A NEW COMPONENT OF THE FEMALE SEX PHEROMONE OF Blattella germanica (L.) (DICTYOPTERA: BLATTELLIDAE) AND INTERACTION WITH OTHER PHEROMONE COMPONENTS

COBY SCHAL, EDINA L. BURNS, RUSSELL A. JURENKA, and GARY J. BLOMQUIST

1Department of Entomology
Cook College, Rutgers University
New Brunswick, New Jersey 08903
2Department of Biochemistry
University of Nevada, Reno
Reno, Nevada 89557-0014

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Abstract—A fourth component, 3,11-dimethyl-2-heptacosanone, was identified as a cuticular contact sex pheromone of the female German cockroach, Blattella germanica. In behavioral assays, higher dosages of 3,11-dimethyl-2-heptacosanone were needed to elicit similar sexual responses in males to those elicited by the major pheromone component, 3,11-dimethyl-2-nonacosanone. A 15:85 blend of the C27 and C29 methyl ketone homologs resulted in a dose-response curve intermediate between that of each of the components alone, indicating independence of activity of each component and lack of synergism. Moreover, the activity of 3,11-dimethyl-2-nonacosanone was not enhanced by female cuticular hydrocarbons. The relationship between sexual responses of males to females and to isolated female antennae, and the amount of cuticular pheromone on whole females was investigated. Cuticular sex pheromone found on females increased with the age of the female, as did the male response to whole females. However, a bimodal male response was elicited by isolated female antennae. Differences between behavioral and analytical assays of pheromone are discussed.

1Present address: Department of Entomology, New York State Agricultural Experiment Station, Geneva, New York 14456.
Key Words—German cockroach, *Blattella germanica*, Dictyoptera, Blattellidae, sex pheromone, behavioral response, contact pheromone, 3,11-dimethyl-2-heptacosanone, 3,11-dimethyl-2-nonacosanone.

INTRODUCTION

The female German cockroach, *Blattella germanica*, produces a nonvolatile three-component cuticular sex pheromone. The most abundant component is 3,11-dimethyl-2-nonacosanone, with lower amounts of 29-hydroxy-3,11-dimethyl-2-nonacosanone and 29-oxo-3,11-dimethyl-2-nonacosanone (review: Nishida and Fukami, 1983). Whole females and isolated body parts of sexually mature females (e.g., antennae, legs, wings) elicit the complete courtship wing-raising response in males, as does each of the three pheromone components alone (Roth and Willis, 1952; Bell and Schal, 1980; Nishida and Fukami, 1983). Grouped females become receptive to courting males four to five days after adult emergence (Gadot et al., 1989).

Nishida and Fukami (1983) noted that excised antennae of individually isolated teneral females and of sexually mature females elicit courtship wing-raising responses in the majority of tested males, whereas antennae from young, sexually immature adult females elicit responses in only a fraction of the males tested. However, gas-liquid chromatography (GLC) of female extracts at five-day intervals suggests that cuticular pheromone in isolated females increases gradually between days 0 and 15 (Schal et al., 1990). This apparent discrepancy between analytical and behavioral observations has not been resolved because the relationship between the amount of pheromone on females at different ages and male response has not been studied. To address this, we examined the amount of cuticular pheromone found on individual females at different ages.

The major hydrocarbon component, from GC-MS analysis of sexually mature *B. germanica* females, is an isomeric mixture of 3,7-, 3,9-, and 3,11-dimethylnonacosane (Augustynowicz et al., 1987; Carlson and Brenner, 1988; Jurenka et al., 1989). Jurenka et al. (1989) and Chase et al. (1990) suggested that the production of the methyl ketone pheromone component results from the sex-specific oxidation of its hydrocarbon analog. Since a mixture of isomers of 3,9- and 3,11-dimethylheptacosane also is found in cuticular washes of females (ca. 2% of the total hydrocarbons) but only the 3,11-dimethyl ketone isomer (3,11-dimethyl-2-heptacosanone) was found (Jurenka et al., 1989), and since neither methyl ketone homolog was found in adult males, we hypothesized that 3,11-dimethyl-2-heptacosanone might act as a fourth pheromone component. We report here on the verification of this hypothesis using behavioral assays and on the responses of males to combinations of the C$_{27}$ and C$_{29}$ methyl ketone components.
METHODS AND MATERIALS

