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Volatile sex pheromone in the female German cockroach

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Abstract. Virgin German cockroach adult females release an attractant that can be extracted with organic solvents and trapped from air blown over sexually receptive females. Behavioral assays with an olfactometer showed that the attractant was produced exclusively by adult females and it elicited behavioral responses in adult males, confirming its function as a female sex pheromone. Using behavioral and electrophysiological assays, we localized the site of pheromone production on the tenth abdominal tergite where an adult female-specific gland is found. Females whose glands were ablated were significantly less attractive to males than sham-operated control females.
Key words. Sex pheromone; German cockroach; sexual behavior; pheromone gland; attractant.

The German cockroach, *Blattella germanica* (L.), is an important pest in association with humans world-wide. The terminal behavioral events of the courtship sequence in this cockroach have been well studied. Male courtship wing-raising responses are mediated by a four-component epicuticular contact sex pheromone produced by the female[1–3]. However, earlier phases of the mate-finding process in the German cockroach are poorly understood. It was reported previously that, in contrast to other cockroach species including *Periplaneta americana*, males of the German cockroach were not specifically attracted to females[1]. Therefore, the current prevailing view is that resource-based aggregations, possibly mediated by aggregation pheromone[4,5] or food, facilitate mate-finding in this species[6,7].

Recently, we observed a 'calling' behavior in the female German cockroach[8]. The behavior is expressed only in sexually receptive virgin females and it is suspended immediately after mating. Similar behavioral postures have been shown or implicated to be associated with pheromone emission in other cockroach species[9–11]. This led us to re-investigate the possibility that a sex attractant might be released by females of this species. We now report on a volatile sex pheromone in the German cockroach that is produced in a female-specific dermal gland on the anterior of the tenth tergite.

Materials and methods

Blattella germanica was maintained at 27 ± 1 °C under a 12 h light:12 h dark photoperiod with rat chow (Purina No. 5012) and water provided ad libitum. Newly emerged adult males and females were collected daily and maintained in separate groups under the same conditions. Where mated females were needed, mating was performed 4 days after the imaginal molt, when females first became sexually receptive[8]. For whole body extraction, the insects were immersed in *n*-hexane twice for 5 min each with gentle vortexing. The extracts were combined and concentrated under a stream of nitrogen. The pheromone was also collected using a head space collection apparatus: high purity air was filtered through Tenax TA and, at 100 ml/min, passed through a glass tube that housed insects which were provided only with water; volatiles emanating from the insects were adsorbed onto Tenax TA. The adsorbed material was then eluted with *n*-hexane.

The biological activity of extracts and air-borne collections was assayed both behaviorally and with an electroantennogram (EAG). For behavioral assays we used a two-choice olfactometer modified from that of Liang & Schal[12]. Briefly, a straight Plexiglas tube (55 cm long and 3 cm ID) was divided vertically along 15 cm of its upwind end and air was drawn through it at 20 cm/min. A single insect was placed in a gated cage at the downwind end 30 min before the start of an assay. Two types of assays were conducted. The first examined the choice of two candidate attractants that were introduced simultaneously at the upwind end of the olfactometer. When live females were used as attractant sources, they were placed in the two halves of a vertically divided tube (10 cm long, 3 cm ID) which was then connected to the upwind end of the olfactometer. Each pair of females was used to assay 3 males. The second assay examined the attractiveness of a particular sample, in which the percentage of insects running upwind was recorded. To examine the responses of different stages of insects to the pheromone, last instar nymphs, 15-day-old adult males, and 6-day-old adult females were used.

EAG activity was recorded from freshly ablated male antennae with Ag-Cl electrodes, amplified with a Grass P16 preamplifier and read-out from a Tektronix 5113 dual beam storage oscilloscope equipped with 5A20N and 5A22N differential amplifiers. One female-equivalent (FE) extract of 6-day-old virgin females was used as a standard and hexane was used as control.

To localize the pheromone gland, 5-day-old females were dissected under a dissecting microscope and various body parts were placed separately into vials containing a known amount of hexane. The dissected body parts of at least 20 females were pooled for each experiment. Ablation of the pheromone gland was performed on day 1 and the attractiveness of operated females was assayed on day 5. Basal oocyte length, a measure of the gonotrophic stage, was measured with an ocular micrometer in a dissecting microscope.

