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Circadian rhythmicity and development of the behavioural response to sex pheromone in male brown-banded cockroaches, *Supella longipalpa*

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Abstract. The effects of age, time of day and pheromone concentration on the responsiveness of the male brown-banded cockroach, *Supella longipalpa* (F.), to female volatile sex pheromone were examined. Male response increased with age and peaked 10 days after the imaginal moult. A diel periodicity of responsiveness was observed, with greater responses in the scotophase. The behavioural response was dose-dependent and its diel periodicity was most obvious when intermediate pheromone concentrations (e.g. 0.01 female equivalents) were used. Higher concentrations obscured the periodicity by eliciting greater responses in the photophase, while the lower concentrations did so by decreasing the response in the scotophase. Following entrainment to a LD 12:12 h cycle, the periodicity of response to 0.01 female equivalents of pheromone persisted for at least 54 h under continuous darkness, further demonstrating a true circadian rhythm.

Key words. Cockroach, *Supella longipalpa*, behaviour, sex pheromone, age, diel periodicity, circadian rhythm.

Introduction

Locomotion, feeding and many aspects of the physiology and behaviour of cockroaches are under endogenous control (Saunders, 1982; Sutherland, 1982; Page, 1990). However, little attention has been given to the circadian nature of sexual behaviour and particularly to the responses of cockroaches to pheromones. The response of male German cockroaches, *Blattella germanica*, to the female contact sex pheromone is lower in the photophase than in the scotophase (Bell *et al.*, 1978). For the American cockroach, *Periplaneta americana*, Hawkins & Rust (1977) showed that the response of males to female volatile sex pheromone exhibits a diel periodicity when a low pheromone concentration is used, but high concentrations disrupt the period icity. Although these results suggest an endogenous timing mechanism, a circadian rhythm of male sexual response, as shown in several moth species (Shorey & Gaston, 1965; Baker & Carde, 1979; Castrovillo & Carde, 1979), has not been demonstrated in any species of cockroach.

The age-dependence of response to pheromones has been reported in cockroaches, as well as in other insects. In *Byrsotria fumigata*, males begin responding to female sex pheromone 3 days after emergence, while females initiate pheromone production on day 4, as demonstrated with bioassays (Bell *et al.*, 1974).

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Similarly, *P.americana* males begin responding on day 7 (Wharton *et al.*, 1954) while females produce detectable amounts of pheromone on day 8 (Hawkins & Rust, 1977).

Virgin females of the brown-banded cockroach, *Supella longipalpa* (F.) (Dictyoptera: Blattellidae), engage in a calling behaviour (Hales & Breed, 1983) during which a volatile sex pheromone is emitted (Smith, 1988). Calling starts on day 4, peaks on day 8 and has been demonstrated to be circadian (Smith, 1988): the behaviour free-runs in continuous illumination as well as in continuous darkness, and it can be phase-shifted. Here, a parallel study was conducted on the male's response to the female sex pheromone. We investigated the development, the nature of the dose-dependent diel periodicity and the circadian rhythm of the responsiveness.

Materials and Methods

Insects. Supella longipalpa was reared under a LD 12:12 h cycle at 27°C with water and dog food provided *ad libitum*. Newly emerged adult males and females were collected daily and caged in groups in separate ventilated rooms under the same conditions.

Pheromone. A stock solution of sex pheromone was obtained by extracting 1000 8-day-old virgin females in groups of five with hexane. The solution was concentrated under nitrogen and then diluted to the appropriate treatment concentrations (see Fig. 3). The concentration of pheromone was measured in female-equivalents (FE); one FE equalled the extract from a single 8-day-old virgin female. All solutions were stored at −20°C.

Olfactometer. A two-choice Y-tube olfactometer was used. However, because we report responses of males only in the undivided stem of the Y-tube, it was effectively used as a straight tube. Pheromone and control dispensers were introduced at the upwind end of a 115 × 4.5 cm i.d. plexiglas tube. A cage (20 × 4.5 cm i.d) with a rotating metal screen gate was used to release males at its downwind end. Eight such olfactometers were connected symmetrically to a vacuum pump, which provided an air velocity of 20 cm/s through each tube during the assay. At other times, the velocity was set at 250 cm/s to minimize contamination of the tubes with pheromone. The tubes were rinsed weekly with hexane. Fluorescent lights, covered with red photographic filters and always left on in all phases of the experiments, were used to facilitate observation in the dark.

