Circadian rhythms of female mating activity governed by clock genes in *Drosophila*

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The physiological and behavioral activities of many animals are restricted to specific times of the day. The daily fluctuation in the mating activity of some insects is controlled by an endogenous clock, but the genetic mechanism that controls it remains unknown. Here we demonstrate that wild-type *Drosophila melanogaster* display a robust circadian rhythm in the mating activity, and that these rhythms are abolished in *period*- or *timeless*-null mutant flies (*per*^{01} and *tim*^{01}). Circadian rhythms were lost when rhythm mutant females were paired with wild-type males, demonstrating that female mating activity is governed by clock genes. Furthermore, we detected an antiphasic relationship in the circadian rhythms of mating activity between *D. melanogaster* and its sibling species *Drosophila simulans*. Female- and species-specific circadian rhythms in the mating activity of *Drosophila* seem to cause reproductive isolation.

Most organisms show circadian 24-h rhythmicity in their behavior and physiology. Various behavioral phenomena in insects are controlled by an endogenous clock (1). A core oscillator mechanism of circadian rhythm and feedback loops, involving several clock genes including *period* (*per*) and *timeless* (*tim*), control locomotor activity and eclosion of the fruit fly, *Drosophila melanogaster* (2–6). Mutants of *D. melanogaster* with defective feedback loops (7–10) provide the means of studying the relationship between behavioral rhythms and circadian clock genes.

Mating by animals is the most important and fundamental process to select the best partner and to produce progeny. Insects in particular show daily rhythms in mating activity (11–17), and these are controlled in some by an endogenous clock (14, 15). Mating activity of the fruit fly *Drosophila mercatorum* shows the daily rhythms of the mating activity under 12-h/12-h light/dark (LD) cycles (16), and several *Drosophila* species show the daily rhythms of male courtship under LD cycles (17). However, the genetic mechanism(s) that modulates mating rhythm in these insects remains unknown.

The present study shows that wild-type *D. melanogaster* display a robust circadian rhythm in mating activity that is governed by clock genes and that females are responsible for generating the mating rhythms. Furthermore, we found that the mating activities of *disconnected* (*disco*) mutants, which have a severe defect in the optic lobe and are missing lateral neurons, are arrhythmic. We also identified an antiphasic relationship in the circadian rhythms of the mating activity between *D. melanogaster* and its sibling species, *Drosophila simulans*.

Materials and Methods

Fly Strains. *D. melanogaster* wild-type strains (Canton-S and OGS-4, Ogasawara, Japan), rhythm mutants [*period*^{01} (*per*^{01}), *timeless*^{01} (*tim*^{01}), and *disconnected*^{3} *forked* (*disco*^{3} *f*)], transgenic flies (*hsp-per* s13) carrying heat shock (HS)-inducible *per* coding sequences (18), *forked* (*f*) mutant, and *D. simulans* wild-type strains (Og, Ogasawara, Japan; and Ots, Otsuki, Japan) were grown on glucose/yeast/cornmeal medium under a 12:12 LD cycle at 24.5 ± 0.5°C. Lights on occurred at Zeitgeber time (ZT) 0 and lights off occurred at ZT12. Virgin males and females were collected without anesthesia within 8 h after eclosion. They were maintained separately in vials at 24.5 ± 0.5°C in LD cycles until the experiments.

Mating Activity Analysis. Mating frequency as an indicator of mating activity was determined as follows. After five males and five females were crossed, we dissected out the female reproductive organs and calculated mating frequency as a percentage of the number of inseminated females divided by the number of dissected females. The mean mating frequencies then were calculated after several replications. To free-run in constant darkness (DD), vials (2.5 cm in diameter × 9.5 cm in height) containing either 5 males or 5 females were placed in light-tight boxes at 24.5 ± 0.5°C until use. Mating frequency in the dark was measured as described by Sakai *et al.* (19). Lids of boxes containing vials of virgin males and females were removed under safelight in a darkroom. Groups of five males were transferred into vials containing five females and immediately replaced in light-tight boxes. Mating frequency in light also was determined in a room that was illuminated 50–100 lux at the level of the vials. Males and females were allowed to mate for 15 or 25 min.