Results and discussion

Virgin female *Blattella germanica* initiate calling on day 4 and exhibit maximal calling on days 6–7. While 100% of virgin females call before they oviposit an infertile egg case, all females cease calling immediately after mating, long before they produce an egg case[8]. When given a choice between live virgin and mated females in the olfactometer, 80% of the males chose the virgin whereas only 20% chose the mated females (fig. 1A), indicating that males used air-borne signals to identify the virgin female to which they preferentially oriented ($p < 0.001$, binomial test, $n = 30$). Oocyte size, which provides an accurate measure of the physiological stage in adult female *B. germanica*[13], was not significantly different in the two groups of females (1.72 ± 0.05 mm for virgin and 1.78 ± 0.10 mm for mated females, $p > 0.1$, *t*-test). These results suggest that, at the same stage in the gonotrophic cycle, only sexually receptive virgin females and not mated females release volatile chemicals that attract males.

Using the headspace collection system, we collected volatile materials emanating from virgin and mated females separately and compared them in olfactometer assays. Again, the collection from virgin females was significantly more attractive to males than that from the mated females: 93.3% of the males (28 out of 30) chose the collection from virgin females while only 6.7% chose odors released by mated females (fig. 1B). This result showed that the volatile pheromone can be trapped with adsorbents and it agrees well with the results obtained with live females. Together, these results conclusively show that volatile chemicals are released specifically by sexually receptive virgin females but not by mated females. The dependence of attractant release on the virgin status of the female argues that these chemicals may facilitate mate-finding in *B. germanica*. The behavioral results clearly document active orientation by males to sexually receptive, calling virgin females.

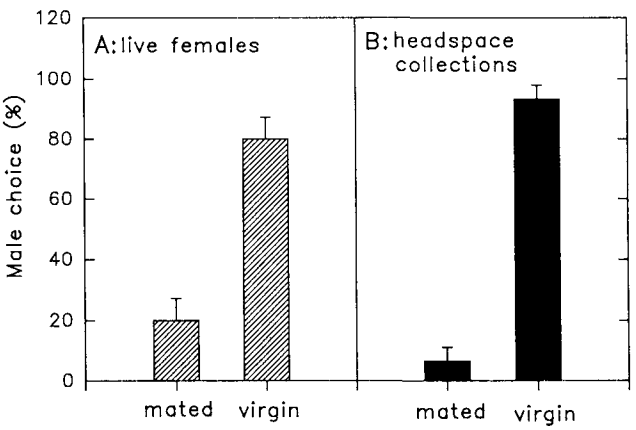

Figure 1. *A* Male choice between live virgin and mated females. Virgin and mated females at similar physiological stages were tested (oocyte length: 1.72 ± 0.05 mm for virgin and 1.78 ± 0.09 mm for mated, $n = 5$ in each group, $p > 0.1$, *t*-test). *B* Male choice between headspace collections from virgin and mated females. A pheromone concentration of 1 female-day-equivalent (FDE) was used. Oocyte length increased from 1.65 ± 0.05 to 1.98 ± 0.05 mm in virgin females and from 1.55 ± 0.04 to 2.14 ± 0.02 mm in mated females during the 24-h headspace collection. Error bars represent SEM.

The sex- and stage-specificity of the behavioral responses were assayed with 0.5 female-day-equivalents (FDE) of the headspace-collected pheromone. Adult males exhibited significantly greater responses to the female pheromone than did nymphs or virgin adult females (fig. 2A). Production of the pheromone was also sex- and stage-specific, as whole-body extracts of virgin females contained significantly more pheromone than extracts of nymphs or adult males (fig. 2B). Production of pheromone by virgin females and maximal responses by adult males support the notion that these volatile chemicals act as sex pheromones.

In addition to the pheromone reported here, a number of other semiochemicals have been described in *B. germanica*[14]. An aggregation pheromone, which includes volatile attractant components[5], is produced by all life stages in association with feces[4] and it exhibits interspecific and intergeneric patterns of attraction[15,16]. The contact sex pheromone, which is produced by both virgin and mated females[17,18], is responsible for the terminal courtship behaviors induced upon contact[1,2]. Dispersion-inducing substances secreted from the salivary gland[19] also act upon contact. A specific volatile attractant is also produced by males to mediate close-range interaction between the sexes[20]. However, unlike all the pheromones mentioned above, the sex pheromone we report here is only produced by females and is clearly most attractive to males. Our discovery of this novel pheromone resulted from the observation of a calling behavior in virgin females. We also employed a highly sensitive olfactometer assay[12] and took extreme caution in using females at the appropriate physiological state because the amount of pheromone released by the female declines after mating (this report) and possibly after oviposition in both virgin and mated females, as suggested by the absence of calling[8].

