Site of Pheromone Production in Female *Supella longipalpa* (Dictyoptera: Blattellidae): Behavioral, Electrophysiological, and Morphological Evidence

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ABSTRACT The site of sex pheromone production in female *Supella longipalpa* (F.) was localized to the fourth and fifth abdominal tergites. Behavioral assays of males with hexane extracts of various female body parts showed that these tergites were significantly more attractive than any other region of the body. Both behavioral and electroantennogram assays confirmed that the fourth and fifth tergites contained significantly more sex pheromone than any other tergites. Cuticular pores occur on all tergites, but their density is highest on the lateral margins of the fourth and fifth tergites. Each pore is connected via a long duct to modified epidermal cells, suggesting that these structures are involved in pheromone production and in its release while the female engages in calling behavior.

KEY WORDS sex pheromone, pheromone gland, *Supella longipalpa*

IN MANY INSECTS, especially in the Lepidoptera, glandular modifications of epidermal cells of the integument are usually involved in sex pheromone production by adult females (Percy-Cunningham & MacDonald 1987). However, in other insects, the location and morphology of pheromone-producing glands may vary, even among closely related species. In immature and adult cockroaches, specialized, highly differentiated glands have been described on the abdominal tergites (Roth 1969, Brossut & Roth 1977), sternites (Liang 1956, Dimeo et al. 1978, Sreng 1984), and in association with tracheae (Brossut 1983) and the head (Brossut 1973). Their products have been described in several species (Roth & Alsop 1978; Brossut 1983; Sreng 1979, 1990). In sharp contrast, female sex pheromones have been elucidated in only two species, *Periplaneta americana* (L.) and *Blattella germanica* (L.) (see review by Schal & Smith 1990), but to date, no female sex pheromone glands have been localized. The digestive tract, tergum, sternum, genital atrium, and the antennae have been implicated as regions associated with sex pheromone production in several species. However, no studies have combined behavioral, electrophysiological, and morphological techniques in a systematic search for sites of pheromone production in female cockroaches.

Supella longipalpa (F.) females initiate calling on the fifth or sixth day after the imaginal molt, and they call during the scotophase under a 12:12 or a 16:8 (L:D) photoperiodic regime at 27°C (Chon et al. 1990; Smith & Schal 1990a, 1991). Calling behavior is characterized by elevated tegmina and wings, a recurved abdomen, and occasional expansion of the genital atrium (Hales & Breed 1983). Smith & Schal (1990a) showed that the onset of pheromone production was on day 4, before calling behavior was observed, and that calling on day 5 was associated with the release of a volatile sex pheromone that elicited orientation and courtship responses in males. Both calling and pheromone production are suspended after mating (Smith & Schal 1990b). Herein we present studies that localize the site of pheromone production–release in *S. longipalpa*.

Materials and Methods

Insects. Newly emerged (day 0) adult *S. longipalpa* males and females were separated daily from a colony and maintained at 27°C under a 12:12 (L:D) photoperiod (dark phase 0900–2100 hours) with access to pelleted Purina dog chow (#1780) and water. Males were maintained under the same conditions but in a separate walk-in chamber in which olfactometer trials were conducted. Male *S. longipalpa* exhibit the greatest responses to the female sex pheromone during the scotophase (Liang & Schal 1990a). Therefore,

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behavioral assays were conducted between 4 and 10 h after the onset of the dark phase.

Dissections. Smith & Schal (1990a) showed that, although 4-day-old females do not call, they contain detectable (by behavioral assay) amounts of pheromone. Therefore, to minimize contamination of various female body parts by released pheromone, we used extracts of 4-d-old females for all the behavioral assays. Day 8 females were dissected and extracted for electroantennogram (EAG) studies.

Under a dissecting microscope, females were separated into various body parts as needed. To avoid contamination, different fine forceps and insect pins were used to dissect each body part. The dissecting tools were washed in acetone before each dissection.

Extractions. Virgin females were killed by freezing 1 h before the onset of the scotophase. Whole females or individual body parts were extracted for 5 min in 0.4 ml n-hexane in a 1-ml conical microreaction vial with occasional vortexing. The tissue was removed carefully, and the tube was purged with nitrogen and stored at −20°C for up to 4 d.

Each sample was evaporated to approximately 100 μl under nitrogen and dispensed with a 50-μl Hamilton syringe onto a filter paper (2 by 1 cm, Whatman no. 1) attached to an insect pin. Each dispenser was used for only one assay.