Behavioural assay procedure. A single male was placed in the cage and allowed to acclimate to the air flow for 30 min. The gate was opened and the pheromone and control dispensers were immediately introduced upwind. Males that ran out of the cage within 180 s were recorded as responsive. In control trials, dispensers were loaded with hexane only. Males were discarded after a single trial.

The dispensers were 50 mm^2 triangles of filter paper (Whatman No. 1) onto which was loaded 50 µl of a pheromone solution or hexane control. The loaded dispenser was attached to a cork (No. 0) with an insect pin and kept inside a glass vial before use. To minimize variation in pheromone release rate, each loaded dispenser was used successively to test only four males.

Experimental design. Development of responsiveness to female pheromone was examined in 1-, 2-, 3-, 4-, 5-, 7-, 10- and 30- day old males with two concentrations of pheromone (0.01 and 1 FE) in a randomized block design. Twenty-four males of each age were tested with each dosage of pheromone and sixteen males of each age were tested with the control. Because males respond maximally in the middle 6 h of the scotophase (see Results), all tests were conducted during this period.

To examine the diel periodicity of the response with various pheromone concentrations, the 24-h day was divided into eight 3-h periods. During the middle of each period, sixteen 25–30-day-old males were tested with each of six pheromone concentrations (10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1 and 10 FE) and eight males were tested with the control in a completely randomized design.

To demonstrate a circadian rhythm of responsiveness, the 24-h day was divided into twelve 2-h periods. Males were entrained to a LD 12:12 h cycle as nymphs and for at least 10 days from emergence. Their responses to 10^{-2} FE of pheromone were assayed in the middle of every time period, first in the entrainment LD conditions for 24 h and then in continuous darkness for 54 h. Every 2 h, sixteen males were tested with pheromone and eight with the control dispenser in a randomized

design. As before, each male was tested only once and discarded after use. The experiment therefore assayed responses of populations of males exposed to identical conditions, rather than the changes in responsiveness of individual males.

Results

Development of responsiveness to pheromone

The percentage of males responding to 0.01 and 1 FE increased with age (Fig. 1). Males did not respond to 0.01 FE until day 3 and their response to this concentration peaked on day 10, whereas 1 FE stimulated 30% of 1-day-old males and 100% of 5-day-old males to run out of the cage. No decline in response was observed in males up to 30 days of age and 100% of 150-day-old males responded to 0.01 FE (data not shown). Control tests were conducted to evaluate the effects of age on the spontaneous running activity independently of pheromone stimulation. No significant changes were observed (Fig. 1).

Dose-dependent diel periodicity of responsiveness to pheromone

Spontaneous running activity of males exhibited a diel periodicity in control tests in which no pheromone was used (Fig. 2). Therefore, the responses to pheromone were corrected with the following formula, modified from Baker & Cardé, 1984):

$$\% \text{ response} = \frac{\% \text{ response to pheromone} - \% \text{ response to control}}{100\% - \% \text{ response to control}} \times 100$$

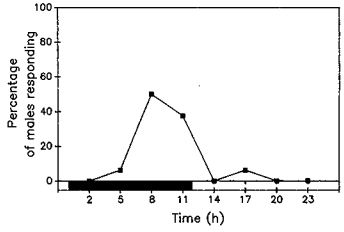

Fig. 2. Responses of males to control dispensers loaded with solvent. Dark bar represents the scotophase, $n = 8$ males.

The 'pheromone-specific responses' of males to six concentrations of pheromone in eight time periods of the LD 12:12 h cycle are presented in Fig. 3. For each time period, responsiveness increased with pheromone concentration. The concentrations needed to elicit a response in 50% of the males (RD_{50}) at various times of the LD cycle were determined by extrapolation from the dose–response curves at each time period. It changes smoothly over the 24-h cycle and is about 100 times higher in the photophase than in the scotophase (Fig. 4).

