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Circadian Rhythms in the Mating Behavior of the Cockroach, *Leucophaea maderae*

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Abstract Mating behavior of small populations of virgin males and females of the cockroach *Leucophaea maderae* were continuously monitored via time-lapse video recording in controlled laboratory conditions. The time of onset of copulation was found to be rhythmic in a light cycle of 12 h light alternated with 12 h of darkness, with the peak of mating behavior occurring near the light to dark transition. This rhythm persisted in constant dim red illumination and constant temperature. In constant conditions, the period of the rhythm was slightly less than 24 h, with a peak of copulation during the late subjective day. These data demonstrated that mating behavior is gated by a circadian clock. When males and females were taken from light cycles that were 12 h out of phase, a bimodal rhythm was observed with one peak in the males' late subjective day and a second peak of equal amplitude in the late subjective day of females. The results indicated that circadian systems in both males and females contribute to the circadian rhythm in copulation. Bilateral section of the optic tracts (OTX) of both males and females abolished the rhythm, but the rhythm persisted when OTX females were paired with intact males or when OTX males were paired with intact females. Furthermore, when OTX males or OTX females were paired with intact animals that were 12 h out of phase, a bimodal rhythm was still observed. These results suggested that the circadian pacemaker in the optic lobes of both male and female cockroaches participates in the control of mating, but that a pacemaker outside the optic lobes is also likely involved. Finally, it was shown that the female's olfactory response (measured by electroantennogram) to components of the male sex pheromone exhibited a circadian rhythm, but the data suggested the peripheral olfactory rhythm is not likely to be involved in the rhythm of mating behavior.

Key words *Leucophaea maderae*, circadian rhythm, cockroach, copulation, electroantennogram, mating behavior, olfactory, sex pheromone

Initiation of a mating sequence is a relatively complex process that depends on precision in both the temporal and spatial coordination of the behavior of 2 individuals. This coordination is critical for obvious reasons—to copulate, the individuals must physically be in the same location at the same time. Temporal coordination is also important, both in the short term for regulation of the stereotypic behavioral sequence that leads to selection of a mate and successful copulation and in the longer term for determination of when

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in the course of a day mating will take place. Numerous species, vertebrate and invertebrate, exhibit daily rhythms in mating, and in a few instances, this daily rhythm has been shown to be under the control of a circadian clock (e.g., Walker, 1979; Tychsen and Fletcher, 1971; Eskes, 1984; Sakai and Ishida, 2001; Silvegren et al., 2005). Restriction of mating behavior to a specific time of day presumably confers a selective advantage as a result of evolutionary pressures to reduce predation and to limit the amount of time (energy) expended in searching for a mate. Temporal regulation has also been suggested as a mechanism for reproductive isolation (Miyatake et al., 2002; Sakai and Ishida, 2001).

In insects, spatial coordination appears in many cases to be mediated by a system of chemical signals and behavioral responses to those signals. Males or females of the species release a volatile sex pheromone that attracts the opposite sex to their location. Daily temporal coordination has often been attributed, at least in part, to circadian regulation of the timing of release of these chemical signals and/or the circadian regulation of the responsiveness of the receiver to these signals. For example, in the brown-banded cockroach, *Supella longipalpa*, results have shown that pheromone release by the female (calling) and the behavioral responsiveness of the male to female sex pheromone both exhibit a circadian rhythm (Liang and Schal, 1990; Smith and Schal, 1991). Furthermore, a circadian rhythm of male responsiveness to female sex pheromone has been shown in the cockroach *Periplaneta americana* (Zhukovskaya, 1995). Similar rhythms in either female calling behavior or male responsiveness to pheromone have also been reported in a variety of moth species (summarized in Steel and Vafopoulou, 2002).

Although these findings implicate circadian regulation of pheromone communication in the temporal organization of mating, little is known about the precise role of these rhythms in the control of mating or about the physiological underpinnings of the rhythms themselves. For example, it is plausible that rhythms in response to sex pheromone could arise from circadian regulation of the sensitivity of olfactory receptors. This suggestion is bolstered by the fact that circadian rhythms in olfactory responses in the antennae to food-related odors (measured by electroantennogram [EAG]) have shown that the circadian clock can regulate sensitivity of olfactory receptors in both fruit flies and cockroaches (Krishnan et al., 1999; Page and Koelling, 2003). However, in other insects (moths), it appears that receptor sensitivity is not rhythmic (e.g., Rosén et al., 2003), suggesting the behavioral response rhythms either reflect rhythmicity in the central processing of the pheromone signal or may simply be due to rhythms in overall levels of locomotor activity.

The cockroach *Leucophaea maderae* may be a useful system for investigations into the circadian regulation of reproductive behavior. The anatomy and physiology of the circadian system has been well-studied (reviewed in Page, 1990; Homberg et al., 2003), and the mating behavior has been described in some detail (Roth and Barth, 1967; Sreng, 1993). In *Leucophaea*, the male is responsible for production and release of volatile sex pheromones. Initiation of reproductive behavior is characterized by male calling via release of sex pheromone from his sternal glands. The receptive female subsequently approaches the male, and following initial tactile stimulation through antennal contact, there is a period of antennal fencing. The male raises his wings providing access to glandular secretions from the tergal glands. The female mounts the male and feeds on the tergal gland secretion. The female's position on the male's back gives the male the opportunity for insertion of the phallomere, and when insertion is successful, copulation begins. Thus, success in mating is seen to involve both initiation of the courtship sequence by the male and an appropriate receptive response by the female.

In the present article, we show that mating behavior (specifically copulation) in *Leucophaea* is gated by a circadian clock and that circadian clocks of both sexes contribute to the timing of the rhythm of copulation. Furthermore, we present evidence that the well-established circadian pacemaker of the optic lobes is involved, although it appears that circadian oscillators outside the optic lobes may also substantively contribute. Finally, we show that the olfactory response in the female antennae to stimulation by components of the sex pheromone exhibits a circadian rhythm, but that this rhythm appears to have little or no impact on mating.

MATERIALS AND METHODS

Animals

Experimental animals were young adult virgin males and females, collected from laboratory colonies of *L. maderae* maintained on a 24-h light cycle, LD 12:12, controlled by electronic timers. Colonies were housed either in environmental chambers or incubators that

maintained temperature at 25 °C. Food and water were available ad libitum. To obtain virgin adults, cockroaches were isolated within 12 h of the imaginal molt. Males and females were housed in separate containers until the beginning of the experiment, which typically occurred within 7 to 14 days after isolation (females reach sexual maturity in about 10 days). In all experiments, females were marked with small spots or stripes of white paint on the pronotum. Additionally, males were marked in 4 of the experiments. In all but 1 case, the markings allowed the identification of individual animals. In the one experiment, marking simply identified the phase of the LD cycle as described below.

At the beginning of each experiment, 10 to 15 virgin male and 10 to 15 virgin female cockroaches were placed in a plastic container (31.8 cm L × 24.1 cm W × 15.2 cm H) housed inside a light-tight box within an environmental chamber and maintained at 25 °C. In nature, the species is gregarious and forms large colonies, so investigations of mating behavior in populations, rather than pairing individuals, seemed appropriate (Cornwell, 1968). Vaseline Petroleum Jelly was used to line the wall's container to prevent cockroaches escaping. Food and water were available ad libitum. Animals were continuously monitored over the course of the experiment by videotaping in time lapse (1 frame being recorded every 0.2 or every 0.4 s) utilizing a Panasonic Time-Lapse Video Recorder coupled to an infrared-sensitive CCD (Sony XC-77, New York) camera. Each frame of the tape contained a time and date stamp. Constant, very dim red/infrared illumination for the videotape was provided by a darkroom safelight (15 W tungsten filament lamp) equipped with a filter that limited wavelengths to greater than 600 nm (Kodak 1A or GBX-2, Rochester, New York). Light intensity was adjusted with a rheostat giving a final light intensity at the floor of the plastic tub of less than 0.05 $\mu E\text{-}m^{-2}\text{-}sec^{-1}$ (measured with a LiCor Photometer, Lincoln, NE). One set of experiments was carried out in a light cycle that consisted of 12 h of white light (15 W fluorescent lamp, 2.0 $\mu E\text{-}m^{-2}\text{-}sec^{-1}$) alternated with the 12 h of dim red light. Experiments typically lasted 10 to 12 days and were ended when all or most of the virgin females had mated. Most females only mated once during an experiment, though in all experiments multiple matings by females did occur. Tapes were reviewed, and the times of onset and offset of copulation were recorded.