HS Treatment of an *hsp-per*. The transgenic line, *hsp-per* s13, carrying a HS-inducible copy of the *per* gene in a background *per*^{01} mutant strain, shows rhythmic locomotor activity at high temperature under free-running conditions (18). We measured the mating activity of *hsp-per* females and Canton-S males on day 2 of DD after 7 days' entrainment in LD. The *hsp-per* females were HS at 37°C for 9 consecutive days from ZT10 to ZT11 (HS+ experiment). Non-HS *hsp-per* females (control females) were kept at 24.5 ± 0.5°C for 9 consecutive days (HS− experiment). Canton-S males also were maintained at 24.5 ± 0.5°C until use. Flies were allowed to mate for 20 min.

Statistical Analysis. The distribution of most of the data was not normal, despite arcus sine (ARCSIN) transformations. Although distribution was normal in some of the data, heteroscedasticity was evident. Hence, data were analyzed by using the Kruskall-Wallis test followed by the Mann–Whitney *U* test for multiple comparisons among all time points of the mating experiment. All significant differences in the figures passed the sequential Bonferroni test (20) except for Fig. 1*A* and Fig. 3 *C* and *D*.

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Abbreviations: LD, light/dark; ZT*n*, Zeitgeber time *n*; DD, constant darkness; CT*n*, circadian time *n*; HS, heat shock.

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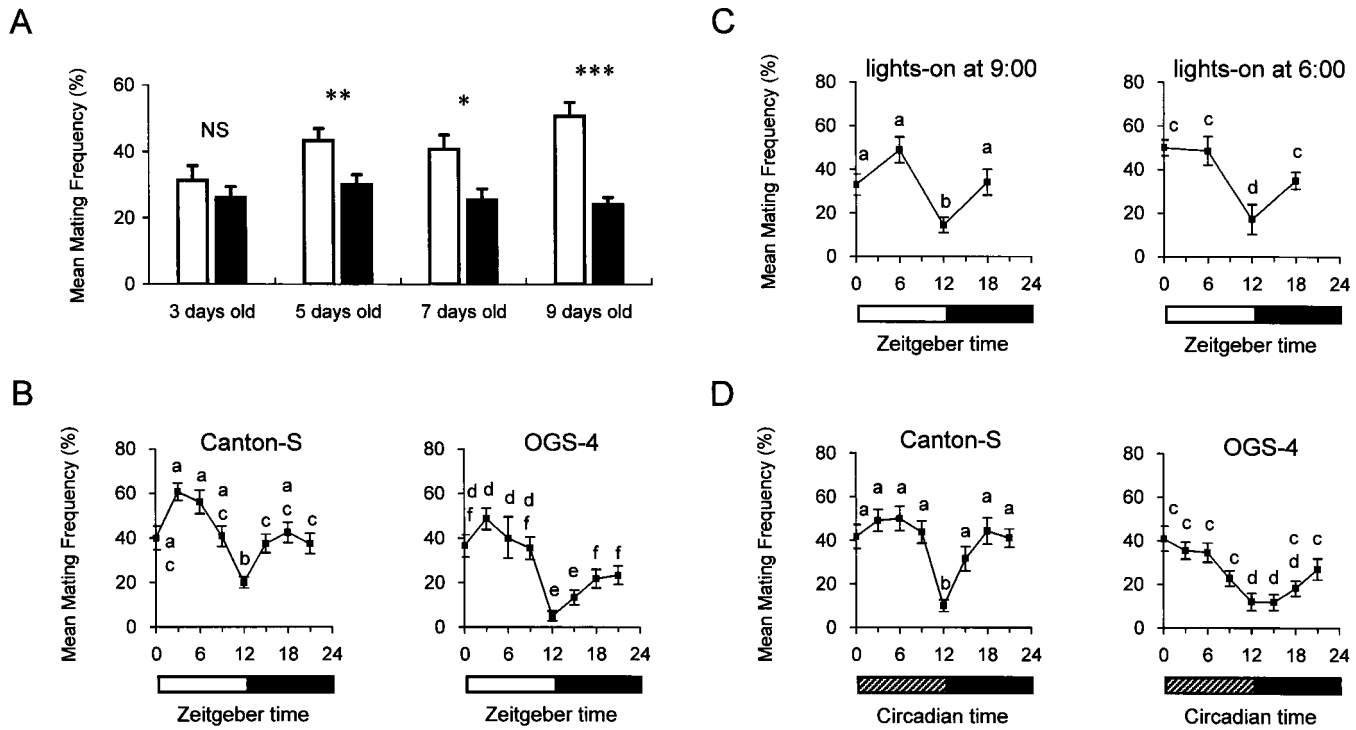

