Time, love and Species

Takaomi Sakai[1,2,*] & Norio Ishida[2,3,4]

1. Institute for Behavioral Sciences, Gunma University School of Medicine, 339-22, Maebashi, 371-8511, Japan.
2. Clock Cell Biology, National Institute of Advanced Industrial Science and Technology (AIST), Institute of Molecular and Cell Biology (IMCB), 1-1, Higashi, Tsukuba, 305-8566, Japan.
3. Department of Biomolecular Engineering, Tokyo Institute of Technology, 4259 Nagatsuda, Yokohama, 226-8501, Japan.
4. Center for Interdisciplinary Research, Tohoku University, Aramaki, Aoba-ku, Sendai 980-8578, Japan.
* Present address: Institute for Behavioral Sciences, Gunma University School of Medicine, Maebashi, Gunma, 371-8511, Japan. (sakait@med.gunma-u.ac.jp)

Correspondence to: Prof. Norio Ishida, Ph. D.,
Clock Cell Biology,
National Institute of Advanced Industrial Science and Technology (AIST),
Institute of Molecular and Cell Biology (IMCB),
1-1, Higashi, Tsukuba, 305-8566, Japan.
FAX +81-298-61-9999,
E-MAIL n.ishida@aist.go.jp

Abstract

Physiological and behavioral phenomena of many animals are restricted to certain times of the day. Many organisms show daily rhythms in their mating. The daily fluctuation in mating activity of a few insects is controlled by an endogenous clock. The fruitfly, *Drosophila*, is the most suitable material to characterize the genetic basis of circadian rhythms of mating because some mutants with defective core oscillator mechanism, feedback loops, have been isolated. *D. melanogaster* wild-type display a robust circadian rhythm in the mating activity, and the rhythms are abolished in *period* or *timeless* null mutant flies (*per[01]* and tim[01]), the rhythms are generated by females but not males. *Disconnected* (*disco*) mutants which have a severe defect in the optic lobe and are missing lateral neurons show arrhythmicity in mating activities. Thus, the lateral neurons seem to be essential for the circadian rhythm in mating activity of *Drosophila*. Furthermore, an anti-phasic relation in circadian rhythms of the mating activity was detected between *D. melanogaster* and their sibling species *D. simulans*. The Queensland fruit flies or wild gypsy moth also show species-specific mating rhythm, suggesting that species-specific circadian rhythms in mating activity of insect appear to cause a reproductive isolation.

1. Introduction

Most organisms from prokaryotes to humans show the circadian, 24-hour rhythmicity, in their behavior or physiology. While only limited behavioral or physiological data are available for most organisms, in the fruitfly *Drosophila melanogaster*, a large amount of genetic approach is available on the studies of the circadian rhythms. In D. melanogaster, mutations at *period* (*per*) and *timeless* (*tim*) loci were found from the arrhythmic locomotor activity and eclosion rhythms [1, 2], and the subsequent cloning of these genes allowed a molecular approach to the *Drosophila* rhythm system [2–5]. Both *per* and *tim* undergo rhythmic transcription and show circadian oscillation in mRNA [6], and their gene products (PER and TIM, respectively) also show circadian oscillation in their abundance [7, 8]. TIM heterodimerizes with PER, which is likely important for clock function [9]. The circadian expression of *per* or *tim* mRNA required the fragment including an E-box (CACGTG) sequence [10–12]. Two basic helix-loop-helix (bHLH) transcription factors, CYCLE (CYC) and dCLOCK (dCLK) [13, 14], are known to bind to and activate the transcription through the E-box by forming a heterodimer [15, 16]. *Doubletime* (*dbt*) encodes a casein-kinase I homolog that has been suggested to regulate *per* phosphorylation and accumulation [17, 18]. These genes mentioned above are believed to regulate the core oscillator in circadian clock. Furthermore, *Drosophila* clocks display conservation with mammalian clocks at the sequence level, suggesting that mammalian clocks are similar to *Drosophila* clocks [19, 20].