Insects. Cockroach nymphs that hatched within four days of each other were reared in 2-liter glass jars and fed dry Purina dog chow and water. Newly emerged (day 0) adult males and females were separated daily and kept in groups until use. Both nymphs and adults were kept at 27°C under a 12:12 light-dark photoperiod.

Extraction, Separation, and Quantification of Pheromone. Cuticular lipids of females (10 per group) were extracted with two 3-ml hexane washes, each for 5 min. Two internal standards, 14-heptacosanone (100 ng) and 1-hexacosan-1-ol (45 ng), were included during extraction to allow quantification by GLC. The hexane washes were combined and most of the solvent removed under N₂. The extracts were separated on TLC plates (silica gel 60F-254; EM Science) developed twice in hexane-diethyl ether (93:7 v/v). Fractions were scraped into test tubes and extracted with diethyl ether.

The fraction containing 29-hydroxy-3,11-dimethyl-2-nonacosanone was reduced to dryness under N₂, and 100 μl redistilled acetyl chloride was added for 30 min to acetylate the pheromone component. Samples were analyzed on a HP 5890A GLC equipped with a splitless injector and a flame-ionization detector, and interfaced with a HP 3390A integrator. Injection in isooctane or methyl chloride (methyl ketone and hydroxy methyl ketone, respectively) was made onto a 15 m × 0.53 mm ID SPB-5 column programmed from 60°C to 250°C at 20°C/min and then to 320°C at 3°C/min. The injector and detector were maintained at 330°C.

Test Compounds. Synthetic racemic 3,11-dimethyl-2-nonacosanone (80% pure, 1.9% 3,11-dimethyl-2-heptacosanone) and 29-hydroxy-3,11-dimethyl-2-nonacosanone were obtained from Drs. A.W. Burgstahler and R. Nishida. The natural pheromone components have the (3S,11S) configuration, but all the synthetic stereoisomers are active at the same concentration in behavioral assays (Nishida and Fukami, 1983). Other compounds were obtained from extracts of sexually mature females and separated by TLC as described above. The C₂₇ and C₂₉ methyl ketone components (85% of the methyl ketone fraction; 15% unknown compounds) were separated by GLC and cold-trapped in 30-cm capillary glass tubes using the GLC procedure described above. Each was more than 93% pure with less than 0.5% of the other homolog.

The highest concentration of each test compound or blend for behavioral assays was calibrated by GLC against an external standard and then serially diluted in CCl₄.

Bioassay. Male response was tested using a modification of the assay developed by Roth and Willis (1952) and adopted by Nishida and Fukami (1983). An antenna of a male Supella longipalpa cockroach was excised and
mounted on a glass Pasteur pipet and a 5-μl solution of test compound in CCl₄ was applied to its distal 1 cm. The antenna was used immediately to test the sexual responses of 30 males 14–30 days old that were housed individually in 9-cm-ID × 5-cm-deep glass beakers and supplied with dog food and water. A positive response consisted of turning away from the stimulus and raising the tegmina and wings to a 90° angle within 30 sec. Any male that failed to respond to both a test compound and later to the antenna of a six-day-old female was discarded. Since isolated males are most responsive in the scotophase (Bell et al., 1978), bioassays were conducted hourly between hours 2 and 10 of a 12-h scotophase. The results were subjected to a maximum likelihood probit-log dose analysis (SAS, 1985).

All treatments were systematically arranged so that low, medium, and high dosages were equally represented in the early, middle, and late portions of the scotophase.