Fig. 1. Mating activities at different times of day in *D. melanogaster*. Males and females of the Canton-S strain were allowed to mate for 15 min. Error bars show SEMs. (*A*) Comparisons of mating activity between day (ZT3, white bars) and night (ZT12, black bars) in flies of different ages (3–9-days-old). The Mann–Whitney *U* test compared mating frequency. NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. $n = 30$ for each bar. (*B*) Daily changes in mating activities of Canton-S and OGS-4 strains on day 9 under 12:12 LD cycles. Values with the same superscript letters were not significantly different according to multiple pair-wise comparisons of the Mann–Whitney *U* test among all time points. $n = 21$–26 per point. The white and black bars below the graphs denote when lights were on and off, respectively. (*C*) Daily changes in mating activity of Canton-S on day 7 under different 12:12 LD cycles (lights on at 9:00 and 6:00, respectively). $n = 14$–18 for each point. (*D*) Circadian rhythms in mating activities of Canton-S and OGS-4 strains on day 2 of DD. Hatched portion of bar below graph indicates subjective day, and black portion indicates subjective night. $n = 20$–23 for each point.

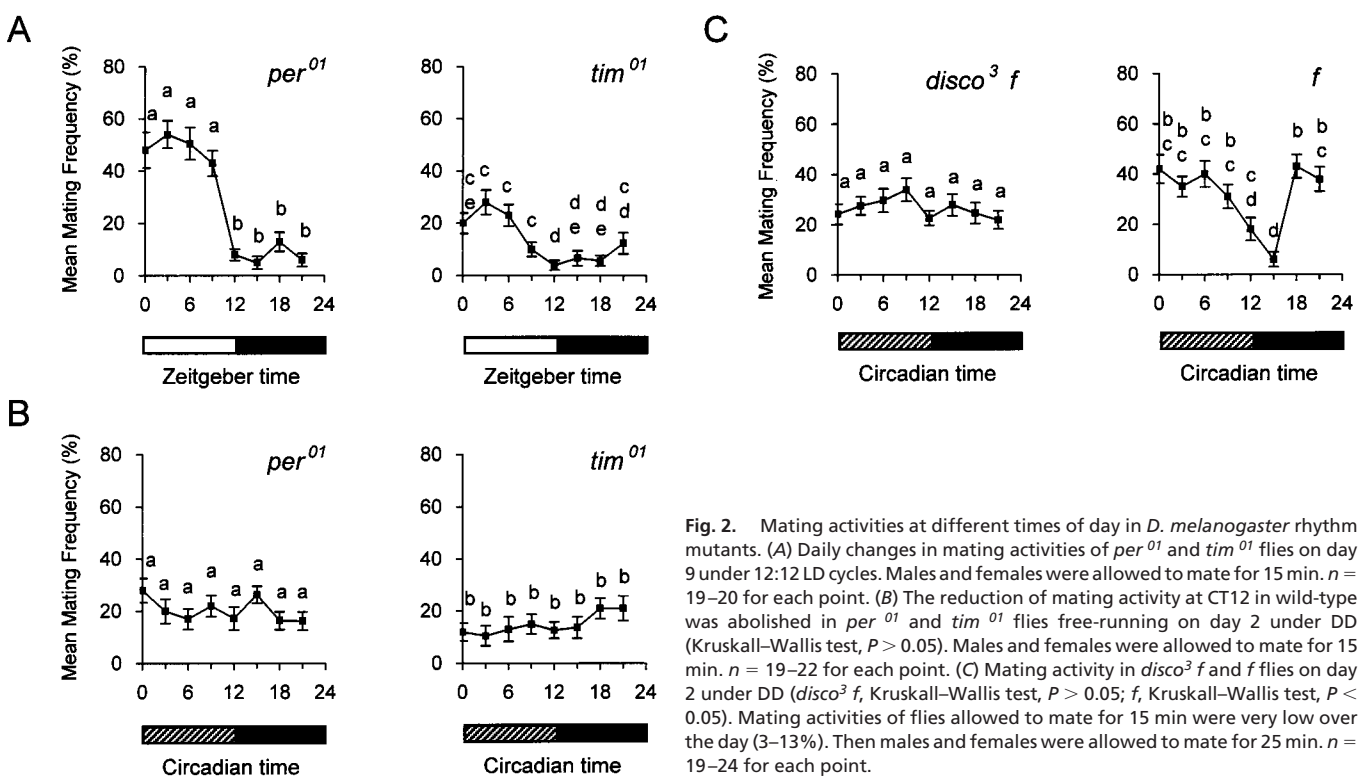

Fig. 2. Mating activities at different times of day in *D. melanogaster* rhythm mutants. (*A*) Daily changes in mating activities of *per* ^{01} and *tim* ^{01} flies on day 9 under 12:12 LD cycles. Males and females were allowed to mate for 15 min. $n =$ 19–20 for each point. (*B*) The reduction of mating activity at CT12 in wild-type was abolished in *per* ^{01} and *tim* ^{01} flies free-running on day 2 under DD (Kruskall–Wallis test, $P > 0.05$). Males and females were allowed to mate for 15 min. $n = 19$–22 for each point. (*C*) Mating activity in *disco*^{3} *f* and *f* flies on day 2 under DD (*disco*^{3} *f*, Kruskall–Wallis test, $P > 0.05$; *f*, Kruskall–Wallis test, $P <$ 0.05). Mating activities of flies allowed to mate for 15 min were very low over the day (3–13%). Then males and females were allowed to mate for 25 min. $n =$ 19–24 for each point.