Each experimental treatment was repeated at least once, and usually there were 3 to 4 replications. Since the data were consistent between replications, they were combined for reporting results.

Mating-Time Data Analysis

Histograms of copulatory events as a function of time were plotted using standard scientific graphics software (Sigma Plot 2001, Aspire Software, International, Ashburn, VA). Data were also plotted and analyzed with the aid of a circular statistics software package (Oriana version 2.02a, Kovach Computing Services, Anglesey, Wales). For unimodal data, Rayleigh's Uniformity Test was used to determine if the data were clustered around a single mean value. This analysis also calculates a mean vector. The direction of the vector indicates the average phase with respect to real time, and the length of the vector is a measure of phase coherence. Average phase is reported as real time (CST) or zeitgeber time (ZT) where ZT 0 is the time of the dark-to-light transition of the prior light cycle. Rayleigh's Test is not appropriate for cases in which the data are nonrandom but are bimodally distributed. Therefore, in those experiments in which males and females came from different light cycles, Rao's Spacing Test was used. Data are reported as means ± standard deviations.

Surgery

Sectioning of the optic tracts was carried out under CO_2 anesthesia. A small section of cuticle was removed from the head capsule to gain access to the optic tract. After removing the ocellus, the optic tract was visible and cut with fine microscissors. Damage to the antenna and antennal nerves was avoided. After cutting the optic tract, the square of cuticle was placed in its original position and sealed in place using low-melting-point wax. Previous experiments have shown this surgery can be done without impact on the animal's ability to detect and behaviorally respond to odors (Page and Koelling, 2003). The success of the surgery could be visually confirmed at the time of the operation. However, the optic tracts consistently regenerate in 3 to 5 weeks (Page, 1983), and given the duration of the present experiments (typically just over 14 days), there was some concern that regeneration might have occurred in a few of the animals. Therefore, we recorded the locomotor activity of all optic-tract-sectioned animals for 10 to 14 days at the end of each experiment to confirm arrhythmicity. Out of 188 animals that underwent optic-tract section, we identified 6 in which visual inspection of the data and chi-square periodogram analysis showed evidence of rhythmicity in the first 7 days of activity recording. These animals were excluded from the data analysis.

EAG Recording

EAGs were recorded from restrained virgin females using the method described in Page and Koelling (2003). Animals were anesthetized briefly with CO_2 and taped to the underside of a petri dish lid with the head protruding through a hole in the lid. A piece of tape behind the head prevented the animal from pulling it back through the hole. The antenna was threaded through 2 small hooks made of silver wires. One wire, near the distal end of the antenna, served as the active electrode, whereas the second wire, located a few mm from the scape, served as the indifferent electrode. Contact between the antenna and the wire was made with ECG electrode cream (Grass Instruments, Berkshire, UK). Animals were maintained in light-tight boxes at a constant temperature. The signal from the antenna was led to an amplifier (Gain: X100, Bandpass: 0.1 Hz to 200 Hz) then to a computerized data acquisition system (Superscope II, GW Instruments, Inc., Somerville, MA).

To deliver the stimulus, a half cylinder fashioned from Tygon tubing (0.9 cm diameter) was taped over the antenna. The stimulus delivery system consisted of a constant stream of humidified air flowing in one end of the half cylinder (at the distal end of the antenna) with a vacuum tube at the other to remove odorants. The air stream (~250-300 ml/min) was bubbled through deionized water, run through a carbon filter, a flow gauge, and then into a solenoid-controlled valve that switched between 2 output ports. Stimulus chambers were vials whose tops were fitted with two 16-gauge hypodermic needles—one for flow into the chamber and the other for flow out. One port from the solenoid valve served to provide a stream of clean air through a blank (empty) stimulus chamber throughout the interstimulus interval. On command from a programmable timer, the solenoid was activated, directing the stream of air through the active stimulus chamber containing the odorant. Outputs from the blank and active stimulus chambers were combined to a single line that blew over the antenna. Stimulus pulses of 0.5-s duration were given once per hour. Odorants ethyl acetate, 3-hydroxy-2-butanone (acetoin), and 3-methyl-2-butenoic acid (senecioic acid) were diluted in mineral oil.

Locomotor Activity

Circadian rhythms of locomotor activity were monitored as described in previous publications (e.g., Page, 1983). Animals were housed in running-wheel cages in individual light-tight boxes. Rotation of the wheel activated a magnetic reed switch. Switch closures were counted by a computerized data acquisition system (Actimetrics, Wilmette, IL) and analyzed using Clocklab software. The presence or absence of rhythmicity was determined using the chi-square periodogram. Phase was evaluated by a linear regression of activity onsets. Activity monitors were housed in an environmental chamber maintained at a constant temperature of 25 ± 0.5 °C.

RESULTS

Circadian Rhythm in Olfactory Response to Pheromone Odors

We were interested in determining whether or not female cockroaches exhibited a circadian rhythm in olfactory response to components of the sex pheromone that is released by the male and whether or not the rhythm was similar in phase to the rhythm in response to stimulation with food-related odors (Page and Koelling, 2003). We tested 2 odors, 3-hydroxy-2-butanone (acetoin) and 3-methyl-2-butenoic acid (senecioic acid), which are 2 of the 3 behaviorally active components of the male sex pheromone of *L. maderae* (Riviere et al., 2003). Figure 1 shows the circadian rhythms in EAG amplitude in response to these 2 odorants at a dilution of 10^{-1}. We made recordings in 8 animals that lasted 4 or more days with acetoin. Six of these animals showed clear rhythms in EAG amplitude. Four recordings were made measuring the response to senecioic acid, and 3 of these were clearly rhythmic. For both compounds, the peak olfactory response occurred early in the subjective day and the minimum response was near dusk (Fig. 1). The phase of the rhythm relative to the prior LD cycle was essentially identical to that obtained using ethyl acetate as the stimulus odorant (Page and Koelling, 2003).

Rhythm in Mating Behavior in LD

Previous observations have suggested that mating behavior in *Leucophaea* is initiated by a female olfactory response to male sex pheromone (Sreng, 1993). In support of this suggestion, we found that when a female's antennae are removed, the female will not mate. We paired 10 virgin males with 10 virgin females whose antennae had been severed near the base. Over the course of the next 12 days, no copulatory events were observed. The results indicate that