Mating is the most important and fundamental behavior to select the best partner and to produce progeny. Many organisms show daily rhythms in their mating activity [21–27]. In molluscs, *Aplysia fasciate*, mating was strongly modulated by 1/day oscillation under light-dark (LD) cycles, and the level of mating activity rose through the night and reached a peak in the early morning [25]. Nile grass rats (*Arvicanthis niloticus*) from tropical Africa mate before daybreak under LD cycles [26]. The daily rhythms of mating in some organisms are controlled by the endogenous clock. In the unicellular ciliate, *Paramecium bursaria*, cells of these exhibit high mating reactivity in the light phase and low mating reactivity in the dark phase under LD cycles. After they are transferred to constant light (LL, 1000 lux), they continue to show a clear circadian rhythm of mating reactivity for several days [28]. A mutant clone that did not show the mating rhythmicity in LL was isolated from the parent stock of *Paramecium bursaria*. The arrhythmicity in LL was inherited cytoplasmically, and the rhythmicity in LL was recovered by injection of cytoplasm from the wild-type cell [29]. These results suggest that the cytoplasmic factors seem to control the circadian rhythm of mating reactivity in the unicellular ciliate. Furthermore, some insects (e.g., Queensland fruit fly, mosquito and moth) show circadian rhythms of mating activity under constant dark (DD) or LL [23, 30, 31] However, genetic mechanisms which modulate such a mating rhythm was unknown in such unicellular organism or insects. *Drosophila* are the most suitable material to characterize the genetic basis of circadian rhythms of mating because some mutants with defective the core oscillator mechanism, feedback loops, involving several clock genes such as *per* and *tim* have been isolated [1–4]. Furthermore, we have a large amount of data of mating behavior in *Drosophila*. The next section explains a *Drosophila* mating system.

2. Mating system of *Drosophila*

Mating behavior in *Drosophila* has been intensively studied since it was first described by Sturtevant [32]. Spieth [33] gave more detailed descriptions of the mating behavior of 101 species and subspecies. Mating of *Drosophila* consists of sequential elements of characteristic behavior and its sequence analysis has been extensively studied, particularly in *D. melanogaster* [34–36]. Many investigators described courtship elements in a male and a female of this species. First the male orients to the female; he takes up and maintains a position near the female with his body axis oriented directly toward her. If she moves, he follows. He spreads one of his wings and vibrates near the female. Then the male licks the female's genitalia after which he may attempt to copulate. A receptive female accepts the male attempted copulation, and they copulate. However, non-receptive females show a variety of rejection responses including decamping, wing flicking, kicking or fending off with the legs, curling of the abdomen or extruding the vaginal plates.

In the courtship sequence between males and females, flies utilize the signaling system between the sexes to make mating successful. Like many insects, both chemical and acoustic signals play important roles in successful *Drosophila* mating. Female sex pheromones are well known as chemical stimulants; species-specific chemical substances have been determined in some *Drosophila* species [37–40]. Such stimulants emitted by females are one of the most important signals to elicit courtship by males. Males, furthermore, use female sex pheromones to distinguish among species [41, 42]. The acoustic signal is also species specific, and females use it to distinguish among species [43–49]. Thus the chemical signals and

the auditory signals play active roles in triggering and controlling courtship in *Drosophila* [50–52].

Furthermore, there have been some interesting mating experiments involving vision. Grossfield [53] reported that many species show a characteristic degree of reduction in the rate of mating success in the dark as compared with that in light. He called this phenomenon 'light-dependency of mating'. Many investigators reported that male visual mutants of *D. melanogaster* show low courtship activity [51, 54, 55]. Sakai et al. [56, 57] reported that males, but not females, predominantly contribute to the light-dependency of mating in the *D. melanogaster* species subgroup and that *D. melanogaster* males perceive female movement as a visual signal during courtship. These reports indicate that light stimuli facilitate male courtship activity and that males utilize the visual signals mediated by light while mating. However, the role of vision in female mating activity is unknown yet.