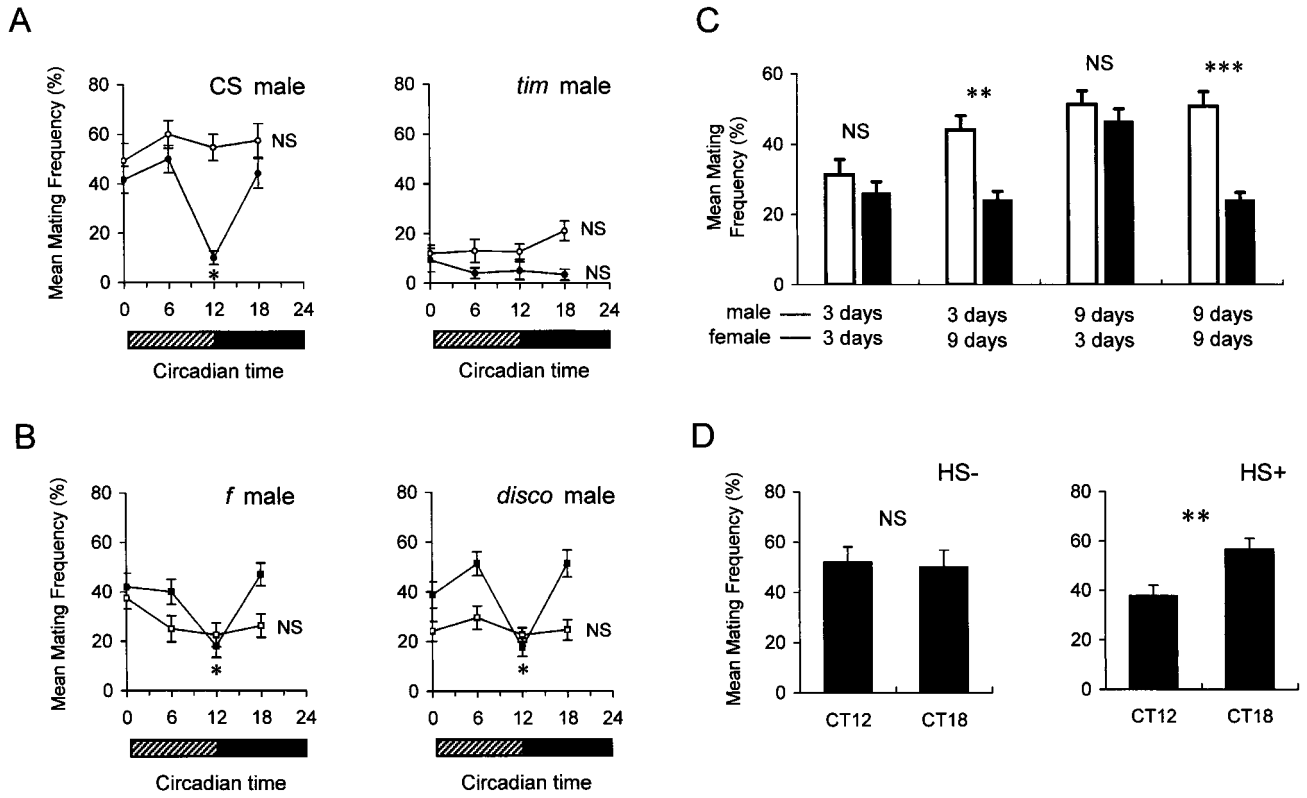

Fig. 3. Mating frequency at different times of day in crosses of *D. melanogaster* wild-type and rhythm mutants under DD. (*A*) Canton-S or tim^{01} males were paired with tim^{01} (open circles) or Canton-S (filled circles) females for 15 min. $n = 12$–24 for each point. NS, not significant (Kruskall–Wallis test, $P > 0.05$); *, time when mating activity was significantly lower than at other times by pair-wise comparisons of the Mann–Whitney *U* test. (*B*) *f* or $disco^3$ *f* males were paired with $disco^3$ *f* (open squares) or *f* (filled squares) females. Males and females were allowed to mate for 25 min. $n = 16$–20 for each point. (*C*) Comparisons of mating activities between day (ZT3, white bars) and night (ZT12, black bars) in crosses of *D. melanogaster* Canton-S 3- and 9-day-old flies. Three-day-old males were paired with 9-day-old females, and 9-day-old males were paired with 3-day-old females. Data showing that males and females were the same age are shown in Fig. 1*A*. Males and females were allowed to mate for 15 min. $n = 25$–30 for each bar. Mating frequencies between day and night were compared by using the Mann–Whitney *U* test. NS, not significant; **, $P < 0.01$; ***, $P < 0.001$. (*D*) Canton-S males were allowed to mate with *hsp-per* females for 20 min. NS, not significant; **, $P < 0.01$. $n = 15$–18 for each bar.

Results and Discussion