Y Olfactometer and Behavioral Assays. An all-glass, 2-choice Y olfactometer was used for behavioral assays (see Smith & Schal 1990a). The single arm of the Y tube was 74 cm long with a diameter of 2.5 cm. A Plexiglas cage (15 cm long) with a wire screen gate was attached to the downwind end of the tube. Air flow was provided by suction through a pump attached to the Plexiglas cage and exhausted out of the room. Each of the two choice arms was 31 cm long, and both formed a 90° angle at the branch point. The distal ends of both tubes accepted the dispensers loaded with extracts or hexane only.

Males exhibit maximal responses to female extracts after they are 10 d old (Liang & Schal 1990a). Five males (30–60 d old) were used for each assay. Males were placed in the cage at the downwind end of the Y tube 20 min before each trial. An air stream of 30 cm/s was maintained through each tube throughout the acclimation and test periods. Each trial consisted of a comparison of a female extract with a hexane control and was replicated at least nine times. In some trials, extracts of different body parts from the same female were tested against each other to determine which body region had the greatest activity. If no response occurred within 5 min after introduction of the filter paper dispensers, the extract was replaced with a whole-body extract of a 4-d-old female. Lack of response to this extract eliminated the trial from statistical analysis; a positive response to the second extract was used to indicate lack of activity in the first extract.

The number of males that became active and the number that chose the treatment or control was recorded. Because pheromone concentration affects both the percentage of males that are activated and the percentage exhibiting the correct choice (Liang & Schal 1990b), the latter parameter was used to present the results of the behavioral assays. We also recorded the time taken (latency) by the first male in each trial of five males to reach 10 cm past the choice point. Each male was used once or twice with at least 48 h separating each trial.

Every 2–3 d, the Y tubes were washed with acetone and baked at 350°C overnight.

The numbers of males choosing each of the two choices in replicated trials were used in statistical analyses of the behavioral data using Student's t test. In the figures, we use percentages for clarity.

Electroantennogram Studies. The EAG apparatus was similar to that described by Roelofs & Comeau (1971). The recording electrode was a silver–silver chloride wire inserted into a Pasteur pipette whose tip was filled with saline. The isolated antenna was supported in wax with its base immersed in saline and constantly exposed to a stream of clean air. Three groups, each consisting of five females, were dissected and the various body parts extracted. Filter paper loaded with five female body part equivalents (FE, see Liang & Schal 1990a,b) was placed into a pipette and used in three or four stimuli of a single male antenna. Each stimulus consisted of a puff of 3 ml of air delivered by syringe through the pipette.

The EAG response was calculated by subtracting the response in mV to a control puff from that to the treatment.

Morphological Studies. For scanning electron microscopy (SEM), the abdominal tergites were fixed in 2% glutaraldehyde, then in 1% osmium tetroxide, and dehydrated in an ethanol series. After critical-point drying, they were sputter-coated and examined in a Hitachi S450 SEM. For SEM of the undersides of tergites, fresh tergites were sonicated and then fixed.

For counting cuticular pores, abdominal tergites 1–7 were removed, freed from underlying tissues, treated in warm 10% KOH overnight, washed in distilled water three times for a total of 3 h, dehydrated in an ethanol series (50, 70, 95, and 100%), and mounted in Permount. Cuticular pores on the lateral portions of tergites were counted at 400× using a grid (0.28 × 0.18 mm) in the ocular of a light microscope.

Results and Discussion

Behavioral Studies. On average, each calling bout lasted 18.8 ± 4.6 min ($\bar{x}$ ± SEM) (n = 5 females) between changes in the female's posi-