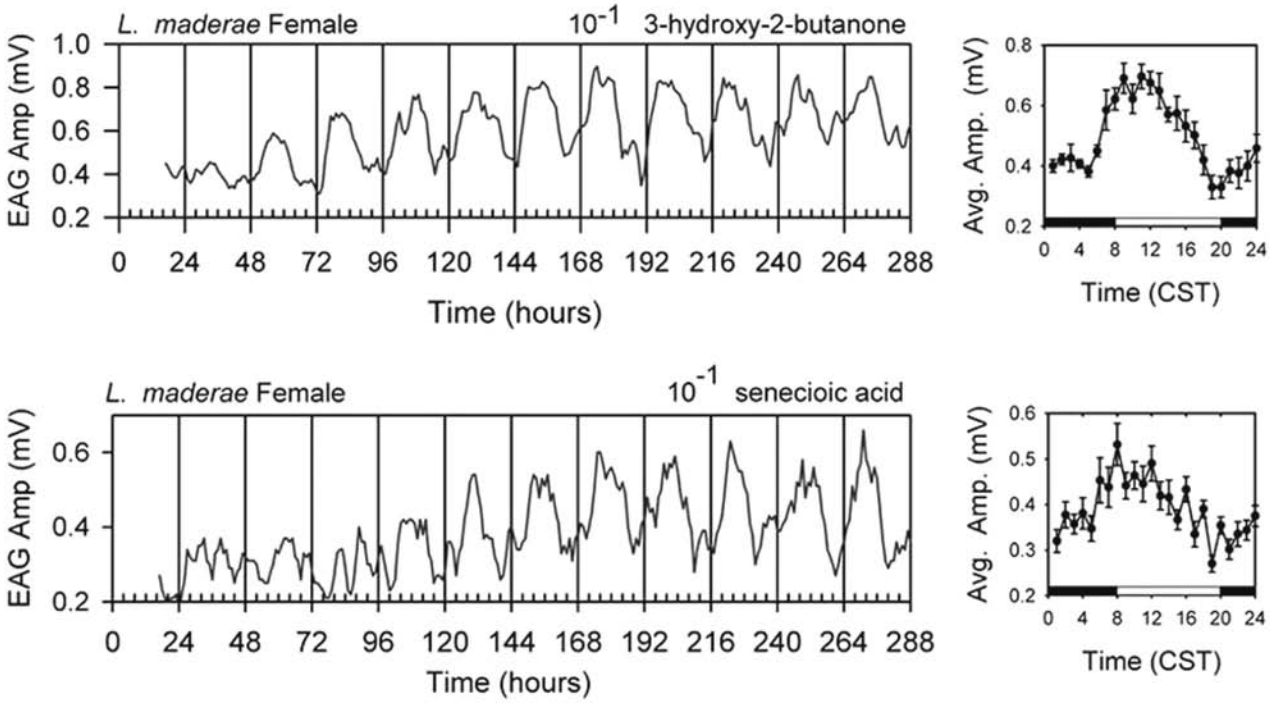

Figure 1. Electroantennogram (EAG) amplitude rhythms. Free-running rhythm of EAG amplitude in response to pulses of 3-hydroxy-2-butanone (upper panels) and senecioic acid (lower panels) given once per hour for 12 consecutive days. Odorants were diluted in mineral oil to 10^{-1}. Recording was done in constant darkness and constant temperature (25 °C). The panels on the left plot the amplitude (3-point moving average) of the EAG recorded each hour in darkness, whereas the panels on the right show the average waveform determined by average EAG responses as a function of time of day (mean ± SEM). In right-hand panels, the light cycle to which the animals had been entrained prior to recording is illustrated by the light and dark bars at the bottom of the graph (LD 12:12, lights-on at 0800 h). For both odorants, peak responses occurred near subjective dawn and troughs were near subjective dusk.

input from the sensory receptors on the antennae are critical to mating.

Since the female's antennal response appeared to be crucial to the initiation of mating behavior, and since the female's peripheral olfactory sensitivity to pheromone is rhythmic (Fig. 1), we wanted to determine if mating behavior was also rhythmic and, furthermore, whether the phase of the peak of the rhythm coincided with the time of peak olfactory sensitivity to components of the sex pheromone. Virgin males and females were placed in the recording chamber and maintained in a light cycle that consisted of 12 h of white light alternated with 12 h of dim red light with the transition from light to "dark" occurring at 2000 CST, and the time of onset and offset of copulation was determined from time-lapse videotapes. As shown in Figure 2, mating activity was strikingly periodic, with the peak of mating activity taking place during the 2 h prior to and 1 h after dusk. The mean phase of copulation onset was 19.2 ± 3.95 CST (ZT 11.2) with 27 of the 31 copulatory events (between a total of 24 males and 24 females) occurring between 1200 and 2400 h (ZT 4 to ZT 16). The duration of copulation ranged from 40 min to 6.5 h, with an average duration of 2.7 ± 2.00 h (mean ± standard deviation). This is consistently shorter than the duration of copulation that we measured in experiments conducted in constant conditions (see below). The duration of copulation was not correlated with the time of its onset ($p = 0.44$).

We were somewhat surprised that the peak of mating behavior occurred prior to lights-off and that copulation most frequently occurred in the photophase, since locomotor activity in *Leucophaea* is strongly nocturnal and the onset of peak activity measured in isolated, individual animals in running wheels in LD 12:12 coincides with the onset of darkness (e.g., Roberts, 1962). These results suggest that mating behavior in LD phase leads locomotor activity. In contrast, results obtained in experiments conducted in constant conditions that are reported below show no significant difference in activity onset

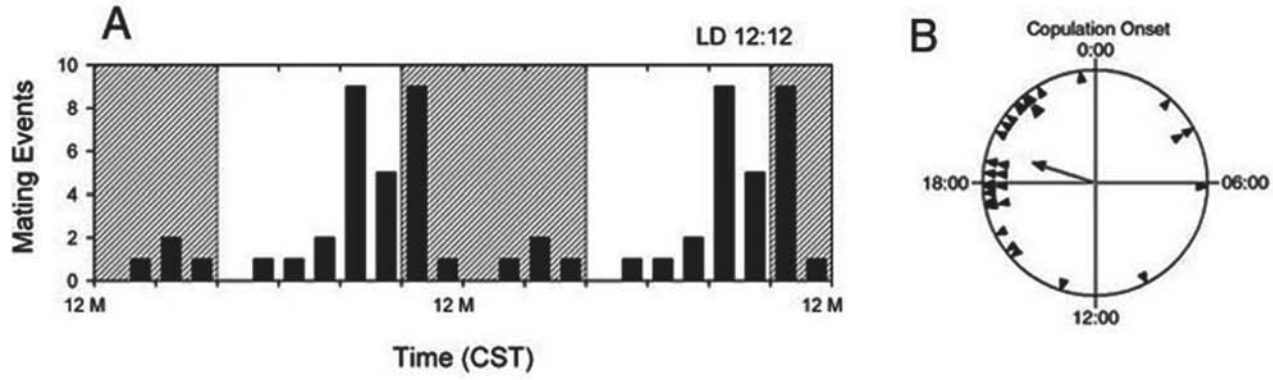

Figure 2. Diurnal rhythm of copulation in a light cycle. (A) Plots the number of copulation onsets as a function of time of day. In this and subsequent histograms, each bin is 2 h in width and the data are "double plotted." The hatched bars show the period of the scotophase (2000-0800 h). (B) Shows the data plotted on a circular time scale. Each triangle represents the time of onset of a copulation event. The arrow shows the mean vector (phase) of the mating events. A peak of copulation onset occurs near the transition of light to dark.

and peak mating time. Thus, the results in LD may be due to a differential masking effect of light.

Circadian Rhythm in Mating Behavior

To demonstrate that the rhythm in copulation was an endogenously driven circadian rhythm, virgin males and females that had been entrained to an LD 12:12 light cycle (lights-off at 2000 CST) were housed together in constant, dim red light (at constant temperature). Figure 3A shows the distribution of mating time over 10 to 12 days of recording from 3 separate experiments, each involving 10 virgin males and 10 virgin females. During the experiment, we observed 27 copulatory events. The onset of copulation was clearly dependent on time of day, with the peak in mating occurring in late subjective day. Copulation was restricted to about 12 h of the day, with 25 of 27 (93%) copulatory onsets occurring between 1200 CST (ZT 4) to 2400 CST (ZT 16). Figure 3B shows the same data with time of copulation events plotted for each day of recording in constant conditions and reveals a clear circadian rhythm in time of copulation. Regression analysis indicated the free-running period of the rhythm was slightly less than 24 h (τ = 23.6 h) similar to the free-running period of the locomotor activity rhythm (Barrett and Page, 1989). Figure 3C shows a circular plot of the data. The data cluster is a statistically significant group ($p < 0.0001$, Rayleigh test) with a mean phase of 17.4 ± 3.14 CST (ZT 9.4). The mean duration of copulation was 4.9 ± 3.34 h and ranged from a minimum of 1.1 h to a maximum of 13.4 h.