3. Circadian rhythms in *Drosophila* mating

Ikeda [22] reported that *D. mercatorum* show the daily rhythms of the mating activity under light-dark (LD) 12:12 cycles, and several *Drosophila* species show the daily rhythms of male courtship in LD cycles (Hardeland [21]). Recently, we found that *D. melanogaster* display a robust circadian rhythm in the mating activity controlled by clock genes and which sex is responsible for the generation of the rhythms. [58].

3.1. Daily rhythms of mating activity of Drosophila

In LD cycles, *D. melanogaster* wild-type strain showed a daily rhythm of mating activity in a day (Fig. 1A). The mating activity at ZT12 was significantly lower than at other times in both strains (Fig. 1A). To determine whether or not these rhythms are controlled by an endogenous clock, we measured the mating activities of flies on day 2 of constant darkness (DD) after 7 days of entrainment in LD cycles. The reduction of mating activity at Circadian time (CT) 12 remained intact under DD as well as the result in LD (Fig. 1B). Hence, the mating activity of *D. melanogaster* is under a restricted control of an endogenous clock.

3.2 Circadian rhythms of mating activity governed by clock genes in Drosophila

In contrast to wild type strain under LD cycles, the recovery of the mating activity after 3 or 6 hours from lights-off was not observed in per^{01} and tim^{01} flies [58]. Furthermore, these flies did not show the reduction of mating activity at CT12 in DD. The results indicate that clock genes, *per* and *tim*, affect the circadian rhythm in mating activity of *Drosophila*. Mating activities of the per^{01} and tim^{01} mutants were elevated in the morning. However, mating activity was not elevated in the two mutants under DD. These results indicate that light signals also directly affect mating activity in the morning. Thus, light signal also acts to affect the mating activity directly.

3.3 Pacemaker neurons of the mating activity rhythms in Drosophila

In D. melanogaster, the specific neurons of the optic lobe seem to play a major role as the pacemaker neurons for locomotor activity rhythms because a transgenic line that per expression is restricted to the lateral neurons show rhythmic locomotor activity [59, 60]. Further evidence is provided by studies of *disconnected* (*disco*) mutants that have a severe defect in the optic lobe and are missing lateral neurons [60, 61]. Both locomotor activity and eclosion of the *disco* mutant are arrhythmic under DD [62]. While over 95% of *disco* mutants are arrhythmic in locomotor activity, rhythmic individuals emerge on rare occasions [60] with intact single or some lateral neurons [63]. These results indicate that arrhythmicity in the locomotor activity of *disco* mutants is due to the defective lateral neurons. In our study, *disco* mutants did not show the reduction of mating activity at CT12 as well as the results in per^{01} and tim^{01} flies [58]. Thus, lateral neurons seem to be essential for the circadian rhythm in mating activity of *Drosophila*.

3.4 Females are responsible for the mating activity rhythms in Drosophila.

In order to know whether or not the robust circadian rhythm in mating activity shown in the wild-type is specifically due to males, females or a combination of both sexes, normal females were paired with *disco* males and normal males, and *disco* females were paired with *disco* males and normal males. When normal females are paired with normal or *disco* flies, the mating activity rhythm of such pairs is clearly detected [58]. In contrast, when *disco* females were used for the crossing with normal or *disco* males, mating activity rhythms were completely abolished. It is indicated that females are responsible for the generation of the circadian rhythms in mating activity of *Drosophila*.

3.5. Species diversity of the mating activity rhythms in Drosophila

D. melanogaster and *D. simulans* are morphologically almost identical and genetically very similar and these two cosmopolitan species are sympatric [64, 65]. In LD cycles, the mating activities of *D. simu-*

lans at daytime (ZT0-9) and ZT12 were significantly higher than the other times (ZT15–18) (Fig. 1C), indicating that the mating activity of *D. simulans* is also subject to a daily rhythm, although the profile was distinct from that of *D. melanogaster* (Figs. 1A, C). In constant darkness, the mating activities of *D. simulans* gradually elevated after CT0 and those at CT6–15 were the highest of all (Fig. 1D). In LD cycles, the mating activity during the day was high in both *D. melanogaster* and *D. simulans* (Figs. 1A, 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) (Figs. 1A, C). In DD, the rhythm of mating activity between *D. melanogaster* and *D. simulans* was species-specific in an anti-phasic fashion (Figs. 1B, D). The mating activity rhythm of both strains of *D. simulans* in DD was obviously distinct from that in LD (Fig. 1C, D), suggesting that the mating activity of *D. simulans* is also controlled not only by an endogenous clock but also by light.