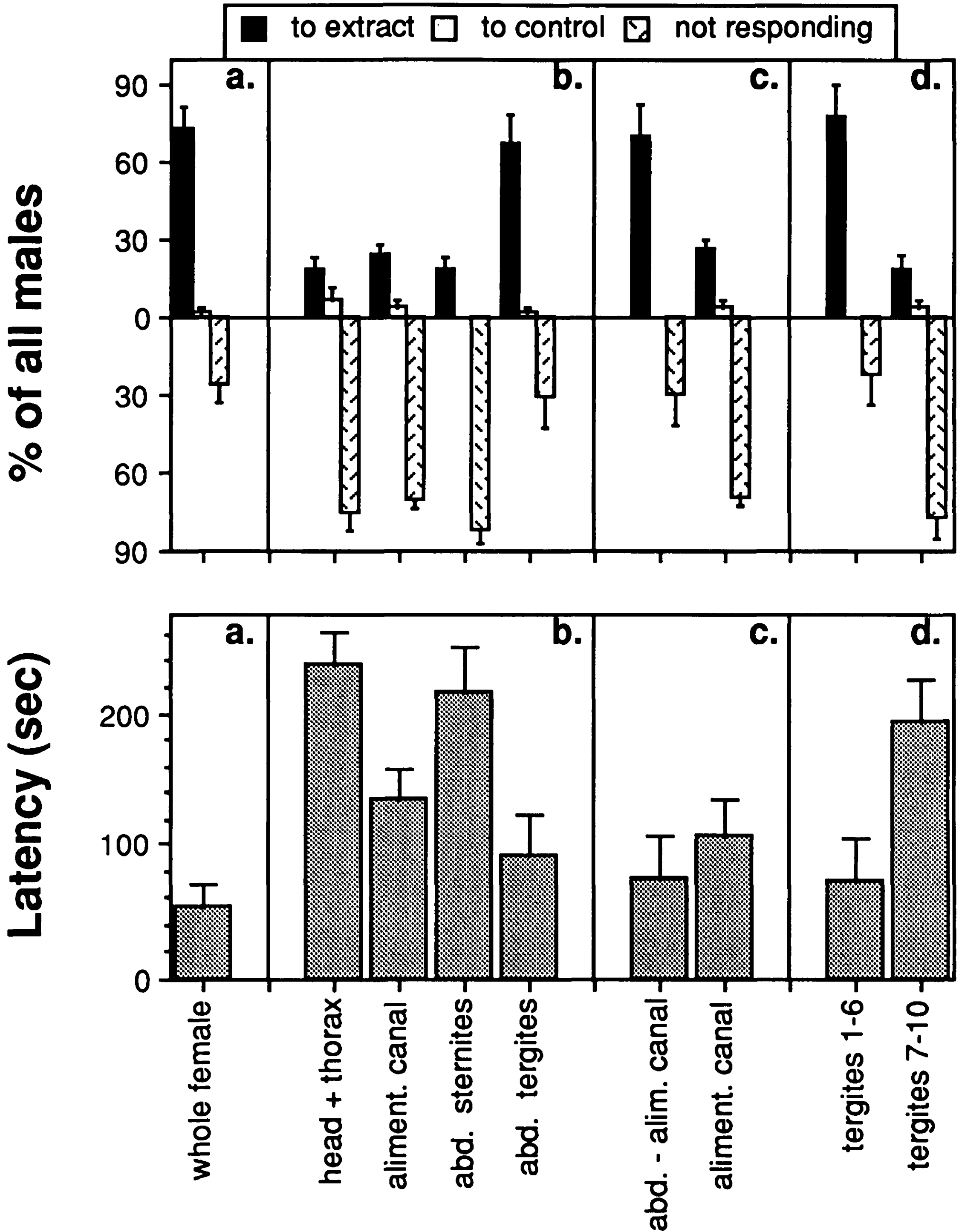

Fig. 1. Behavioral assays with male *S. longipalpa* in a two-choice olfactometer. Extracts of 4-d-old females or of various body parts were assayed against a hexane control. Latency is the time taken by the first male to reach a point 10 cm past the binary choice; it was set at 300 s if no males responded in 5 min. At least nine trials (45 males) were performed in each treatment. Bars represent SEM. Each of experiments (a) through (d) represents responses to a given set of females or their body parts.