To estimate the phase relationship between the time of copulation and the rhythm of locomotor activity, we placed 16 virgin males and 18 virgin females that had been entrained to an LD 12:12 light cycle (lights-off at 2000 h) in running wheels in constant darkness and measured the phase of the free-running activity rhythm for 10 to 14 days. The average phase estimated for day 6 in constant conditions was 17.3 ± 1.71 CST for males and 18.0 ± 1.99 CST for females. These phase estimates were not significantly different from each other or from the time of copulation (ANOVA, p = 0.718), indicating that mating and locomotor activity occur at about the same time of day. However, because of the differences in conditions between mating and locomotor activity measurements (e.g., animals housed in groups vs. isolated individuals), any apparent phase differences or similarities should be viewed cautiously and definitive experiments will require careful behavioral measurements of both general activity and mating under similar conditions.

These data show unequivocally that mating behavior in *Leucophaea* is gated by a circadian clock that free-runs in constant conditions. Furthermore, they indicate that the peak of mating behavior occurs at a time when the responsiveness of the female's olfactory receptors to sex pheromone components is at a minimum.

Both Male and Female Clocks Contribute to the Determination of Mating Time

The discovery of a robust circadian rhythm in the onset of copulation suggested that there was circadian

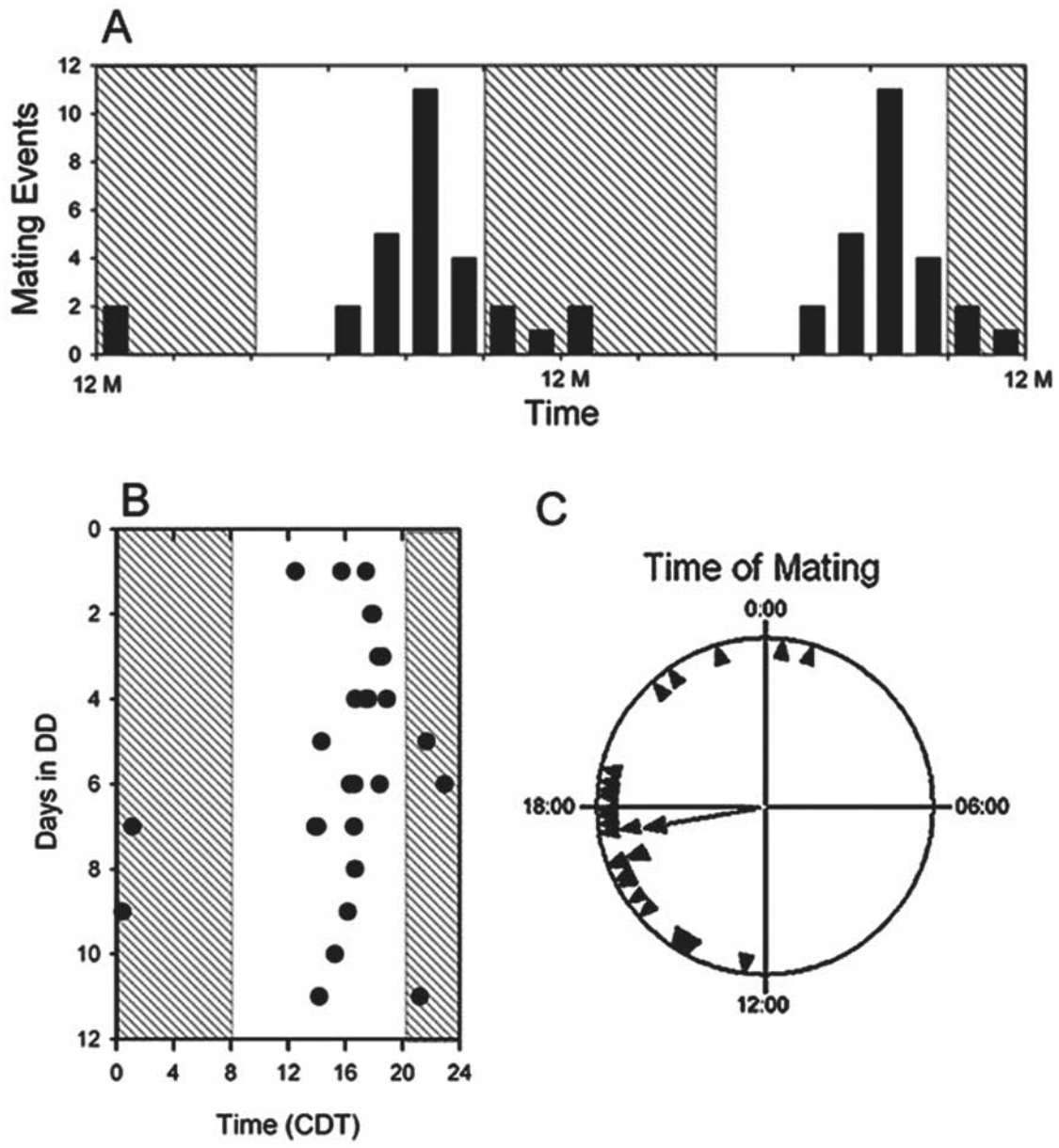

Figure 3. Free-running circadian rhythm of copulation in constant conditions. (A) Double-plotted histogram showing the number of copulation onsets as a function of the time of day. The white and hatched bars overlaying the plot show the projected phases of the LD cycle to which the animals had been entrained prior to the beginning of the experiment. (B) Shows the daily time of copulation events for each day of the 12 days of monitoring demonstrating the persistence of the free-running rhythm of this gated behavior. The hatching indicates the period of darkness of the prior LD cycle. (C) Data plotted on a circular time scale. The distribution of mating showed highly significant clustering around 17.4 CST (arrow). The arrow represents the mean vector (phase).

control of either the male's initiation of mating behavior or the female's receptivity, and we set out to distinguish between these possibilities. Our initial test involved evaluating the time of copulation onset in males and females that had been entrained to light cycles that were 12 h out of phase with one another. Males (n = 20) entrained to LD 12:12 with lights-on at 0800 CST were housed (in constant conditions) with females (n = 20) that had been entrained to LD 12:12 with lights-on at 2000 CST. If the male's clock determined mating time, the peak of mating should occur at about 1700 h, whereas if the female's clock determined mating time, the peak of copulatory activity would be at 0500 h. We found that the distribution of mating appeared to be bimodal ($p < 0.05$, Rao's Spacing Test) with one grouping of mating events