4. Mechanisms to oscillate the mating activity in *Drosophila*

Drosophila has two mechanisms to drive the mating activity; one is a mechanism controlled by a circadian pacemaker consisting of clock genes, and the other is a mechanism that light signal acts to affect the mating activity directly (Fig. 2). Mating activity rhythm in *D. melanogaster* females is under the restricted control of circadian clock genes, and the lateral neurons might be essential for the generation of the rhythm. Flies, especially males, utilize olfactory cues for mating [37, 41, 52] and the circadian rhythm of the olfactory response is robust in *Drosophila* [66]. Olfactory responses of the wild-type were elevated in the middle of the night in LD cycles [66], but mating activities were decreased during the early part of the night (Fig. 1B). Furthermore, the lateral neurons are insufficient to sustain olfactory rhythm [66], but the optic lobe including the lateral neurons seemed to be essential for mating activity rhythm. 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* [37, 41, 52], and the sound produced by male wing vibration, referred to as courtship song, affects female receptivity [43, 47, 52]. 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.

5. Species-specific circadian rhythms of *Drosophila* mating and reproductive isolation

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 [64, 65]. 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.

Two sibilng species in the Queensland fruit flies, *Dacus tryoni* and *D. neohumeralis*, show the difference in time of mating between these two species under LD cycles [24]. In wild gypsy moth, two closely related species, *Lymantria dispar* and *L. monacha*, show the different daily rhythm pattern of pheromone oriented flight activity [31]. Taken together, species-specific daily rhythms of mating behavior in insects may affect the reproductive isolation among sibling species, and the species-specific circadian rhythm controlled by clock genes in insects may lead to the species-specific daily pattern in the mating activity. The transformation experiments, which introduce per gene from other species to *D. melanogaster per^{01}* flies, showed the existence of DNA sequence for the species-specific features of locomotor activity rhythm or male courtship song rhythm in *Drosophila* [67–69]. Several clock genes including *per* and *tim* may also have DNA sequence for the species-specific circadian rhythms in female mating activity of *Drosophila*. Further molecular analyses will clarify the possibility.