To compare male responses elicited by isolated female antennae (above) to the responses elicited by whole females, single live females were introduced successively to two individually isolated males. The latency between contact with the male and male wing-raising was recorded. Males were allowed 30 sec to respond. A total of 54 males were tested using 27 females. The same cohort of females was used daily for seven days between the ages of 0 and 6 days.

RESULTS

3,11-Dimethyl-2-heptacosanone: A New Pheromone Component. We used Supella longipalpa antennae to deliver test compounds to males in order to avoid any confounding effects of conspecific odors. Isolated B. germanica males did not respond to excised antennae of male S. longipalpa mounted on Pasteur pipets. However, when loaded with synthetic 3,11-dimethyl-2-nonacosanone, or when rubbed against the cuticle of a sexually receptive B. germanica female, such antennae elicited in the male a characteristic stilt posture, antennal waving, an abdominal twitch, and a rotational turn accompanied by wing-raising (see Roth and Willis, 1952; Bell and Schal, 1980). Courtship wing-raising exposes a tergal gland on the male, which the female palpates, placing her in position for copulation. Wing-raising is a terminal act of the male’s sexual response requiring chemosensory stimuli, and subsequent acts such as copulatory thrusts can be elicited in males by physical stimuli alone applied to its tergum (mimicking female mounting).

Both synthetic and female-extracted, GLC-separated 3,11-dimethyl-2-nonacosanone elicited similar responses in B. germanica males (Figure 1A). Overlapping fiducial limits at the RD₅₀ and RD₇₅ and similar slopes indicated identical dose–response curves for both materials (Table 1). Female-extracted and GLC-separated 3,11-dimethyl-2-heptacosanone also elicited strong sexual responses
FIG. 1. (A) Responses of *B. germanica* males to various dosages of natural (circles) and synthetic (squares) 3,11-dimethyl-2-nonacosanone and natural 3,11-dimethyl-2-heptacosanone (triangles). At least 75 males were tested with each compound at each dosage, except at 200 ng at which \( N = 30 \) males. (B) Responses of *B. germanica* males to various dosages of the TLC-purified methyl-ketone fraction (circles), a 15:85 blend of natural GLC-separated 3,11-dimethyl-2-heptacosanone and 3,11-dimethyl-2-nonacosanone (triangles), and 2.5 \( \mu g \) TLC-purified cuticular hydrocarbons combined with synthetic 3,11-dimethyl-2-nonacosanone (squares). At least 76 males were tested with each combination at each dosage.

in males. However, the dose–male response relationship with 3,11-dimethyl-2-heptacosanone was significantly different from both the natural and synthetic 3,11-dimethyl-2-nonacosanone, based on nonoverlapping fiducial limits following probit analysis (Figure 1A, Table 1).

**Interactions among Pheromone Components.** The methyl ketone TLC fraction from extracts of sexually mature females (20% 3,11-dimethyl-2-heptacosanone, 64% 3,11-dimethyl-2-nonacosanone) elicited intermediate responses between each of its two major components (Figure 1). The \( \text{RD}_{50} \) dosage was not significantly different from that of 3,11-dimethyl-2-nonacosa-
none alone, but significantly higher dosages of the whole methyl ketone fraction were required to elicit responses in 75% or more of the cockroaches (Table 1). These data suggest that the less active 3,11-dimethyl-2-heptacosanone is responsible for the reduced activity of the total methyl ketone fraction. Combining the two major \( C_{27} \) and \( C_{29} \) methyl ketone pheromone components in a 15:85 ratio resulted in a dose–response curve identical to that obtained with the total methyl ketone fraction (Figure 1B, Table 1). Synergism was clearly not indicated, and it is unlikely that other minor components in this TLC fraction significantly enhance or suppress male responsiveness.