That release of this sex pheromone is specific to virgin females was demonstrated by the ability of both live virgin females and their headspace collections to attract significantly more males than did mated females in the

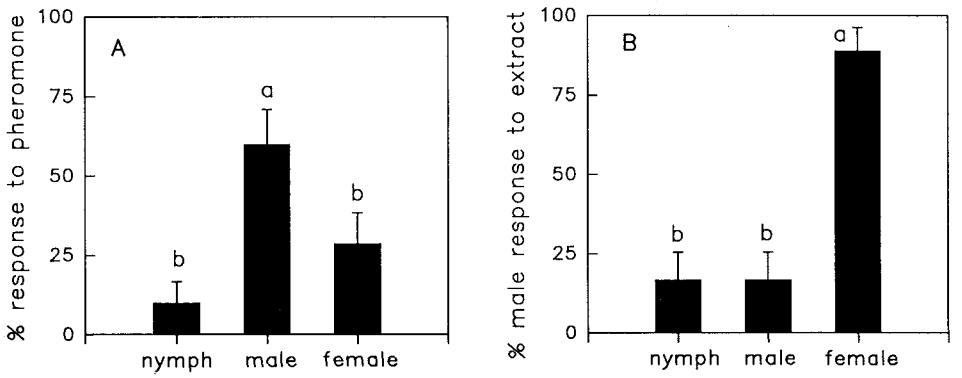

Figure 2. *A* Responses of nymphs, adult males, and adult females to pheromone (0.5 FDE of headspace collection of virgin females). Responses of males were significantly greater than those of nymphs and females ($p < 0.01$, χ^2 test, $df = 2$). *B* Male responses to whole body extracts of nymphs, adult males, and adult females (50 mg body-weight-equivalent was used, which was 1.0, 1.04 and 0.52 individual equivalents for nymphs, males and females, respectively). The female extract was significantly more attractive than the other extracts ($p < 0.001$, χ^2 test, $df = 2$). Error bars represent SEM.

respective assays. This is clearly related to the fact that calling behavior, during which pheromone is emitted, ceases immediately after mating[8]. In another cockroach, *Supella longipalpa*, mating has been shown to terminate both the production and release of female sex pheromones[11]. It remains to be determined whether pheromone production ceases as well in mated *B. germanica* females. German cockroach females become unreceptive after mating and most females do not mate again before ovulation[21]. Whether pheromone production is suppressed or not after mating, it would be advantageous for females to become unattractive to males in order to avoid persistent courtship by males. This is achieved by terminating the release of sex pheromones. Again, the reduction in attractiveness of the mated female could not be accounted for by a reduction in aggregation pheromone or other general odors, because the only difference between the virgin and mated females was their mating status, as we rigorously controlled their physiological age.

Volatile female sex pheromones are known in only a few species of cockroaches[17] and to date, only the sex pheromone of the American cockroach has been identified and confirmed by synthesis. A volatile sex pheromone in the German cockroach was suspected by Volkov et al.[22] who showed that traps containing filter paper that had been contaminated by virgin females trapped more adult males than untreated control traps, and that both treatments trapped few females. However, sex-specificity of production of these materials was not demonstrated. Also, because they were unable to obtain activity from the organic solvent extract, Volkov et al.[22] instead used a water extract of the filter paper for subsequent attempts at isolation of the pheromone. Because the volatile components of the aggregation pheromone of the German cockroach are highly soluble in water[5], attraction of cockroaches to the water extracts could, at least in part, be attributed to the aggregation pheromone which is produced by both immatures and adults. Hexane, which extracts little aggregation pheromone[5], was used to extract the sex pheromone in the present study. Although other volatile attractants, such as those associated with salivary secretions and feces, could also be extracted during this process, the specificity of male resonses to hexane extracts of virgin females and specificity of pheromone production by adult females indicate that the major attractant in the hexane extract is a female sex pheromone.

