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Speciation of Drosophila: A Role for Clock Genes in the Timing of Species-Specific Mating and Locomotor Behavior【Research report】

果蠅的種化：時鐘基因在種的特殊交尾及活動行為的時機角色【研究報告】

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Abstract

Species-specific timing of mating behavior is one of the important factors in speciation which is new barriers to gene exchange in evolution. We previously found that the circadian rhythms of female mating activity differ among various Drosophila species. Here, we found that the mating activity rhythm of D. ananassae differed from that of D. melanogaster.

To evaluate the effect of clock gene products on fly mating activities, we examined the mating activity of D. melanogaster tim0 (timeless null mutant) transgenic fly harboring the heatshock promotor driven-D. ananassae timeless gene (hs-AT tim0). The locomotor rhythm of hs-AT tim0 was changed from diurnal to nocturnal when the timing of D. ananassae TIMELESS induction changed to diurnal. The peak of mating activity of the hs-AT tim0 transgenic flies appeared after heatshock was applied; however, this rhythm could not be entrained by the timing of D. ananassae TIMELESS induction. These data indicate that timeless is not enough for the generation of species-specific mating rhythms and suggests that species-specific mating rhythms require more factors than those required for locomotor activity rhythm. A role of circadian clock genes in Drosophila speciation is discussed.

摘要

種的特殊交尾行為的時機是種化的重要因子之一，也是在演化中基因交換的新障礙。我們之前發現不同種果蠅雌蟲交尾的日週律動大不相同，此研究我們發現D. ananassae與D. melanogaster交尾行為的規律性是不同的。

評估時鐘基因產物對果蠅交尾活動的影響，我們用D. melnnogaster tim0 (無timeless基因的突變體) 又同時帶著來自D. ananassae熱感應促進子的轉殖果蠅檢驗交尾活性，當Timeless蛋白質的刺激發生在白天時，其活動規律性從日行性改變為夜行性。帶有hs-AT tim0果蠅的交尾高峰發生在給予熱處理後，然而這個規律性無法由D. ananassae Timeless蛋白質的刺激而產生導引作用，這些資料顯示timeless基因是不足以產生種的特殊交尾規律性，並推測種的特殊交尾規律性需要比活動規律性更多的因子參與。在果蠅的種化中日週律動時鐘基因的角色扮演，在本文中有討論。

Key words: Circadian, clock genes, mating, speciation, timeless

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Speciation of *Drosophila*: A Role for Clock Genes in the Timing of Species-Specific Mating and Locomotor Behavior

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ABSTRACT

Species-specific timing of mating behavior is one of the important factors in speciation which is new barriers to gene exchange in evolution. We previously found that the circadian rhythms of female mating activity differ among various *Drosophila* species. Here, we found that the mating activity rhythm of *D. ananassae* differed from that of *D. melanogaster*.

To evaluate the effect of clock gene products on fly mating activities, we examined the mating activity of *D. melanogaster tim*^{0} *(timeless null mutant)* transgenic fly harboring the heatshock promotor driven-*D. ananassae timeless* gene *(hs*-AT *tim*^{0}*)*. The locomotor rhythm of *hs*-AT *tim*^{0} was changed from diurnal to nocturnal when the timing of *D. ananassae* TIMELESS induction changed to diurnal. The peak of mating activity of the *hs*-AT *tim*^{0} transgenic flies appeared after heatshock was applied; however, this rhythm could not be entrained by the timing of *D. ananassae* TIMELESS induction. These data indicate that *timeless* is not enough for the generation of species-specific mating rhythms and suggests that species-specific mating rhythms require more factors than those required for locomotor activity rhythm. A role of circadian clock genes in *Drosophila* speciation is discussed.