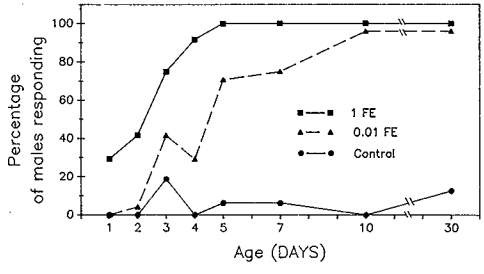

Fig. 1. Responsiveness of *Supella longipalpa* males to 0, 0.01 and 1 FE of pheromone as a function of age (n = 16, 24 and 24, respectively). Day 0 is the day of adult emergence.

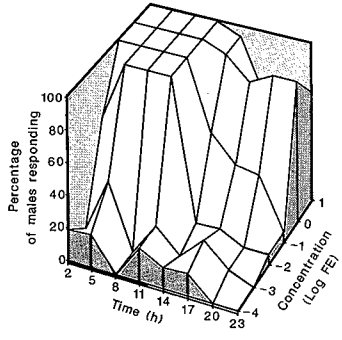

Fig. 3. Responses of *S.longipalpa* males to different concentrations of pheromone at various times of the day. Dark bar represents the scotophase. n = 16 males for each time–concentration combination.

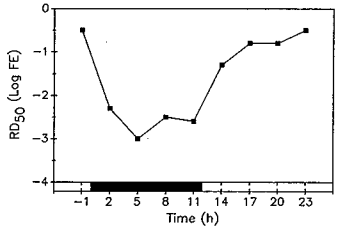

Fig. 4. Pheromone concentrations needed to elicit responses in 50% of tested males (RD_{50}) at various times of the day. The values were generated from Fig. 3 by extrapolation of the response curves for each 3 h time interval after correction by the response of control males.

For any given concentration of pheromone, the response varied with time in the LD cycle (Fig. 3). A diel periodicity was obvious in responsiveness to intermediate concentrations, but it was less evident when higher or lower pheromone concentrations were used. Higher concentrations elicited high percentages of response in both scotophase and photophase, while the response to lower concentrations was low at all times.

Circadian rhythm of responsiveness to pheromone

Males' responses in the first 24 h (normal LD 12:12 h cycle) were similar to those in the diel periodicity experiment (Fig. 3): the percentage

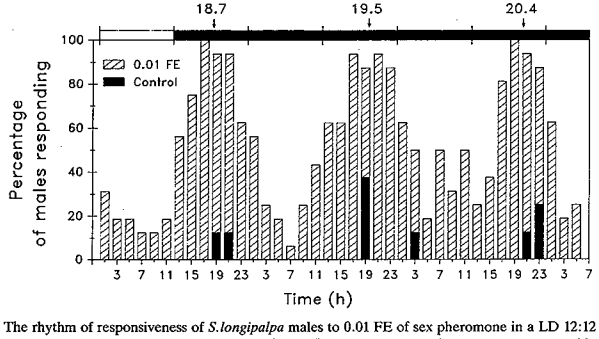

Fig. 5. The rhythm of responsiveness of *S.longipalpa* males to 0.01 FE of sex pheromone in a LD 12:12 h cycle for 24 h and then in continuous darkness for 54 h (n = 16). Numbers above the arrows represent midpoints of each response cycle. The control consisted of a dispenser loaded with solvent only (n = 8). The horizontal white and black bar above represents the light and dark conditions.

responding was high and reached 100% in the scotophase but remained below 20% in the photophase (Fig. 5). The periodicity persisted under continuous darkness for at least 54 h in the absence of external time cues (Fig. 5). More males were stimulated to run in the subjective 'light' period in continuous darkness (36 h after the last light–dark transition) than in the first subjective photophase. This might suggest an increase of response under continuous darkness or the de-synchronization of individual rhythms.

To estimate the free-running period of the rhythm, the midpoint of each of the three response peaks above 50% were calculated. The midpoints of the daily peaks of responsiveness were 6.7 h after lights-off for the first cycle, and 7.5 and 8.4 h after subjective 'lights-off' for the second and third cycles, respectively (Fig. 5). Thus, a consistent period of about 24.8–24.9 h was observed under continuous darkness. Evidently the rhythm of responsiveness is driven by an endogenously timed circadian clock.

The response of control males in the absence of pheromone stimulation was low at all times with activity in the middle of the scotophase or the subjective dark periods (Fig. 5).