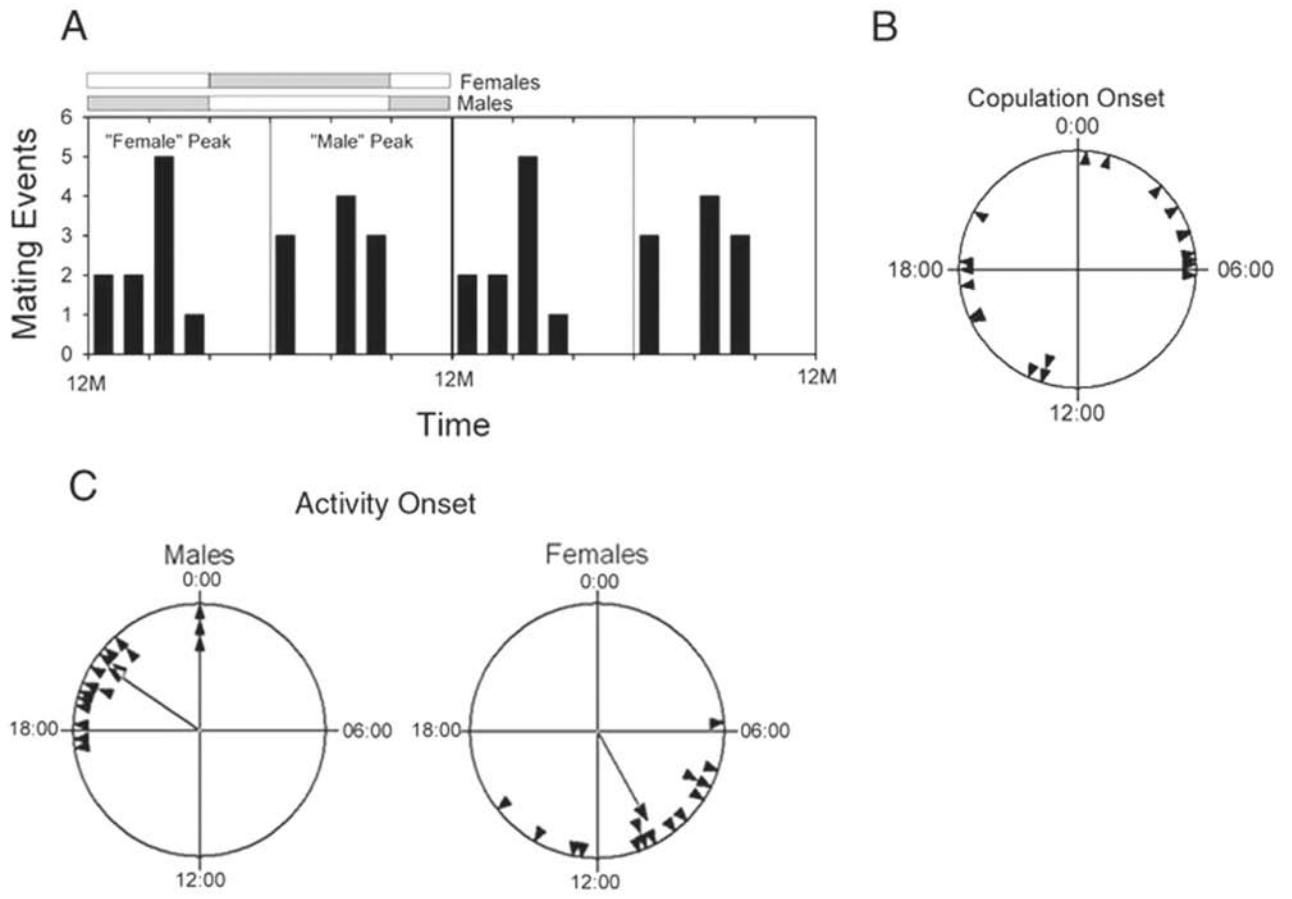

Figure 4. Mating behavior in constant conditions of virgin males and females that had been entrained to light cycles that were 12 h out of phase with one another. (A) Histogram plot of mating events (double plotted). The light cycles to which the females and males had been entrained prior to the beginning of the experiment are shown by the bars at the top of the graph. (B) Circular plot of the data showing the 2 clusters of mating events over the 24-h day. (C) Circular plots of the phase of locomotor activity onsets projected for the last day of monitoring of reproductive activity. Phase estimates were based on records of the locomotor activity rhythm recorded at the end of the experiment. The mean phases of the activity rhythms (arrows) of the males and females were clearly out of phase, and the phases showed no overlap.

("male peak") having a mean phase of 16.3 ± 2.21 CST (n = 10) and the other group ("female peak") having a mean phase of 4.2 ± 1.97 CST (n = 10) (Fig. 4 A,B). These data indicate that the phases of both the male's and the female's circadian clocks contribute to the determination of mating time, and the fact that the numbers of copulations at each phase were equal would suggest that the clocks of the 2 sexes are about equal in influence. Furthermore, it is apparent that neither the male nor female circadian system can prevent mating at an "inappropriate" phase.

We were concerned that "social entrainment" over the course of the experiment might have impacted these results by bringing clocks of mating pairs into the same, common phase relationship. To confirm that the circadian clocks of the males and females were indeed out of phase and that the phase difference was maintained throughout the experiment, we monitored locomotor activity rhythms of most of the individuals in constant darkness following the completion of the mating behavior experiment. Activity onset of locomotor activity rhythms of males (n = 17) and females (n = 15) were found to be, on average, 10.3 h out of phase, and while there was scatter in the phases of both sexes, the phases of the activity rhythms did not overlap (Fig. 4C). The results show that the circadian clocks of males and females did maintain phase separation throughout the period of mating.

Eighteen of the 20 females in the experiment mated, suggesting that the phase difference had essentially no effect on the propensity to mate. There was a difference in the duration of copulation between animals

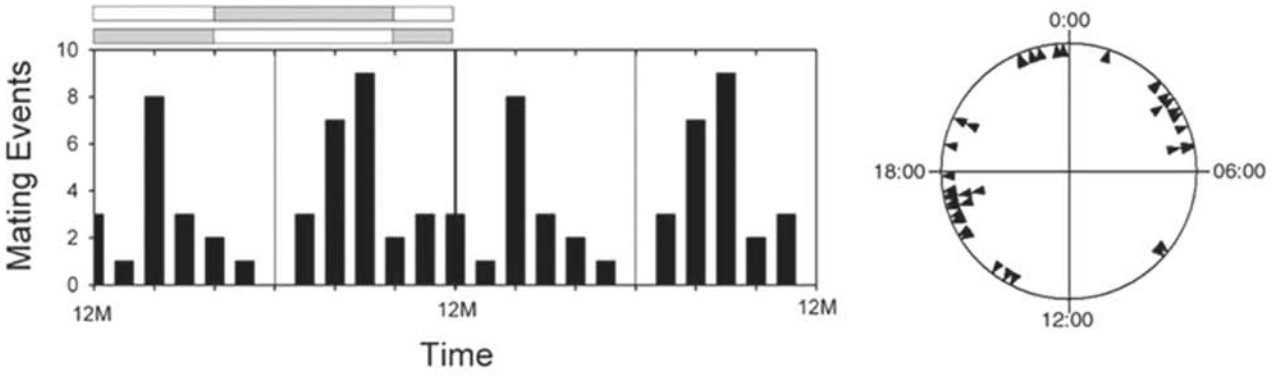

Figure 5. Shows the bimodal distribution of mating in a population composed of virgin males and virgin females where members of each sex had been entrained to 1 of 2 different light cycles that were 12 h out of phase, then placed in constant conditions. In one of the cycles, the light-to-dark transition occurred at 0800 h, and in the other it was at 2000 h, as illustrated by the bars at the top of the histogram plot. A circular plot of the individual mating time is shown at the right. As expected, 2 peaks of copulation occur just prior to the time of lights-off projected from the 2 LD cycles.

Table 1. Summary of Mate Choice in the Mating Preference Test

	Males (0800-2000 h)	*Males (2000-0800 h)*
Females (0800-2000 h)	11	7
Females (2000-0800 h)	8	15

Times in parentheses show the photophase of the prior LD cycle. Numbers in the cells are copulation events between pairs. Chi-square: $p = 0.173$.

mating "out of phase" and those from the previous experiment that were mating "in phase." The duration was about 1.5 h shorter, with copulations between pairs that were out of phase lasting on average 3.3 ± 2.16 h ($p = 0.03$, t test). However, as shown in the next experiment to be discussed, the tendency of copulation to be shorter for animals that are out of phase was not a consistent finding.

Is There a Preference to Mate "In Phase"?

To determine whether or not there was a preference to mate with a partner that was in phase, we placed animals together in which equal numbers of virgin males and virgin females came from the 2 light cycles. As expected, there were 2 peaks in the mating behavior (Fig. 5). Of 41 copulatory events total, 26 occurred between males and females from the same light cycle and 15 were between animals from different light cycles (Table 1). Thus, there appeared then to be a tendency for individuals to preferentially mate with partners from the same LD cycle; however, the difference was not statistically significant ($p = 0.173$, chi-square test). We also found in this experiment that the average duration of copulation was longer in mating pairs that were out of phase than in pairs that were in phase (4.75 ± 3.46 vs. 3.93 ± 3.24 h), though this difference was also not significant ($p = 0.46$, t test).