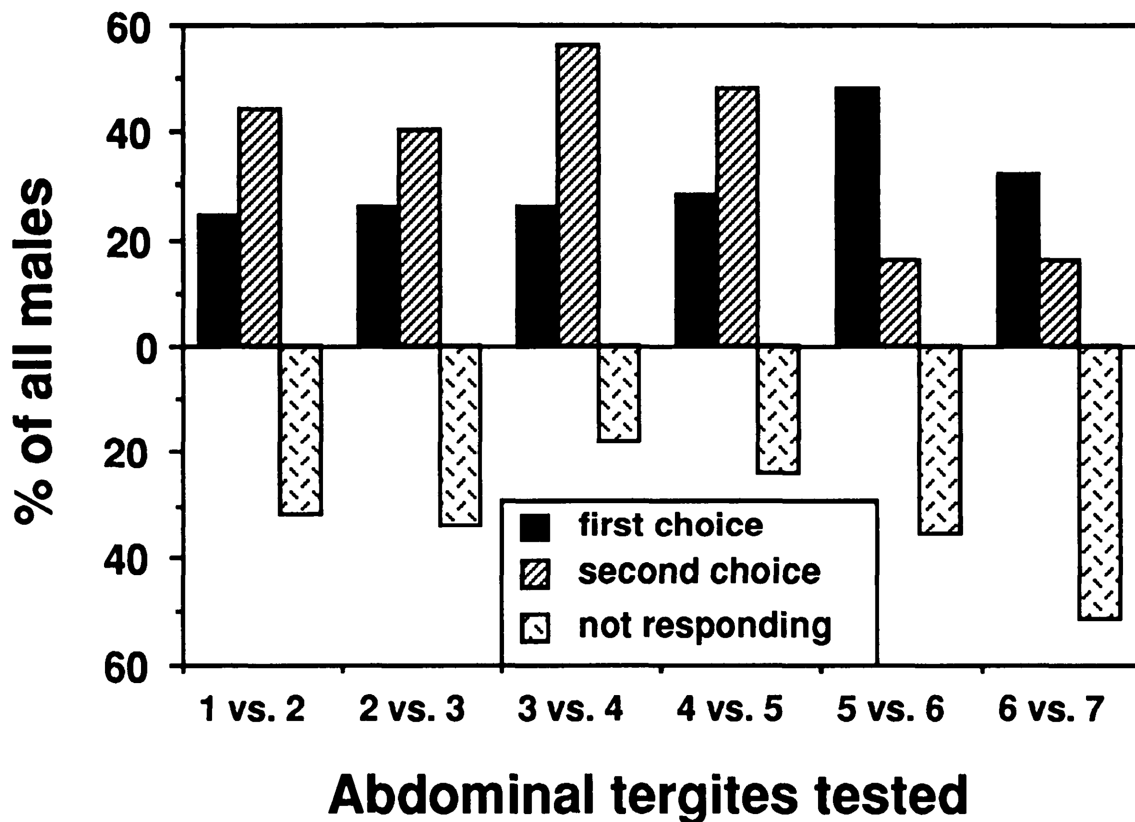

Fig. 2. Behavioral assays with male *S. longipalpa* in a two-choice olfactometer. Extracts of each abdominal tergite from 4-d-old females were assayed against extracts of the adjacent posterior tergite. Ten trials (50 males) were performed in each treatment. Bars represent SEM.

tion, the genital atrium opened an average of 7.6 ± 1.5 times during this period, and the third valves of the genitalia pulsated in the expanded atrium. This posture would suggest that the abdominal tergites (which are exposed during calling), the genitalia or the genital atrium, or the alimentary canal might produce or release the sex pheromone.

We used several criteria to measure male responses in the two-choice olfactometer, including the latency of the response and the percentage of all males that made the correct choice (see Liang & Schal 1990b). Males exhibited active behavioral responses to extracts of 4-d-old virgin females. In 17 trials (85 males) with whole-body extracts, 75% of the males were activated, whereas 25% remained in the chamber for 5 min; 97% of the activated males (73% of all males) chose the female extract, and only 2% chose the hexane control (Student's *t* test, $T = 8.21$, $P < 0.0001$) (Fig. 1a). This compares with 95–100% of males attracted to the whole-body extract of an 8-d-old *S. longipalpa* female (Liang & Schal 1990b, Smith & Schal 1990a). The latency of male response to whole females was only 54 ± 17 s (Fig. 1a).

Bioassays of various female body parts indicated that the greatest activity was in the abdominal tergites (Fig. 1). Extracts of the head and thorax failed to activate and attract >25% of the males ($T = 1.47$, $P = 0.18$ compared with hexane controls). Similarly, only 13, 18, and 24% of all the males were attracted to extracts of the external genitalia (not shown), abdominal sternites, and alimentary tract, respectively. The alimentary canal alone was less attractive than the abdomen from which the alimentary canal had been removed (Fig. 1c). The abdominal tergites, on the other hand, elicited responses from 70% of the males, 97% of the activated males (68% of all males) orienting to the extract (Fig. 1b). The latency of the response to tergite extracts was 91 s compared with 217 and 134 s to sternites and the alimentary canal, respectively.