Various amounts of synthetic 3,11-dimethyl-2-nonacosanone and 2.5 \( \mu \)g cuticular hydrocarbons (TLC fraction) were loaded onto S. longipalpa male antennae. An antenna of a 6-day-old B. germanica female contains 1087 ± 32 ng (mean ± SEM, \( N = 5 \)) of hydrocarbons. The dose–response relationship to these mixtures was not significantly different from the response to 3,11-dimethyl-2-nonacosanone alone (Figure 1B, Table 1).

**Behavioral Responses of Males to Females and to Isolated Female Antennae.** Our assays employed grouped females, which undergo sexual maturation faster than isolated females (Gadot et al., 1989), while Nishida and Fukami (1983) used isolated females. We therefore repeated the dose–response relationship study with antennae of different aged grouped females. Our results clearly corroborate Nishida and Fukami’s (1983): Antennae from newly emerged females elicited responses in nearly 100% of tested males (ca. 85% in their assays), the response declined to 58% by day 3 (nearly 0% by day 4 in their assays) and peaked at 100% on day 6 [day 9 in Nishida and Fukami (1983)]

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**Table 1. Maximum Likelihood Probit Analysis of Six Log-Dosage-Male Response Relationships**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dosage (ng)</th>
<th>RD(_{50}) (95% FL)</th>
<th>RD(_{75}) (95% FL)</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,11-Dimethyl-2-nonacosanone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synthetic</td>
<td>1.2 (0.9–1.5)</td>
<td>3.4 (2.6–4.5)</td>
<td>1.47</td>
<td></td>
</tr>
<tr>
<td>Natural</td>
<td>1.1 (0.7–1.8)</td>
<td>3.7 (2.3–6.6)</td>
<td>1.31</td>
<td></td>
</tr>
<tr>
<td>3,11-Dimethyl-2-heptacosanone</td>
<td>17 (7.0–58)</td>
<td>392 (99–7409)</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Methyl-ketone TLC fraction</td>
<td>2.2 (1.2–3.7)</td>
<td>10 (5.9–22)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>15:85 ( C_{27} ):( C_{29} ) methyl ketones</td>
<td>3.2 (1.0–10)</td>
<td>13 (4.7–94)</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>3,11-Dimethyl-2-nonacosanone + 2.5 ( \mu )g hydrocarbons</td>
<td>2.0 (0.9–3.5)</td>
<td>4.5 (2.6–10)</td>
<td>1.88</td>
<td></td>
</tr>
</tbody>
</table>

*RD\(_{50}\) and RD\(_{75}\): dosages required to elicit wing raising responses in 50% and 75% of tested males, respectively. FL: 95% fiducial limits (in nanograms) associated with the 50% and 75% response levels.
(Figure 2A). Male response remained at or near 100% throughout the female's adult life (data not shown).

Male responses to whole females were significantly different from responses to isolated antennae from females of the same ages (Figure 2A). Eighty percent of tested males responded within 30 sec of contacting a newly emerged female, and the response increased steadily to 100% upon contact with 5-day-old females. The latency of the male wing-raising response to whole females decreased from 8.6 sec (day 0 females) to 5.2 sec (6-day-old females) (Figure 2B). Thus, the patterns of male responses to whole females and to isolated female antennae are different.

**Pheromone Accumulation over Time.** Newly emerged females contained $4.4 \pm 0$ ng, 3,11-dimethyl-2-heptacosanone and $9.3 \pm 0.8$ ng 3,11-dimethyl-2-nonacosanone. In females reared in groups, the amounts of both components increased after emergence in relation to oocyte maturation (Figure 3); on average, females oviposited on day 9 and contained $187 \pm 33$ ng 3,11-dimethyl-2-heptacosanone and $494 \pm 28$ ng 3,11-dimethyl-2-nonacosanone. After day 2, the percentage composition of the two components remained the same (ca. 40:60, respectively) until ovulation. The amount of 29-hydroxy-3,11-dimethyl-

![Graph A](image1.png)

**Fig. 2.** (A) Percentage of *B. germanica* males ($N = 54$) responding within 30 sec of stimulation with whole females ($N = 27$) and isolated female antennae of various ages ($N = 77$). (B) Latency (in seconds) of male response to whole females of various ages.
Fig. 3. (A) Amount of 3,11-dimethyl-2-heptacosanone and 3,11-dimethyl-2-nonacosanone per female of various ages. Each point represents the mean (±SEM) of three to five determinations of 10 females each. (B) Relationship between age and oocyte length in B. germanica (N > 10 for each mean).