Effects of Section of the Optic Tracts

Since severing the optic tracts (OTX), disconnecting the optic-lobe pacemaker from the midbrain, disrupts the circadian rhythms of both locomotor activity (Nishiitsutsuji-Uwo and Pittendrigh, 1968; Page, 1983) and olfactory response in the antennae (Page and Koelling, 2003), we evaluated the effects of cutting the optic tracts bilaterally on the rhythm in copulatory behavior. Following surgery, animals were given 2 to 4 days to recover and were then placed in the video recording chamber. Three experiments were run, each involving 8 to 12 males and 9 to 12 females that had survived surgery. Nineteen of 30 males in the experiments mated, and 26 of 31 females mated. The average duration of copulation was 3.9 ± 2.37 h. The temporal distribution of mating is shown in Figure 6. Following optic-tract section, there was no significant dependence of copulation on time of day ($p > 0.5$, Rayleigh Test; $p > 0.5$ Rao's Spacing Test). The duration of copulation was unaffected (3.9 ± 2.37 h). The results suggest that the circadian pacemaker in the optic lobes is involved in timing the mating activity and drives the rhythm via neural connections made by the optic tracts.

In view of the suggestion that both the male's and female's clocks are involved in timing mating, we next did similar experiments in which only the male's

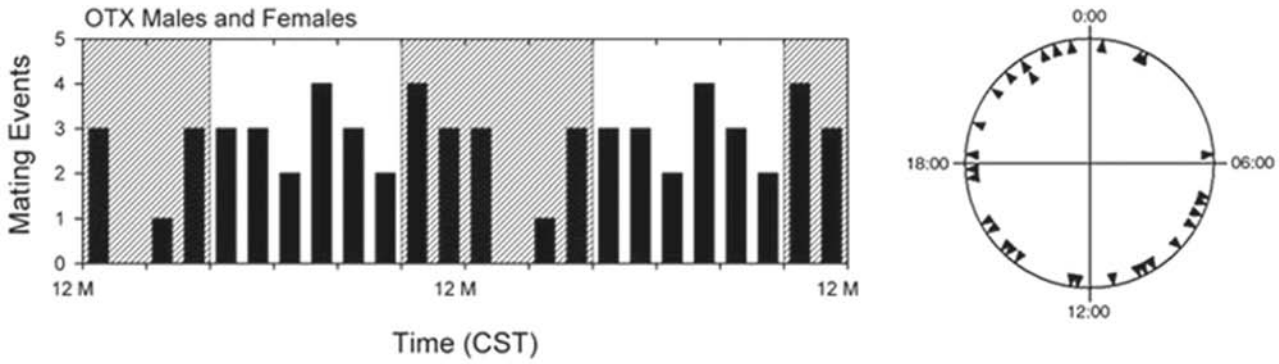

Figure 6. Effects of bilateral section of the optic tracts of both males and females on mating activity recorded in constant conditions. The left panel shows the double-plotted distribution of the times of copulation onset. The hatched bars show the projected scotophase of the light cycle to which both the males and females had been entrained prior to the beginning of the experiment. The right panel shows the circular plot of the data. There was no significant clustering of the mating events, indicating that optic tract section abolishes rhythmicity (Rayleigh Test, $p = 0.536$; Rao's Spacing Test, $p > 0.50$). OTX = section of the optic tract.

optic tracts or only the female's optic tracts were cut. When intact males (n = 21) were paired with OTX females (n = 21), the time of copulation showed a clear, unimodal rhythm that was virtually identical to that observed in intact animals (p = 0.004, Rayleigh Test), with 17 of 19 copulations occurring between 1200 and 2400 CST (Fig. 7, upper panels). The mean phase was 16.8 ± 4.29 CST, with an average duration of 4.4 ± 3.34 h. These values are quite similar to those seen with intact animals. The results indicate that while an intact female optic-lobe pacemaker may play a role, it is not required for timing either the circadian rhythm in mating behavior or the duration of copulation.

Sectioning the optic tracts of males was more disruptive (Fig. 7, lower panels). While there was still a significant clustering of copulatory events (p = .011, Rayleigh Test) with a peak in mating activity in the late afternoon, the mean phase was about 1 h later than normal and the scatter in the data was pronounced (mean phase = 18.4 ± 6.04 CST). Nevertheless, 73% of the mating events occurred in a restricted 12 h period between 1400 and 0200 CST. Interestingly, in this experiment it was more common for both males and females to mate multiple times. Although there were only 34 females (and 32 males) in the experiments, there were 55 copulatory events, with 14 of the females mating 2 or more times. Since female cockroaches are generally less receptive following successful copulation (e.g., Roth, 1964; Montrose et al., 2004), the continued receptivity of the females could suggest that males without optic lobes are impaired in the ability to successfully inseminate females. In this context, it is interesting to note that clock-less male fruit flies have been found to show a substantial decrease in reproductive fitness (Beaver et al., 2002). The possible lack of mating success could not be attributed to a change in the average duration of copulation, which remained near 4 h (3.8 ± 2.29 h).

These results indicate that intact optic tracts in either the males or the females are adequate to drive a circadian rhythm in copulatory behavior. The data are consistent with the hypothesis based on the bimodal rhythms exhibited by intact, but out of phase, males and females that the circadian systems of both sexes participate in the determination of mating time. The results appear to suggest that the circadian clock in the optic lobes of either sex can dictate a time for initiation of copulation and when the optic-lobe clocks of the 2 sexes are out of phase, 2 peaks in copulatory behavior, one driven by the male clock and one by the female clock, are evident.

This hypothesis leads to the clear and testable prediction that 1 of the 2 peaks of the bimodal rhythms in copulation exhibited when the males and females are 12 h out of phase should disappear if the optic tracts of one the sexes is severed. The results of the experiments to test this prediction are shown in Figure 8. The top panels show the times of copulation onset for males (n = 35) and females (n = 35) coming from light cycles that were 12 h out of phase with the optic tracts of the females sectioned. Much to our surprise, the bimodality in the rhythm persisted (in constant conditions), was robust, and was statistically significant (p < 0.01, Rao's Spacing Test). As with intact animals, the peaks of mating behavior were approximately 12 h out of phase and of similar