To determine whether mating activities differ between day and night, we measured the mating frequencies of *D. melanogaster* maintained in 12:12 LD cycles. The mating activities of 3-day-old flies did not significantly differ between day (ZT3) and night (ZT12; Fig. 1*A*). However, the mating activities of 5-, 7-, and 9-day-old flies significantly differed between day and night (Fig. 1*A*).

To determine whether the mating activity of *D. melanogaster* fluctuates over the day, we measured the mean mating frequencies of two strains (Canton-S and OGS-4) at 9 days of age. The mating activities of both *D. melanogaster* strains were similar over the day under LD cycles (lights on at 9:00 and lights off at 21:00; Fig. 1*B*). The mating activities of both strains at ZT12 were significantly lower than at other times (Fig. 1*B*). We confirmed that the rhythms of 7-day-old Canton-S flies were the same under the same LD conditions (Fig. 1*B*). Furthermore, the rhythms of 7-day-old flies that were kept under different LD cycles (lights on at 6:00 and lights off at 18:00) after eclosion were similar to those of 7- and 9-day-old flies kept under LD cycles with lights on at 9:00 (Fig. 1 *B* and *C*). These results indicate that the rhythms of *Drosophila* mating activity become synchronized (entrained) to daily LD cycles. To determine whether these rhythms are controlled by an endogenous clock, we measured the mating activities of flies on day 2 of DD after 7 days of entrainment in LD cycles. The reduction of mating activity at circadian time (CT) 12 remained intact in both strains under DD as well as in LD (Fig. 1 *B* and *D*). These results indicate that the mating activity of *D. melanogaster* is under the restricted control of an endogenous clock.