Bioassays of the tergites indicated that extracts of tergites 1–6 were significantly more attractive than tergites 7–10 from the same females (Fig. 1d): 78% of the males chose the anterior tergites over a hexane control compared with 18% that chose the posterior tergites ($T = 21.43$, $P < 0.0002$). Also, males responded significantly

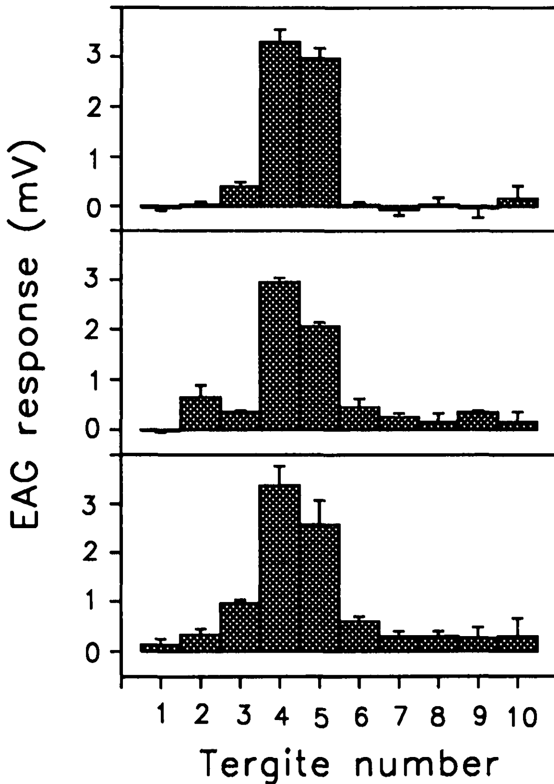

Fig. 3. Electroantennogram responses of antennae of male *S. longipalpa* to extracts of abdominal tergites from 7-d-old females. Responses to control stimuli were subtracted from responses to extracts. Each graph represents the average of three responses of a male antenna to a series of extracts. Different male antennae were used for each graph. Bars represent SEM.

faster to anterior than to posterior tergites (73 ± 31 versus 193 ± 33, $P < 0.05$).

Direct comparisons of successive tergites confirmed that activity increased posteriorly to the fifth tergite but decreased in the sixth and seventh tergites (Fig. 2).

Electroantennogram Studies. The EAG responses to extracts of individual abdominal tergites confirmed that the fourth and fifth tergites contained the highest pheromone concentration (Fig. 3). The responses to extracts of either tergite were generally greater than 2.5 mV, and the fourth tergite appeared to contain more pheromone than the fifth tergite. Responses to all other individually tested tergites as well as to all sternites tested together were usually <0.5 mV.

Morphological Studies. Fig. 4a shows a SE micrograph of cuticular pores, which are numerous on the abdominal tergites. The pores have internal diameters of ≈0.25 μm, distinguishing them from the larger diameters of the cuticular sensilla. The pores generally occur at the base of surface scales and lead to tubular structures that penetrate the cuticle. Fig. 4b shows the underside of an abdominal tergite after sonication. Cuticular pores open through long ducts to modified epidermal cells.

Both light and SE microscopy indicated that the cuticular pores were distributed throughout the surface of tergites, but at much higher densities on the lateral regions of tergites than in the medial region. The densities of pores on the lateral regions of tergites 1–7 were measured under a light microscope at 400× in six females. The mean number of cuticular pores increased from 186 ± 28 per mm^2 on the first tergite to 1,036 ± 55 pores per mm^2 on the fifth tergite, and decreased to 146 ± 14 pores per mm^2 on the seventh tergite (Fig. 5).

Sites of Pheromone Production in Other Cockroaches. A similar association of cuticular pores with pheromone production or storage has been shown in male *Nauphoeta cinerea* (Olivier) (Sreng 1979, Menon 1981) and related members of the subfamily Oxyhaloinae (Sreng 1984). *N. cinerea* males produce a multicomponent sex pheromone (Sreng 1990) which is apparently released from these pores. Our preliminary work indicates that, as in *N. cinerea* males, large glan-