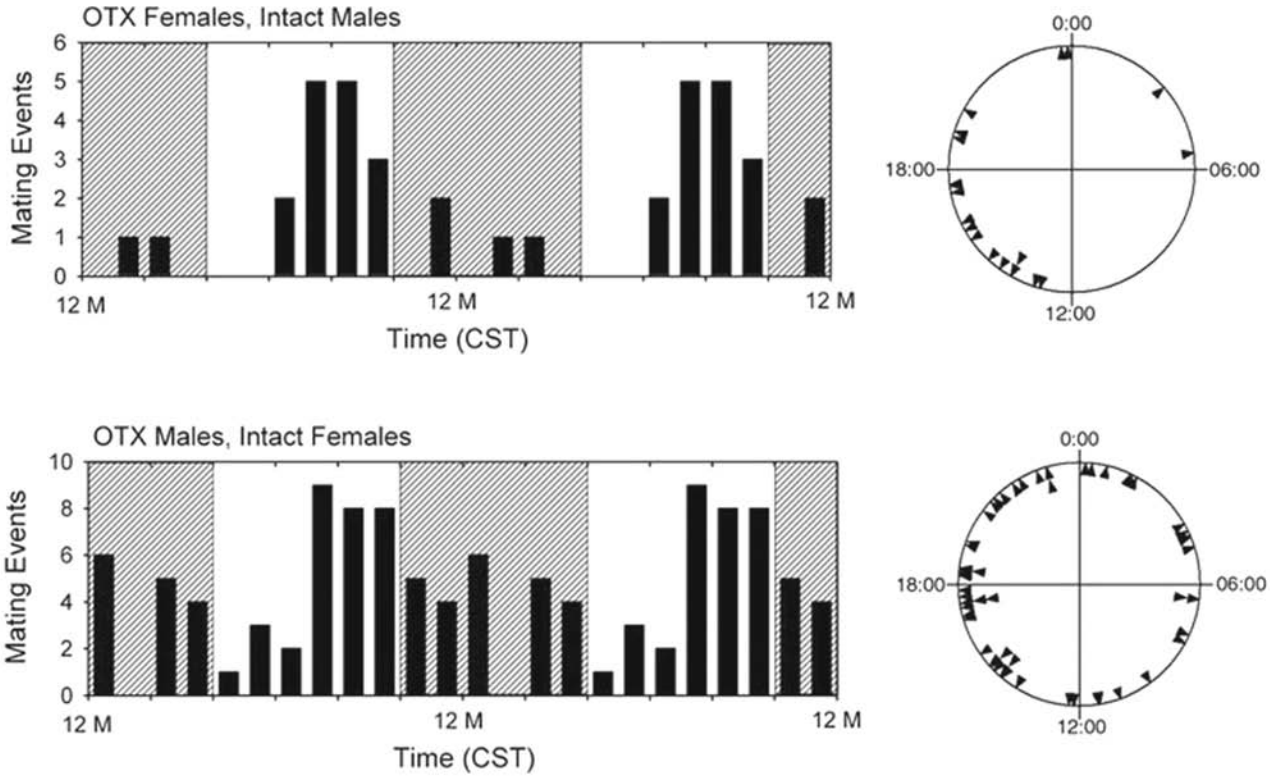

Figure 7. Effects of section of the optic tracts of either females (top panels) or males (bottom panels) on mating activity recorded in constant conditions. The hatched bars overlaying the histograms show the projected scotophase of the light cycle to which both the males and females had been entrained prior to the beginning of the experiment. Section of the optic tracts of females alone (upper panels) has little or no effect on either the phase or amplitude of the free-running rhythm of copulation onset (cf. Fig. 3). Section of the optic tract of males (lower panels) disrupts the rhythm causing substantial scattering in the time of copulation onset; however, a statistically significant clustering of mating events persists. OTX = section of the optic tract.

amplitude. Thirteen copulation events took place between 0000 and 1200 CST, with a mean of 6.6 ± 2.28 ("male peak"), and 11 occurred between 1200 and 2400 CST, with a mean of 19.0 ± 1.75 ("female peak").

In the next experiment, we combined intact females ($n = 35$) with OTX males ($n = 33$). Sectioning the optic tracts of the males was again more disruptive than when the surgery was done on females, and there is substantially more scatter in the data (Fig. 8, lower panels). However, the copulatory events were still not random, with a broad peak of copulation that appears to exhibit a bimodal distribution; the most robust peak occurred at an appropriate phase predicted by the females' phase, and there is an indication of a second peak of slightly lower amplitude appropriate for the males' phase. The statistical analysis was ambiguous, reflecting the "sloppiness" and lack of well-defined bimodality in the data. While the Rao Spacing Test was not significant ($p > 0.10$), the Rayleigh Test indicated a significant, unimodal clustering of the mating events ($p = 0.016$). Twenty of the females mated, and of these, 9 mated 2 or more times.

DISCUSSION

The results presented here clearly show that the reproductive behavior of *L. maderae* is gated by a circadian clock and provide some insight into the mechanisms by which this control is exerted. On the other hand, some features of the data do not readily yield to straightforward interpretation.

As noted above, based on current models of mating behavior in *Leucophaea*, circadian rhythms in mating could arise from a variety of sources. In *L. maderae*, this could include circadian regulation of calling and sex pheromone release by males, circadian rhythms of behavioral response to sex pheromone due to rhythms in receptor sensitivity or central processing in females, or finally, it could be due to rhythms in general levels of activity in either males

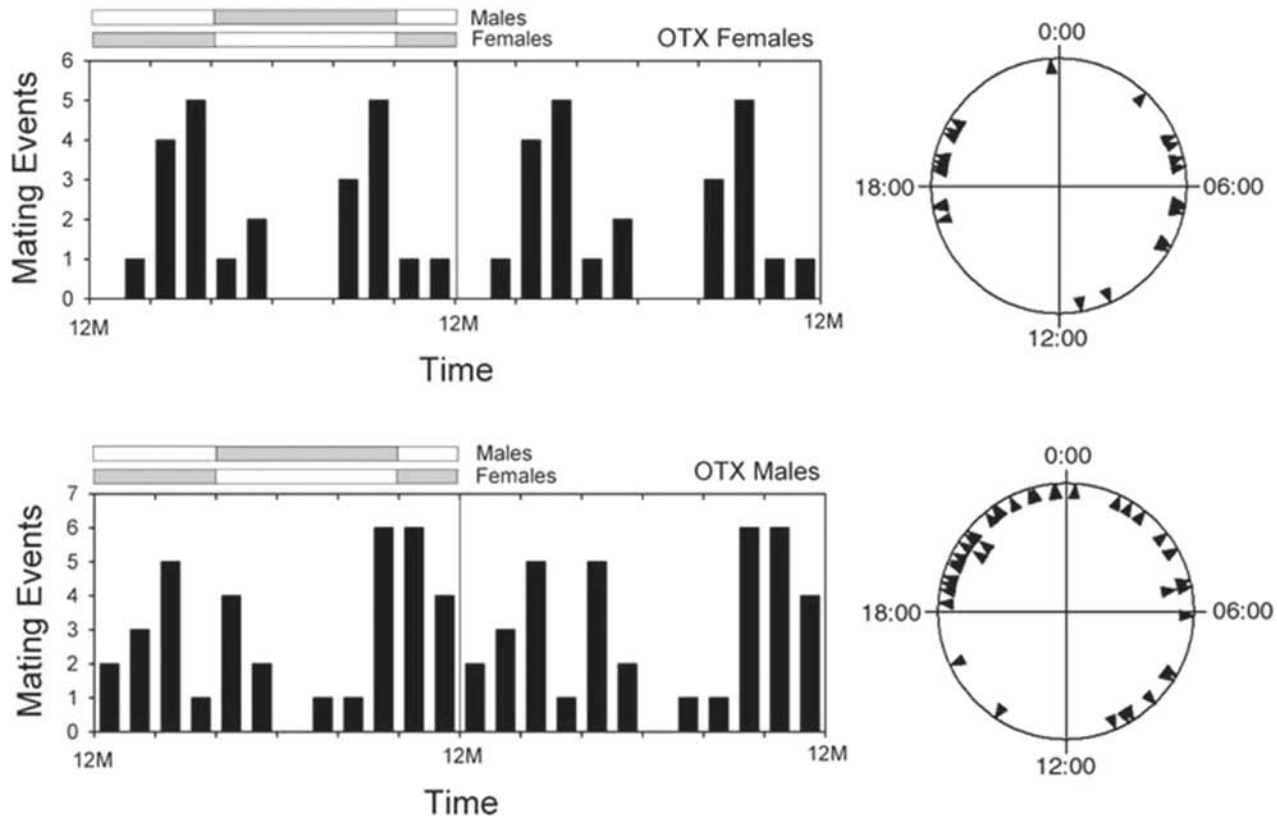

Figure 8. Effects of section of the optic tracts of either females (top panels) or males (bottom panels) on mating activity when sexes had been entrained to light cycles that were 12 h out of phase (note that the male and female light cycles are reversed in this experiment relative to Fig. 4). Copulation events were recorded in constant conditions. Bars at the top of each histogram, which are "double-plotted," show the light cycles to which the animals had been entrained prior to the beginning of the experiment. Section of the optic tracts of females had little or no effect on the predicted bimodal distribution of mating behavior. Section of the optic tracts of males was again more disruptive, but a rhythm persisted and appeared to be weakly bimodal. OTX = section of the optic tract.

or females that would modulate the propensity to copulate.