Discussion

Sexual responses to pheromones are integrated behavioural patterns that result from interactions in the central nervous system between peripheral sensory inputs and internal states. Variations in response intensity could arise either peripherally with changing sensitivity of receptors or centrally due to changes in internal physiological states that set the behavioural response threshold. Since peripheral olfactory and taste receptors do not usually exhibit diel periodicities of sensitivity (Payne *et al.*, 1970; Hall, 1980), the periodicity of the expressed response of males to female pheromone (Fig. 3) is attributed to temporal variations of response threshold within the CNS. This threshold (e.g. the concentration to which 50% of males respond) changes smoothly across the diel and is higher in the photophase (Fig. 4). The increased response with age could also be explained by decreases in central response thresholds during sexual maturation (also see Silverman, 1977). However, in some insects, electroantennogram (EAG) responses change with age (Seabrook *et al.*, 1979) and haemolymph borne factors modulate the sensitivity of peripheral sensory neurones (Davis, 1984). Such phenomena have not been reported in cockroaches.

The responses over time reported here are based upon populations of males rather than on individuals; each male was used only in a single trial. This type of measurement is common to most studies on responses to pheromones (see Baker & Cardé, 1979), because it is not feasible to observe such patterns in individual insects due to adaptation and habituation effects (Bell *et al.*, 1974, 1978). Since individual males were entrained and presumably synchronized to the same conditions, we infer that the population response measured here represents the patterns of responsiveness of individual males.

Variation in the responsiveness of the population reflects changes in internal states over time and differences among individuals at specific times of the day. The abrupt transitions from low to high responses with increasing pheromone concentration at any given time of the day (Fig. 3) indicate that the differences among individuals are relatively small and therefore contribute to the sensitivity of this behavioural assay.

With pheromone concentrations either higher than 1 FE, or lower than 0.01 FE, the responses are similar in the photophase and in the scotophase, and the periodicity is not obvious (Fig. 3). Responsiveness to pheromone shows a clear diel periodicity only when 0.1 and 0.01 FE are used. Using two concentrations of female sex pheromone, Hawkins & Rust (1977) also found that in *P.americana* the high concentration resulted in a loss of diel periodicity of male response, but the periodicity was expressed at low pheromone concentration. Similar results were reported in a lepidopteran, *Pseudaletia unipuncta* (Turgeon *et al.*, 1983). Clearly, even lower concentrations would have concealed the periodicity in both cases.

The dependence of the diel periodicity of a behavioural response upon stimulus intensity can be readily explained in terms of the relationship between stimulus intensity and a central response threshold (Hall, 1980). When pheromone concentration is either very high or very low, it is always above or below a cycling response threshold in both scotophase and

photophase. The insects therefore respond (to high concentrations) or do not respond (to low concentrations) throughout the day, resulting in the loss of periodicity. A response periodicity appears only when the stimulus intensity is below the fluctuating threshold at certain times of the day and above it at other times. As noted by Baker & Cardé (1984), it is important to use the appropriate pheromone concentrations to obtain maximal separation of response in photophase and scotophase. The intermediate concentration in our study (0.01 FE) best reveals the diel periodicity of male response (Fig. 3) and it was therefore used in the experiment demonstrating the circadian nature of male responsiveness to female pheromone (Fig. 5).

Although we have not directly demonstrated that the patterns of response of individual cockroaches are circadian, persistence of the response periodicity in continuous darkness with a free-running period of about 24.8 h provides strong evidence for endogenous control (see Saunders, 1982). A circadian rhythm of behaviour is presumably advantageous. Closely related species of cockroaches might use similar or the same components of sex pheromone as do insects in other taxa (Tamaki, 1985). For instance, components of the sex pheromone of the American cockroach attract other closely related species of cockroaches (Seelinger, 1985). In such cases, periodicity of mating behaviour may be important in reproductive isolation of sympatric species (Cardé & Baker, 1984). The response of *S.longipalpa* males peaks at 6.7 h after lights-off in a LD 12:12 h cycle (Fig. 5). This coincides well with female calling which peaks about 7.5 h after lights-off (Smith, 1988). The combined effect of these behaviours in both sexes would result in a distinct rhythmicity of mating activity. More studies are needed to elucidate the importance of chemical communication in species isolation in cockroaches.

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