To know whether the mating-activity rhythms of *Drosophila* are controlled by circadian clock genes, we measured the mating activity in $per^{01}$and tim^{01} flies that lack rhythms in adult emergence and locomotor activity (7–10). In contrast to the two wild-type strains under LD cycles, these mutant flies did not recover mating activity within 3 to 6 h from lights off (Fig. 1*B* and Fig. 2*A*). When the flies were allowed to mate for 15 min, mating activities in these rhythm mutants were not reduced at CT12 in DD (Fig. 2*B*). When per^{01} flies were allowed to mate for 25 min, mating activity over the day was high (37–50%) and not reduced at CT12 (data not shown). These results indicate that the circadian clock genes, *per* and *tim*, affect the circadian rhythm of *Drosophila* mating activity. Mating activities of the per^{01} and tim^{01} mutants were elevated in the morning (Fig. 2*A*). However, mating activity was not elevated in the two mutants under DD (Fig. 2*B*). These results indicate that light signals also directly affect mating activity in the morning.

In *D. melanogaster*, the specific neurons of the optic lobe seem to play a major role as pacemakers for locomotor activity rhythms, because a transgenic line in which *per* expression is restricted to the lateral neurons has rhythmic locomotor activity (21, 22). Further evidence is provided by studies of *disconnected*

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(*disco*) mutants that have a severe defect in the optic lobe and are missing lateral neurons (21, 23). Both locomotor activity and eclosion of the *disco* mutant are arrhythmic under DD (24). The present study found that mating activities of the *disco*, like those of the per^{01} and tim^{01}, mutants were not reduced at CT12 (Fig. 2*C*). As the *disco* mutants studied here ($disco^{3}$ *f*) included the *forked* (*f*) mutation, we used *f* mutants as a control. The mating activities of the *f* mutants were reduced at CT15 like those of the wild-type OGS-4 strain (Fig. 2*C*). Although over 95% of *disco* mutants are arrhythmic in locomotor activity, rhythmic individuals emerge on rare occasions (21) with intact single or some lateral neurons (25). These results indicate that arrhythmicity in the locomotor activity of *disco* mutants is a result of the defective lateral neurons. Taken together, our results suggest that arrhythmicity in the mating activities of *disco* mutants is also caused by defective lateral neurons.

The specific role of sex that may be involved in the circadian rhythm of mating activity can be investigated in rhythm mutants. To determine whether the robust circadian rhythm in mating activity shown in the wild-type is caused specifically by males, females, or a combination of both sexes, Canton-S females were paired with tim^{01} or Canton-S males, and tim^{01} females were paired with tim^{01} or Canton-S males. Mating-activity rhythm was abolished in tim^{01} females crossed with Canton-S males (Fig. 3*A*). In contrast, mating-activity rhythm was undetectable regardless of which females were mated with tim^{01} males (Fig. 3*A*). The mating activity of such pairs was very low over the day, suggesting that the *tim* gene or the genetic background of the tim^{01} mutant was responsible for low courtship activity of the mutant males. We performed the same experiments with crosses of *f* and $disco^{3}$ *f* flies (Fig. 3*B*). When *f* females were paired with *f* or $disco^{3}$ *f* males, mating activity was reduced at ZT12 in both types of males, and rhythms of such pairs were obvious (Fig. 3*B*, filled squares). In contrast, when $disco^{3}$ *f* females were crossed with *f* or $disco^{3}$ *f* males, mating-activity rhythms were abolished (Fig. 3*B*, open squares). These results demonstrated that females are responsible for generating the circadian rhythm of mating activity in *Drosophila*. The findings suggested that females need lateral neurons to generate these rhythms and that a female-specific circadian clock suppresses mating activity at CT12.