The Role of Circadian Rhythms in Locomotor Activity

It is well established that the optic lobes contain the circadian oscillators that drive the circadian rhythm of locomotor activity in *Leucophaea* and that section of the optic tracts or ablation of the optic lobes results in loss of a detectable activity rhythm (Page, 1990). Data presented here show that severing the optic tracts of females alone has essentially no effect on the mating rhythm, and therefore this rhythm is independent of the circadian rhythm in general locomotor activity in the female. Section of the optic tracts in males is more disruptive and results in substantial variability in mating times; however, a significant rhythm persists. Finally, cutting the optic tracts of both males and females completely abolishes rhythmicity. The results suggest that the optic lobes do participate in timing the circadian rhythm in mating behavior but that a circadian rhythm of locomotor activity in one sex or the other is dispensable. This latter conclusion is consistent with the observation that when males and females are 12 h out of phase, a bimodal rhythm of mating behavior persists in constant darkness. This indicates that the circadian regulation of locomotor activity does not exclude mating at an "inappropriate" inactive phase in either sex—both sexes can be induced to mate during phases of either activity or inactivity. The data indicate that the circadian rhythms of the 2 behaviors (locomotor activity and mating) are

temporally and physiologically separable and suggest, therefore, that they are functionally distinct.

The Role of the Circadian Rhythm of Olfactory Sensitivity

Interestingly, the temporal distribution of mating behavior appears to be completely independent of the sensitivity of the olfactory receptors in the antennae of the female that respond to components of the male sex pheromone. While the amplitude of the olfactory response peaks in the late subjective night/early subjective day, this is the period within the circadian cycle where mating is absent. In contrast, the minimum olfactory response is in the late afternoon/early subjective night and coincides with the peak of mating behavior. In addition, when females are paired with males that are 12 h out of phase, mating occurs with about equal frequency at both the projected peak and trough of the antennal olfactory response rhythm of the female. One is forced to conclude that whatever the function of the circadian modulation of olfactory sensitivity, it has little or no impact on the timing of mating behavior (at least under the conditions of our experiments). Furthermore, it has been shown that section of the optic tracts abolishes the circadian rhythm in EAG amplitude (Page and Koelling, 2003), but we found that optic-tract section in females does not affect the rhythm in mating behavior. The results are difficult to reconcile with the hypothesis that the rhythm in mating arises as a consequence of the rhythm of olfactory sensitivity in antennae.

On the other hand, there have been clear demonstrations in other cockroach species that indicate the behavioral attraction to sex pheromones of the opposite sex does exhibit a circadian rhythm (e.g., Liang and Schal, 1990; Zhukovskaya, 1995). The comparable experiment has not yet been done in *Leucophaea*, but it is possible that female attraction to pheromone is governed by the circadian system through a central mechanism that is independent of regulation of peripheral sensitivity. In this context, it is interesting to note that there are 2 species of moths where information is available on both the EAG response and the behavioral response to sex pheromone stimulation. In these cases, the results suggest that there is a behavioral response rhythm despite a constant EAG response to pheromone stimulation throughout the day (Payne et al., 1970, Linn et al., 1995; Rosén et al., 2003), suggesting a central rather than peripheral mechanism in regulating rhythmic responses to pheromone.

The Role of a Potential Circadian Rhythm in Male Calling Behavior

In at least one other cockroach species, it has been shown that pheromone calling behavior is regulated by a circadian clock. In this case, females of the species *Supella longipalpa* exhibit a circadian rhythm in calling behavior that persists in constant darkness (Smith and Schal, 1991). In addition, in both *Periplaneta americana* (Seelinger, 1984) and *Blatella germanica* (Liang and Schal, 1993), calling has been shown to be diurnal in a light cycle. Whether or not *Leucophaea* males exhibit rhythmic calling is unknown. However, based on the observation that female phase appears to be a significant determinant of mating time, it is not likely that a calling rhythm in males, if it exists, can exclusively account for the circadian rhythm in copulation. Nevertheless, detailed behavioral and biochemical analysis of male calling and pheromone release would be useful and informative.

Function and Localization of the "Mating Clock"

The data seem quite clear in showing that circadian clocks in both the males and females contribute to the determination of the time of mating since, in constant darkness, the phase of the timing of copulation onset is explicitly dependent on the phase of the light cycle(s) to which both males and females had been entrained. Thus, we suggest that "mating clocks" are located in both males and females. For both sexes, the clocks contribute to determining when copulation will begin. How the regulation occurs is unclear, and given the differing roles of the males and females in mating behavior, it is plausible that the clocks play differing roles between the sexes. In the simplest scheme based on current understanding of the behavior (Sreng, 1993), one could suggest that the male's clock controls behaviors that initiate mating (e.g., calling and pheromone release) while the female's clock controls receptivity to the male's advances. However, in this scheme, mating would only occur when initiation and receptivity coincide in phase. This is observed, in fact, in the moth *Spodoptera littoralis*. In this moth, mating is virtually absent between males and females that are out of phase, indicating that clocks of both sexes are critical and that phase coincidence is important for copulation

(Silvegren et al., 2005). In contrast, in *Leucophaea*, placing the clocks of males and females out of phase simply adds an additional peak of mating activity. The difference between the 2 species is interesting. One plausible explanation is that moths are typically solitary and rely heavily on long-distance chemical communication for finding mating partners. In contrast, *L. maderae* is a gregarious species and when housed in close proximity, which likely mimics conditions in the field (Cornwell, 1968) as well as in the rearing colonies in the laboratory, it may be able to support a more opportunistic mating strategy. Furthermore, under these conditions of proximity, the volatile male sex pheromone may be dispensable (or continuously present) so that either the male or the female can initiate clock-controlled mating through other chemical or tactile signals. For example, contact pheromones that can elicit courtship responses are common in cockroach species (Gemeno and Schal, 2004). As long as both sexes exhibited a constant level of receptivity throughout the circadian cycle, then the circadian pacemakers of each could open a gate that would lead to the initiation of mating behavior and copulation. Further detailed behavioral and physiological studies will be necessary to sort out the potential mechanisms.

Given the well-established role of the optic lobes as the locus of "the" circadian clock in *Leucophaea*, we hypothesized that the optic-lobe pacemaker in either the males or the females or both would be the source of the timing signal for control of the circadian rhythm of mating. The results of the experiments involving sectioning the optic tracts as a test of the involvement of the optic lobes do not yield to any simple version of this hypothesis. Severing the optic tracts of both males and females abolishes the rhythm in the onset of copulation (though the duration of copulation was unaffected), suggesting some involvement of the optic lobes. In contrast, cutting the optic tracts of only the female has little or no effect and, in particular, did not abolish the "female-driven" peak of mating activity in out-of-phase animals. Cutting the optic tracts of the males had significantly more disruptive effects on the timing of mating activity; however, a rhythm in mating persisted between animals that were in phase or out of phase and appeared to be (weakly) bimodal in the out-of-phase group. Overall, the results suggest intact optic tracts of one or the other sex are necessary for expression of a rhythm in mating and that the optic lobes are more important in males than in females, but for both sexes, circadian clocks outside the optic lobes may participate. One alternative to the conclusion that a clock outside the optic lobes plays a role is that there are 2 output pathways from the optic lobe pacemakers—one involving neural connections via the optic tracts and the other relying on some unidentified humoral signal—that play a role in regulating the rhythm in mating behavior. We consider this interpretation less likely, but it cannot be completely ruled out by the present data.

In summary, the data are compelling in the demonstrations that the circadian system regulates a circadian rhythm of copulatory behavior and that circadian clocks of both sexes contribute to the timing of mating behavior. The results also strongly suggest that this behavioral rhythm is independent of the rhythm in olfactory response to sex pheromone in the antennae and probably do not arise as a consequence of the circadian rhythm of locomotor activity. Finally, the circadian pacemaker of the optic lobes is involved in temporal regulation of mating, but the data suggest timing systems outside the optic lobes also play a role.

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