Critical to our claims of a new female sex pheromone in *B. germanica* is the localization of a specific gland responsible for the production of this pheromone. Among cockroaches, only in the brown-banded cockroach, *Supella longipalpa*, has the female sex pheromone gland been identified. The pheromone is produced by isolated glandular units, each containing a single secretory cell, occurring primarily on the fourth and fifth tergites[23]. In the American cockroach, most of the sex pheromone activity was found in the female alimentary canal but the secretory cells have not been located[24]. We used 5-day-old females which had just initiated calling[8] to localize the site of the sex pheromone gland in *B. germanica*. Only minor behavioral and EAG responses were elicited in males by hexane extracts of the female head, thorax, sternites, ovipositor, and cerci. In contrast, extracts of the abdominal tergites and alimentary canal showed significantly greater activity (fig. 3A). Because food odors can be extracted from the alimentary canal, and the rectum produces the aggregation pheromone[4], the alimentary canal was not examined further for sex pheromone activity. Rather, we focused our attention on the abdominal tergites from which no attractants have been described in female *B. germanica*. A comparison of the anterior and posterior tergites showed significantly greater behavioral and EAG activities in the latter, and the responses to tergites 7–10 were comparable to responses to the extract of all tergites

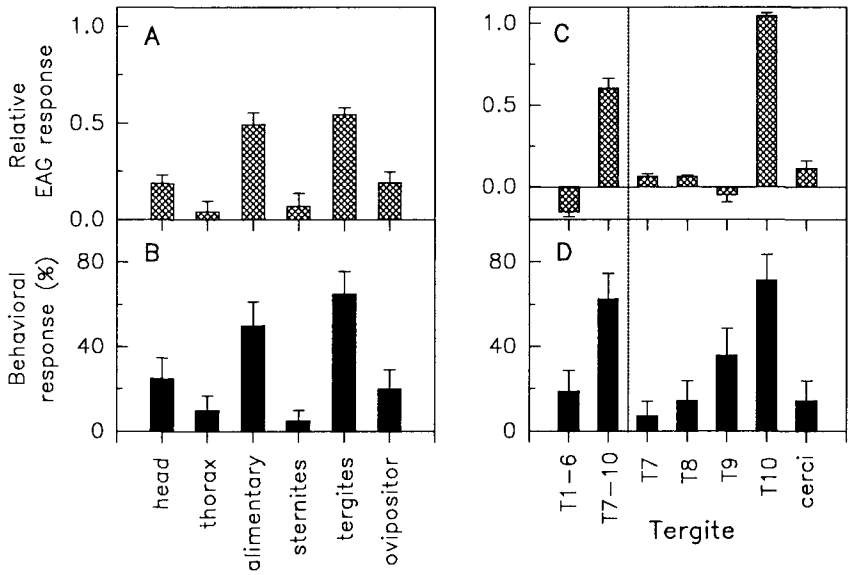

Figure 3. Localization of the site of sex pheromone production with behavioral and EAG assays. For EAG responses, the control was subtracted from the total response and the responses to treatments are represented relative to a standard 1 FE stimulus. Error bars represent SEM. *A* and *B* Activity of body parts. *C* and *D* Activity of tergites. Dashed line in *C* and *D* separates two sets of females. Error bars represent SEM.

combined (fig. 3B). Further analysis of the posterior tergites indicated that the tenth tergite contained the highest amount of pheromone (fig. 3B). Scanning electron microscopy revealed the presence of a female-specific gland on the anterior of the tenth tergite. In virgin females this gland contains highly active secretory cells, suggesting that this gland might be responsible for production of a volatile sex pheromone[25].

To confirm this, the attractiveness of females whose tenth tergite had been ablated was compared in choice assays with sham-operated females in which a similar region on the eighth tergite was removed. The results showed that while the sham-operated females attracted 86.7% of the males, the operated females attracted only 13.3% ($n = 30$), indicating that ablation of the glandular region on the tenth tergite significantly reduced the attractiveness of live females to males ($p < 0.001$, binomial test). Both groups of females exhibited calling behavior and both reached similar stages of oocyte maturation. These results provide conclusive evidence that the gland on the last tergite produces the volatile sex pheromone in female *B. germanica*. At rest, the gland is concealed by the ninth tergite, but it is partially exposed during the calling behavior (pers. obs.). The exposure of the gland during calling may facilitate the release of pheromone, but the exact mechanism of pheromone release in this cockroach is yet to be investigated.

This new sex pheromone has significant potential in the control of German cockroach populations. Monitoring traps that are currently in use are inefficient because they have a very limited capture space and they do not sample cockroaches that retreat to deep refugia[26]. This pheromone can also act as an attracticide to enhance the efficacy of insecticides, which is often limited by the inherent repellency to cockroaches of many insecticide formulations. With the advent of fungi, viruses, bacteria and entomophagous nematodes as biological control agents, this pheromone can be of immense utility to attract cockroaches to infection stations.

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