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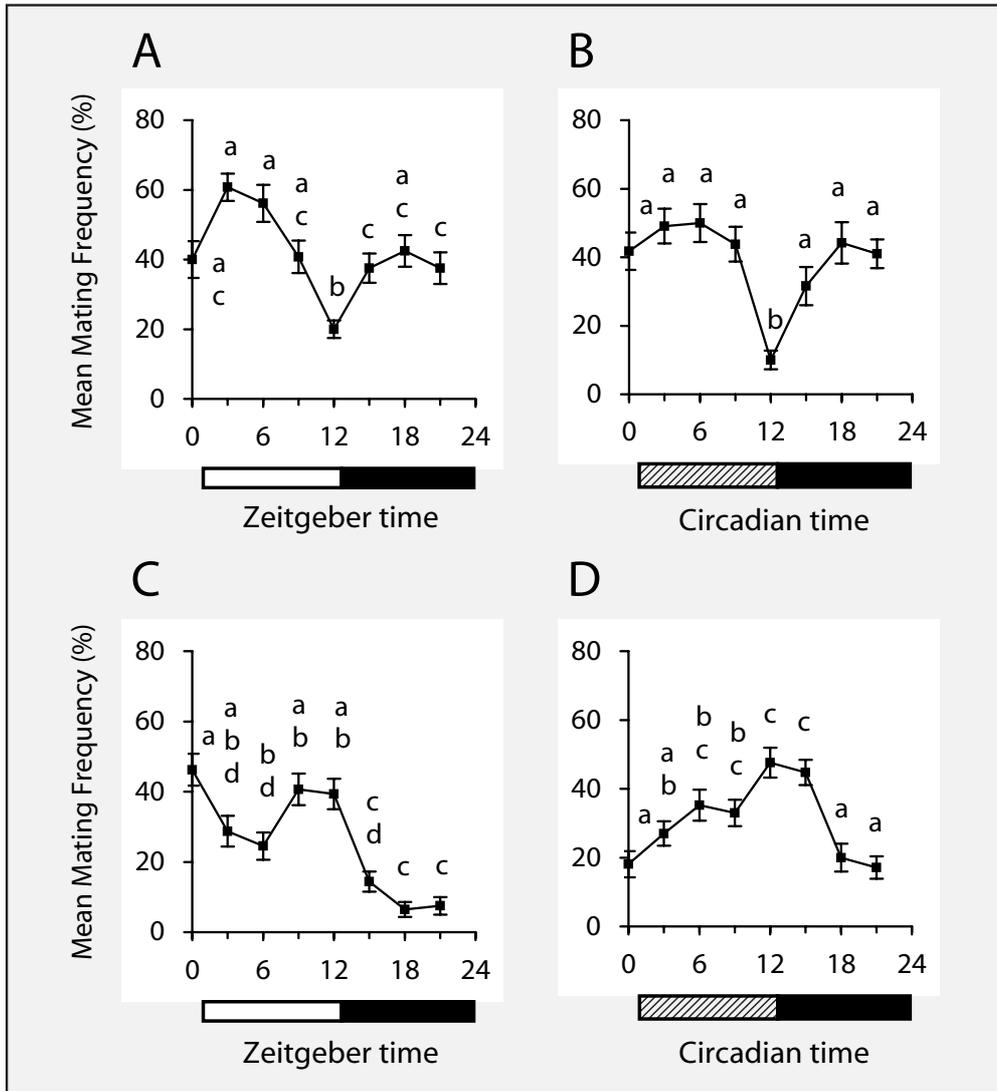
Fig 1. Mating activities at different times of day in D. melanogaster and D. simulans. Error bars showed the SEMs. The values with the same superscript letters were not significantly different by pair-wise comparison of the Mann-Whitney U test as multiple comparisons among every time points. All significant differences in the figures pass the sequential Bonferroni test. (A) Daily changes in mating activity of D. melanogaster Canton-S strain on day 9 under LD 12:12 cycles. (B) Circadian rhythms in mating activity of Canton-S on day 2 under DD. (C) Daily changes in mating activity of and D. simulans Og strain on day 9 under LD 12:12 cycles. (D) Circadian rhythms in mating activity of Og on day 2 of DD.
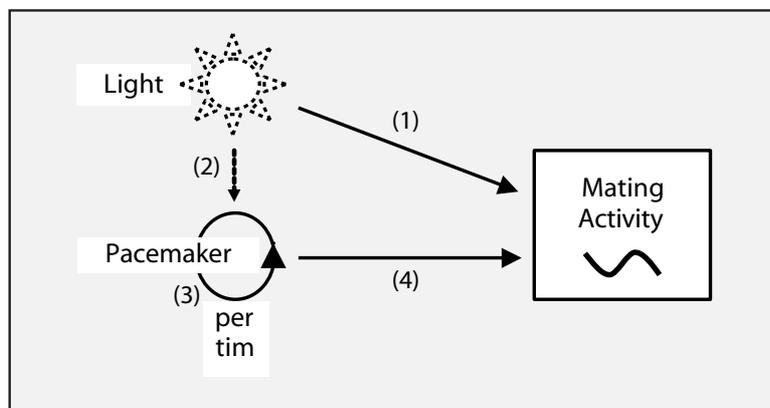
Fig 2. A model of daily fluctuation in mating activity of Drosophila. In this model, light acts to affect mating activity directly (1) or indirectly through the entrainment pathway (2). The clock pacemaker (3) and the output pathway (4) control the circadian rhythms in female mating activity.

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