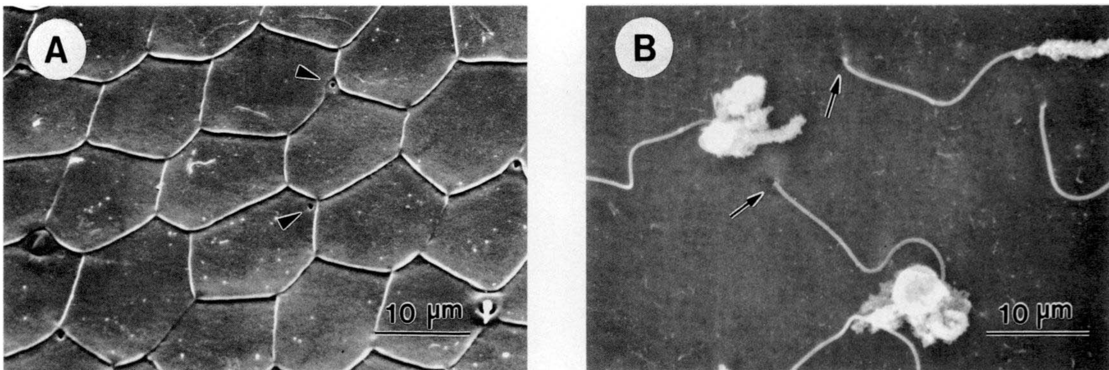

Fig. 4. Scanning electron micrographs of (A) the lateral margin of the fourth tergite, and (B) the underside of the sonicated tergite showing the cuticular duct. Arrows indicate cuticular pores in (A) and the insertion of the ducts into the cuticular pores in (B).

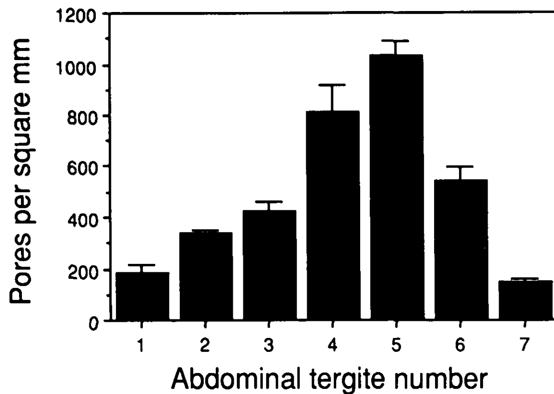

Fig. 5. The density of cuticular pores on the lateral region of tergites one through seven. The number of pores was determined in a grid (0.28 by 0.18 mm) in the ocular of a light microscope; $n = 6$ females.

dular cells beneath the cuticle are connected to the cuticular pores via long ducts, and we hypothesize that the ducts serve to transport the pheromone to the cuticular surface.

Using behavioral assays with various body parts of virgin *P. americana* females, it was first concluded that the sex pheromone was produced in the head (Sturckow & Bodenstein 1966). However, Sass (1983) showed that both components of the pheromone, periplanone-A and -B, could be extracted from female alimentary canals. More recent work has documented a specific calling stance, presumably associated with pheromone release, in which females expose the genital chamber and anal region by lowering the seventh abdominal sternite (Seelinger 1984). It remains unknown whether specific glands are exposed during this behavior, or whether pheromone produced in the midgut is simply released through the anus.

In *Byrsotria fumigata* (Guérin), the Cuban burrowing cockroach, removal of the ovaries (Barth 1962), colleterial glands (Moore & Barth 1976), and the digestive tract did not interfere with pheromone production. Using wax plugs inserted into the genital atrium and electrocautery of the lining of the genital atrium, Moore & Barth (1976) showed that females producing pheromone (determined with behavioral assays) possess active columnar epithelium along the roof of the atrium, whereas in cauterized nonproducers, cells appeared smaller.

In insects that use modified cuticular lipid components as sex pheromones, a logical site of sex pheromone biosynthesis is the epidermal cells, particularly the oenocytes, which are involved in cuticular lipid production (Blomquist et al. 1987). Females of the German cockroach, *B. germanica*, produce a contact pheromone consisting of at least four components (Nishida & Fukami 1983, Schal et al. 1990). Although antennal fencing is sufficient to elicit courtship responses in males, the pheromone is not limited to female antennae but is found on all cuticular surfaces. However, the site of synthesis has not been localized. Moreover, large amounts of pheromones can be extracted from noncuticular internal sources, including the hemolymph (unpublished observations), suggesting a complex route of externalization of these hydrocarbon derivatives (Schal et al. 1991).

Our report is the first to localize a sex pheromone gland in female cockroaches using behavioral, electrophysiological, and morphological studies. We are currently elucidating the chemical structure of the *S. longipalpa* sex pheromone, which will facilitate a direct examination of the site of pheromone production using analytical and radiochemical procedures.

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