2-nonacosanone increased from 3.2 ng per female on day 3 to 11.6 ng on day 9 (data not shown).

DISCUSSION

Sex Pheromone Blend of B. germanica. Nishida and Fukami (1983) summarized work on three components of the female sex pheromone of B. germanica. We now add a fourth component, 3,11-dimethyl-2-heptacosanone, to this blend. This component is second only to 3,11-dimethyl-2-nonacosanone in mass on the cuticle of sexually receptive females (Figure 3A). However, it is at least an order of magnitude less effective than 3,11-dimethyl-2-nonacosanone (Figure 1A, Table 1). The mass-activity relationship among the four components is as follows: 3,11-dimethyl-2-nonacosanone is most abundant, followed by
3,11-dimethyl-2-heptacosanone (Figure 3A), 29-hydroxy-3,11-dimethyl-2-nonacosanone and, lastly, 29-oxo-3,11-dimethyl-2-nonacosanone (data not shown). The methyl alcohol is most active, followed by the C\textsubscript{29} and C\textsubscript{n} methyl ketones and, lastly, 29-oxo-3,11-dimethyl-2-nonacosanone (Figure 1) (Nishida and Fukami 1983). Small amounts of unknowns in the TLC methyl ketone fraction may comprise other pheromone components. Moreover, the suggestion that the methyl ketone components are formed through oxidation of the respective branched alkanes by a 3,11-dimethyl specific enzyme (Jurenka et al., 1989; Chase et al., 1990) and presence of 3,11-dimethylheptatriacontane in cuticular extracts of Blattella germanica (Carlson and Brenner, 1988), together suggest that 3,11-dimethyl-2-hentriacontanone will be another pheromone component.

The lower pheromonal activity of the C\textsubscript{27}, as compared with the C\textsubscript{29} methyl ketone component, is predicted from the work of Nishida and Fukami (1983): Shorter or longer carbon chains of the pheromone analog 3-methyl-2-nonacosanone had lower activity. It is expected therefore that the activity of 3,11-dimethyl-2-hentriacontanone, if found on the female’s cuticle, will be lower than that of 3,11-dimethyl-2-nonacosanone.

As noted by Nishida and Fukami (1983), activity of the sex pheromone blend of Blattella germanica is strikingly different from blends in other insects (e.g., Lepidoptera). Each component alone can elicit the complete courtship wing-raising response in the male cockroach, whereas in most insects deletion of components reduces responses (review: Tamaki, 1985). In addition, combining components does not enhance or synergize their activity (Figure 1B). Indeed, the combined activity of the more active 3,11-dimethyl-2-nonacosanone and the less active 3,11-dimethyl-2-heptacosanone is intermediate between the activities of each component alone.

Blattella germanica nymphs and adult males and females have the same cuticular hydrocarbons, although their relative compositions vary (Jurenka et al., 1989). The rationale for combining 3,11-dimethyl-2-nonacosanone with female cuticular hydrocarbons on a S. longipalpa antenna was to verify whether a "species-specific signature" will enhance the activity of the pheromone. Clearly, this was not the case (Figure 1B, Table 1). In the housefly, Musca domestica, Uebel et al. (1976) reported that sex specific methyl- and dimethyl-branched C\textsubscript{27} and C\textsubscript{29} alkanes enhanced the mating strike activity of males when combined with (Z)-9-tricosene, the major sex pheromone component. Male flies possess very small amounts of methyl-branched alkanes compared with mature females (Nelson et al., 1981), and therefore the methylalkanes represent true sex pheromone components. More recent work by Adams and Holt (1987) confirmed that the C\textsubscript{23} alkene is the main sex attractant, but they found that the methylalkanes promote sexual contact and mating as arrestants. Furthermore, Adams and Holt (1987) reported that a C\textsubscript{23} epoxide and ketone present only on the female housefly serve as sex recognition factors. In contrast, each of the
four recognized contact sex pheromone components of the German cockroach elicits the complete courtship wing-raising response in the male cockroach.