Fig. 1*A* shows significant differences in the mating activities of 5-, 7-, and 9-day-old flies between day and night. To determine whether these differences are caused specifically by males, females, or both sexes, 9- and 3-day-old males were paired with 3- and 9-day-old females, respectively (Fig. 3*C*). When males were paired with 9-day-old females, day/night activities clearly differed (Fig. 3*C*). In contrast, these differences were absent when 3-day-old females were paired with 3- and 9-day-old males (Fig. 3*C*). Thus, we concluded that *Drosophila* females contribute to the day/night differences in the mating activities, and suggest that a female-specific circadian clock drives mating activities at least after 5 days of age. In 3-day-old females, the mechanisms to modulate the mating activity may be undeveloped. Alternatively, some sort of mating drive may overwhelm clock control in the youngest females.

To know whether the *per* gene affects the reduction of mating activities at CT12, we measured the mating activity in combinations of both types of *hsp-per* females and Canton-S males. No differences in the mating activities between CT12 and CT18 were detected in the HS− experiment (Fig. 3*D*) as well as in the per^{01} mutant (Fig. 2*B*). However, mating activities at CT12 were significantly lower than those at CT18 in the HS+ experiment (Fig. 3*D*). These results were similar to those from the wild-type (Fig. 1*C*), suggesting that *per* gene expression causes the reduction in mating activity at CT12, and that arrhythmicity in the mating activity of the per^{01} mutant (Fig. 2*B*) is caused by the *per* mutation of female flies rather than by the genetic background of the mutants.

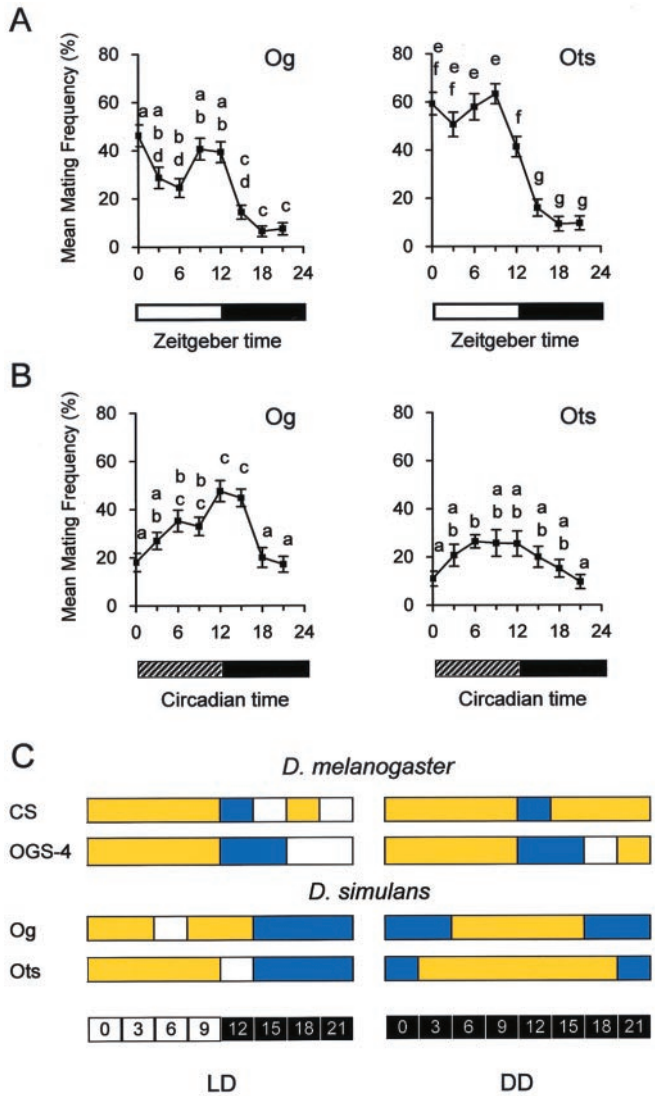

Fig. 4. Mating activities at different times of day in *D. simulans*. (*A*) Daily changes in mating activities of Og and Ots strains on day 9 under 12:12 LD cycles. Mating activities of flies allowed to mate for 15 min were very low over the day (0–3%). Thus, males and females were allowed to mate for 25 min. n = 29–32 for each point. (*B*) Circadian rhythms in mating activity of *D. simulans* on day 2 of DD. Males and females were allowed to mate for 25 min. n = 26–29 for each point. (*C*) Schematic representation of mating-activity rhythms in *D. melanogaster* and *D. simulans*. Yellow portions denote times of no significant differences compared with times of highest-mating activity. Blue portions denote times of no significant differences compared with times of lowest-mating activity. White portions denote other times (average mating activity).

