frequency of visits on the leaf and the time spent in each field (\( n = 20 \)). Logarithmically transformed data were analyzed via a one-way analysis of variance using days and times of day as blocking factors. Because of heterogeneous sample sizes among treatments, means were compared using Tukey’s honest significant difference test. One observation from the mated female treatment was dropped because the female did not mate, although the analysis carried out with this outlier led to the same conclusions.

We also studied male behavioral responses to leaves visited by virgin females in the field. We released 50 male–female pairs of *A. asychis* over 3 days in June 1992 in a barley field near Montpellier. In each trial, a male was observed continuously after being placed on a leaf a few minutes after a virgin female had been released on the same leaf. To avoid interactions between trials, each release was made on a different barley stem.

**Male Search Behavior in a Wheat Patch.** To study male search behavior in a more realistic laboratory environment, we observed male movement in a patch of 64 wheat leaves (6–8 cm tall) planted in potting soil (Fig. 1). Two sets of leaves were used to sample male dispersal: four leaves 4.8 cm from the release point and another four leaves 10.7 cm from the center.

In each set, two randomly selected leaves were exposed to a virgin female while the other two were left unexposed. Exposed and control leaves were each covered with a test tube (10 cm long, 1 cm diameter), with and without a virgin female, respectively. After 2 h, the tubes and virgin females were removed. Ten males were then released simultaneously on the soil surface at the center of the patch. Every 5 min for 2 h after release, the number of males on each of the eight leaves was recorded. The cumulative number of times males were observed on the leaves over a 2-h period was analyzed with an analysis of variance with two factors, distance from the release point and exposure to females.

**Active Period of the Trail Pheromone.** To measure the duration of trail pheromone activity, we studied male response to virgin female traces at different times after the traces were emitted. On the first day, a female was enclosed between the bottom of a 6-cm-diameter plastic Petri dish and the top of a 10-cm-diameter glass Petri dish, thus delimiting a round marked area on the glass dish. After 2 h, the female and the plastic Petri dish were removed, and a male was released at the center of the marked area. Every 2 min for 30 min, we observed whether the male was inside the marked area. Male response was measured as the total number of times males were seen in the marked area (range, 0–15). We tested 20 female traces simultaneously and exposed the same Petri dishes to other males for 24, 48, 72, and 96 h. As a control, the Petri dishes were cleaned with 95% ethanol after the 96-h test, and a male was again released in each dish. We analyzed the effects of female identity and time since a female visit on male response using an analysis of variance. Because sample sizes were equal for each treatment, means were compared with a Ryan–Einot–Gabriel–Welch test (19, 26).

In the previous experiment, changes in male response over time may have been caused either by pheromone decay or by repeated exposure of the substrate to males. To test for the effect of male visits on the behavior of subsequent males, we set up another 20 female-exposed Petri dishes. Ten were tested with males immediately after exposure using the protocol above, and 10 were left unvisited. After, 24 h both sets were tested with males. We used a \( t \) test to analyze the effect of exposure of the substrate to males on male response after 24 h.

**RESULTS**

**Volatile Pheromone.** Males did not respond differentially to odors of virgin females in the olfactometer. The distribution of first choice for the four fields was uniform, and males spent equal amounts of time in all four fields (Table 1). Males showed none of the usual signs of female presence (wing fanning, increase in turning rate, and increase in walking speed) when walking in the air field from virgin females.

**Trail Pheromone.** In the laboratory, male behavior on and around leaves previously exposed to virgin females was different than that on leaves visited by other males, mated females, or unexposed controls (Fig. 2). Males visited leaves exposed to virgin females more frequently (\( F_{3,14} = 17.62, P < 0.0001 \)), and time spent within 9 cm of the leaf was longer for leaves exposed

**Table 1.** Choice frequencies and time spent in four fields of an airflow olfactometer

<table>
<thead>
<tr>
<th>Olfactometer field and odor</th>
<th>Frequency of first choice</th>
<th>Duration, min (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virgin female</td>
<td>7</td>
<td>1.7 ± 0.22</td>
</tr>
<tr>
<td>Pure air 1</td>
<td>9</td>
<td>2.7 ± 0.32</td>
</tr>
<tr>
<td>Pure air 2</td>
<td>8</td>
<td>3.2 ± 0.36</td>
</tr>
<tr>
<td>Pure air 3</td>
<td>6</td>
<td>2.4 ± 0.30</td>
</tr>
</tbody>
</table>

The frequency of first choice for the four fields was uniform (\( \chi^2 = 0.67, P = 0.88 \)). The males spent equal amounts of time in all four fields (\( F_{3,116} = 0.25, P = 0.86 \)).
virgin females on the same leaf if the males walked more than were observed on leaves exposed to females (Fig. 3; when males pursue females before copulation. Although males toward the path of the female. This searching pattern led some males to find and mate the females. Males did not respond to leaves or stems.