Comparison of Analytical and Behavioral Assays of Pheromone. The pattern of pheromone production, as measured by its accumulation on the cuticle was reported previously (Schal et al., 1990). However, it was measured in isolated females at five-day intervals and was not directly related to oocyte maturation. For the present study it was important to track the daily changes in cuticular pheromone on whole females reared in groups. The data indicate that the amounts of each of the three major components gradually increase after the adult molt through at least day 6, in relation to oocyte maturation (Figure 3 and unpublished observations), and reach maximal values near or shortly after ovulation (Schal, unpublished observations). These results support our previous determinations that were conducted on isolated females every five days (Schal et al., 1990). Similar correlations of pheromone production and ovarian development were obtained with the housefly (Dillwith et al., 1983).

In contrast to these findings with whole female extracts, isolated antennae of teneral and 9-day-old sexually mature females elicited strong wing-raising responses in males, while the antennae of 4-day-old females elicited little or no response (Nishida and Fukami, 1983). In order to explain differences between behavioral and analytical results, we repeated Nishida and Fukami’s (1983) behavioral assays using the same colony of cockroaches as we used for analytical determinations (Figure 2). Clearly, the pattern of behavioral responses to isolated female antennae is similar in both studies. In our work, male response declines with female age between 0 and 3 days, but subsequently increases and peaks in 6-day-old females (Figure 2A). The differences between our results and Nishida and Fukami’s (1983) are likely due to different rearing and assay temperatures in the respective laboratories (27°C and 25°C). More importantly, however, our assays were conducted on grouped females whereas their work employed individually isolated females. Schal et al. (1990) showed that juvenile hormone induces production of sex pheromone in B. germanica females, and Gadot et al. (1989) showed that grouping accelerates the gonotrophic cycle by advancing corpora allata activity. Grouping of females would therefore advance pheromone synthesis, resulting in greater male responses to young grouped females than to isolated females of the same age.

Whole newly emerged females contain ca. eight times more 3,11-dimethyl-2-nonacosanone than is required on isolated antennae to elicit responses in 50% of tested males (Figure 1, Table 1). Although antennal fencing alone can stimulate males to perform the wing-raising response, other female body parts also are effective inducers of this response (Roth and Willis, 1952), and the male usually antennates various body parts of young females (Schal, personal observation). Therefore, the response to isolated antennae differs from that to whole
females: Responses increase from 80% to 100% using day 0 to day 5 females, respectively, with no decrease in response as seen with isolated antennae on day 3 (Figure 2A). Although the latency of male response to whole females suggests a bimodality of response, this is not a significant trend (Figure 2B).

Schal et al. (1990) suggested that the response elicited by teneral females is unrelated to the production of 3,11-dimethyl-2-nonacosanone and 29-hydroxy-3,11-dimethyl-2-nonacosanone, as this response also is elicited by teneral males and nymphs (Roth and Willis, 1952; Nishida and Fukami, 1983). The present study supports this hypothesis. Thus, the response to isolated antennae may be the net response to other stimulatory factors on teneral antennae, which dissipate over time, and to pheromone components, which increase over time (Figure 3A). The trough in the response pattern to isolated antennae disappears when whole females are tested, indicating that the amount of pheromone on the antenna of a 3-day-old female is close to the threshold of male response, but the pheromone on the rest of her body is sufficient to elicit a response. A similar situation, where males respond to teneral males and females, but where females become receptive several days later, was described in Nauphoeta cinerea (Schal and Bell, 1983).

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