To determine the mating-activity rhythm in sibling species of *D. melanogaster*, the mating frequencies of *D. simulans* were measured in LD and DD. *D. melanogaster* and *D. simulans* are morphologically almost identical and genetically very similar, and these two cosmopolitan species are sympatric (26, 27). We measured the mean mating frequencies of two strains (Og and Ots) of *D. simulans* by using 9-day-old flies. In LD cycles, the mating activities of these two strains during the daytime (ZT0–9) and ZT12 were significantly higher than at other times (ZT15–18; Fig. 4*A*). We confirmed that the rhythm of 7-day-old flies of the Og strain is the same as that of 9-day-old flies (data not shown). These findings indicate that the mating activity of *D. simulans* is also subject to a daily rhythm, although the profile was distinct from that of *D. melanogaster* (Fig. 4*B*). In DD, the

mating activities of both strains of *D. simulans* gradually elevated after CT0, and those at CT6–15 (Og) or CT3–18 (Ots) were the highest of all (Fig. 4*B*). In LD cycles, the mating activity during the day was high in both *D. melanogaster* and *D. simulans* (Fig. 4*C*). During the night (ZT12–21), however, *D. simulans* frequently mated when the mating activity of *D. melanogaster* was low (ZT12), and *D. melanogaster* frequently mated when that of *D. simulans* was low (ZT18–21; Fig. 4*C*). In DD, the rhythm of mating activity between *D. melanogaster* and *D. simulans* was species-specific in an antiphasic fashion (Fig. 4*C*). The mating-activity rhythm of both strains of *D. simulans* in DD was obviously distinct from that in LD (Fig. 4 *A* and *B*), suggesting that the mating activity of *D. simulans* also is controlled not only by an endogenous clock but also by light.

The results of the present study demonstrate that mating activity is driven by two mechanisms in *Drosophila*. One is a circadian pacemaker consisting of clock genes and the other is the direct effect of light. The mating-activity rhythm of *D. melanogaster* females is under the restricted control of circadian clock genes, and the lateral neurons might be essential to generate the rhythm. Flies, especially males, use olfactory cues for mating (28–30), and the circadian rhythm of the olfactory response is robust in *Drosophila* (31). Olfactory responses of the wild-type were elevated in the middle of the night in LD cycles (31), but mating activities were decreased during the early part of the night (Fig. 1*B*). Furthermore, the lateral neurons are insufficient to sustain olfactory rhythm (31) but the optic lobe, including the lateral neurons, seemed to be essential for mating-activity rhythm according to our results (Fig. 2*C*). Thus, the mechanism that generates the mating-activity rhythms might be independent of that which generates olfactory rhythms. A female sex pheromone attracts male courtship in *Drosophila* (28–30), and the sound produced by male wing vibration, referred to as courtship song, affects female receptivity (28, 32, 33). One explanation for the generation of female mating activity in *Drosophila* is that females show circadian rhythms in pheromone release and/or responses to auditory signals.

The behavioral characteristics of mating, habitat, and breeding season vary in a species-specific manner between *D. melanogaster* and *D. simulans*, thus creating a barrier to interspecific hybridization referred to as reproductive isolation (26, 27). During the night under LD cycles that were similar to conditions in the wild, mating-activity rhythms between these two species differed in a species-specific manner. Such a difference might create an effective barrier against interspecific hybridization during the nighttime in nature.

The species-specific circadian rhythm controlled by clock genes in the female-mating activity of *Drosophila* may affect the daily species-specific pattern of mating activity. Transformation experiments in which the *per* gene from other species was introduced into *D. melanogaster per*^{01} flies have revealed a DNA sequence that encodes the species-specific features of locomotor-activity rhythm or male courtship-song rhythm in *Drosophila* (34–36). Several clock genes including *per* and *tim* also may have DNA sequences for the species-specific circadian rhythms of *Drosophila* female mating activities. The discovery that species diversity of circadian rhythms in the female mating activity of *Drosophila* is controlled by clock genes will provide insight into sexual behavior and speciation.

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