Male Search Behavior in a Wheat Patch. Males released in the wheat patch dispersed from the release point and were logarithmically transformed for analysis. Letters indicate means that differ significantly (α = 0.05).

to virgin females (F_{1,14} = 11.95, P = 0.0004). Date and time of day had no effect on frequency of visits (F_{1,14} = 0.67, P = 0.43; F_{4,14} = 1.16, P = 0.37, respectively) or time spent within 9 cm of the leaf (F_{1,14} = 2.02, P = 0.18; F_{4,14} = 0.48, P = 0.75, respectively). Male behavior on and around leaves previously visited by virgin females was qualitatively different than on other types. Males walked faster on leaves exposed to virgin females, and after moving several centimeters away from exposed leaves, males turned back toward the leaves. On contact with the leaves, males intensively drummed the substrate with their antennae, and 3 out of 10 males fanned their wings, a behavior commonly observed when males pursue females before copulation. Although males slowly palpated unexposed leaves with their antennae, we never observed wing fanning on unexposed leaves.

In the field, males who crossed the paths of virgin females also changed their walking behavior, especially by turning back toward the path of the female. This searching pattern led some males to find and mate the females. Males did not respond to virgin females on the same leaf if the males walked more than 1 cm in front of the females or if they were on opposite sides of the leaves or stems.

Male Search Behavior in a Wheat Patch. Males released in the wheat patch dispersed from the release point and were found on all leaves sampled. At each distance, more males were observed on leaves exposed to females (Fig. 3; F_{1,36} = 13.73, P = 0.0007), but distance did not affect the number of males on leaves (F_{1,36} = 2.33, P = 0.14). The slight decrease in number of males with distance was the same for exposed and control leaves (Fig. 3), so that the interaction between leaf type and distance was not statistically significant (F_{3,36} = 0, P = 1).

Trail Pheromone Active Period. The frequency of visits by males to areas of glass Petri dishes exposed to virgin females declined with time after the female visit (F_{5,114} = 11.87, P < 0.0001). The response was highest just after the female visit, decreased after 1 day, and leveled off after 2 days (Fig. 4). A multiple comparison test distinguished male responses just after the female visit from male responses 1 or more days later. The decrease in male response over time was not affected by previous male visits. Male response after 24 h was the same to Petri dishes exposed to both virgin females and males as to Petri dishes exposed to females only (t_{18} = 0.56, P = 0.58).

Modeling the Probability of Mate Encounter. To explore whether a trail pheromone would be sufficient for mate finding in the field, we modeled a situation where a trail pheromone left on the substrate was the only cue to assist male search and asked how this might affect the probability that a female mates. We assumed that males searched by walking 12 h/day and spent a constant amount of time on a plant stem (the giving-up time) before moving randomly and instantaneously to another stem. However, when contacting the pheromone on a stem,
DISCUSSION

To our knowledge, this is the first demonstration of a trail sex pheromone used for mate searching in any insect species. Male A. asychis visited more frequently and spent more time on and around the leaves previously exposed to virgin females relative to leaves visited by mated females or other males. These results, along with observations of males orienting to paths of virgin females in the field, suggest that virgin females lay the trail sex pheromone while walking. We observed no differences between virgin and mated female walking behavior (e.g., virgin females do not drag their abdomens on leaf surfaces), so it is unclear how females deposit the pheromone. We do not know the chemical structure of the pheromone, but it clearly has a relatively short active period (<24 h).

The pheromone functions when left on the substrate. Because of its short active period, the pheromone is a reliable indicator of the presence of a female nearby (i.e., on the same leaf or stem), and it elicits intensive search by males on marked areas and their surroundings. The main response is that males turn back toward the marked substrate (or in the field, the female path) when they move away from it. This eventually leads males to females, as observed in the field. Males may follow a gradient of intensity, created by the decay of the compound over time, to orient their movements along paths toward females.

The trail sex pheromone of A. asychis resembles recruitment pheromones laid by workers of social insects like ants. Ants follow pheromone trails by walking in wavy trajectory to keep the area of maximum pheromone concentration between the tips of their antennae (27), a response similar to, although apparently more precise than, that of A. asychis. Substrate-borne pheromones have also been found in a number of fruit flies (Tephritidae). In these insects, females lay oviposition-deterring pheromones on fruit in which they oviposit to prevent overcrowding during the development of their progenies (28). In some species like Rhagoletis pomonella or Ceratitis capitata, males contacting this oviposition-deterring pheromone increase their giving-up times on marked fruit, though without altering their locomotory behavior on and around the marked fruit (29). In these species, males typically produce a volatile sex pheromone to attract receptive females (30) and are able to detect them visually over relatively long distances (i.e., 50 cm). Presumably, males using the oviposition-deterring pheromone as an indicator of the presence of nearby females would also encounter unreceptive females and attempt "rapes" (31, 32). Although the authors conservatively called this cue a "chemical arrestant" (29), it seems that males of R. pomonella and other fruit flies may use oviposition-deterring pheromones as sex pheromones.

Substrate-borne chemicals often elicit "arrestment" or "area restricted search" (33) characterized by an increase in turning rate and a decrease in walking speed. Parasitoid arrestment in response to substrate-borne kairomones (chemicals from the host) has been observed in a number of species (33, 34), but it is thought to be a complementary step to olfactory attraction at a distance, increasing foraging activity in the area where hosts are detected (33, 35). Ants like Novomessor spp. use trail pheromones for recruitment over long distances and volatile pheromones only for short-range recruitment (36). In A. asychis, the trail sex pheromone is apparently not associated with an airborne sex attractant. Males did not respond to virgin females in the olfactometer, and field observations confirmed this lack of response at a distance. Our attempts to capture native or released males on virgin female baited traps in the region of Montpellier and in Colorado were also unsuccessful. We cannot, however, reject completely the possibility of a volatile pheromone with a very small active space or a pheromone that would be emitted in other circumstances. Substrate-borne semiochemicals in the...
form of trails or patches are more commonly found in walking or crawling animals [e.g., spiders (37) and snakes (38)] than in flying organisms [the best examples of long-range volatile sex pheromones are from moths and butterflies (39)]. We have seldom observed A. asychis move by flying, either in the laboratory or in the field. Instead, males and females move by walking on the plants and on the ground and occasionally jump in a nondirected fashion.

The major effect of the trail pheromone on male behavior is to increase the time they spend on stems with females. In our laboratory wheat patch experiment, males searched all leaves but remained longer on leaves that had been exposed to virgin females. Given a walking speed of 10 cm/min (X.F., unpublished data), males could traverse a 75-cm mature wheat stem in 7.5 min. However, such an extensive search would only be necessary in systems devoid of any sex pheromone. In our field observations, virgin females walked (and thus deposited their pheromone) on most of the stem. Thus, a 2-min giving-up time, sufficient for a male to visit more than a quarter of a mature stem, is probably enough to assess accurately the presence (or absence) of a virgin female.

Would a trail sex pheromone be sufficient for mate finding in the field? Given the range of densities in wheat fields around Montpellier, our model demonstrates that the trail pheromone could result in most females being mated, even with males moving randomly among stems. Early in the season when male density is low (~0.3 male per m²), 50% of the virgin females would remain unmated after 2 days. However, the low probability of mating when parasitoid density is low early in the season may be counterbalanced by shorter giving-up times on the younger, smaller cereal stems. Later on, with 2.2 males per m², nearly 100% of 2-day-old females would be mated for all of the giving-up times we modeled. Our model also predicts that the time required for a female to be encountered by a male would be short enough to result in most females being mated for the majority of their lives. At low density, a female would have a 95% probability of being encountered and mated within 5 days, whereas at higher densities, the same probability is reached within a single day. Even if longevity were much lower in the field than in the laboratory [where females live 38.0 ± 6.6 days (mean ± 95% confidence interval)], our model suggests that most females would oviposit as virgins only for short periods and consequently would not produce male-biased sex ratios. Thus, Allee effects caused by low mating success, and the resulting overabundance of males and low population growth, would not be expected in established populations of A. asychis.

On the other hand, both probability of a female mating and the time required for her to encounter a male were very sensitive to low male density (Fig. 5). Population density is often low during invasions; thus, we expect that parasitoids like A. asychis may suffer from Allee effects after introduction into new environments because of the difficulty of finding mates.

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