Key words: Circadian, clock genes, mating, speciation, *timeless*

Introduction

The behaviors of most organisms are subject to rhythms that are controlled by an endogenous circadian clock (Dunlap, 1999; Sakai and Ishida, 2001). Clock genes including *period* (*per*), *timeless* (*tim*), *clock* (clk), and *cycle* (cyc) and their products constitute the core of the circadian mechanism. The sexual receptivity and reproductive behaviors of insects, for example courtship songs, and mating and

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ovipositor activities, are related to circadian mechanisms (Hardeland, 1972; Kyriacou and Hall, 1980; Sakai and Ishida, 2001; Sheeba *et al.*, 2001; Tanber *et al.*, 2001). The locomotor activities of virgin queen ants are rhythmic whereas those of mated queens become arrhythmic when they lay eggs, but rhythmicity is restored after the eggs are deposited (Sharma *et al.*, 2004). Clock genes of the melon fly may cause reproductive isolation through a change in the time of mating (Miyatake *et al.*, 2002).

The rhythms of *Drosophila* mating behaviors are controlled by circadian clock genes and are especially attributed to the female clock (Sakai and Ishida, 2001). Female circadian rhythms in mating activity are also species-specific, and this might constitute one source of reproductive isolation that allows *Drosophila* to avoid sympatric hybridization. The mating behavior rhythms of *D. melanogaster* and *D. simulans* are different and antiphase (Sakai and Ishida, 2001).

We also reported that the *timeless* gene product is highly conserved between *D. melanogaster* and *D. ananassae*. *Timeless* cDNA of *D. ananassae* rescues the arrhythmic locomotor induction or activity of the *D. melanogaster timeless* null mutant, tim^{01} (Nishinokubi *et al.*, 2003).

In the present study, we examine whether the mating activity rhythm of the *D. melanogaster* tim^{01} mutant can also be rescued by introducing the *D. ananassae tim* gene. We also determined whether the locomotor behavior rhythm of *D. melanogaster* tim^{01} transgenic flies carrying the *tim* gene from *D. ananassae* is entrained by the timing of induction for the TIMELESS protein.

Materials and Methods

***Animals*:** Flies grown on glucose-molasses-yeast-cornmeal were maintained at 25 ± 0.5°C under a 12-h light-dark (LD) cycle of lights on at 09:00 and lights off at 21:00. Transformant flies carrying *D. ananassae tim* cDNA were generated using P-element-mediated methods as described by Nishinokubi *et al.* (2003).

***Mating activity assays*:** Flies maintained as described above for 7 days after eclosion were transferred to constant dark (DD) conditions for 2 days at 25°C. Transformant flies were heat-shocked for 30 min circadian time ((CT) 10.5-11.0) at 37°C every day. We then analyzed the mating frequency of 9-day-old adult flies that were allowed to mate for 20 min as described by (Sakai *et al.* 1997; Sakai and Ishida, 2001).

***Locomotor assays*:** We tracked the movements of flies that were individually housed with medium, using infrared sensors and a *Drosophila* activity monitor (Trikinetics, Waltham, MA) placed in an incubator under DD at 25 ± 0.5°C except when heat shock was applied (37°C for 30 min at either CT 10.5-11.0 or CT 22.5-23.0 daily). Signals from the sensors were summed every 30 min using a computer.

***Western blotting*:** Flies were entrained at 25 ± 0.5°C under LD conditions, then transferred to DD for 2 days. Fly heads were collected on dry ice every 3 h and Western blotted as described by Nishinokubi *et al.* (2003).

Results

We examined whether the expression of the *D. ananassae* TIMELESS protein can entrain the locomotor rhythm of *D. melanogaster* tim^{01} flies. We determined the locomotor activity rhythms of heat-shocked transgenic tim^{01} flies carrying *D. ananassae tim* cDNA. The transgenic flies heat-shocked for 30 min at CT 10.5-11.0 every day under DD conditions became rhythmic and moved about during the subjective day (Nishinokubi *et al.*, 2003) (Fig. 1). On the other hand, flies that were heat-shocked at CT 22.5-23.0 became active during the subjective night (Fig. 1). Heat shock initially increased

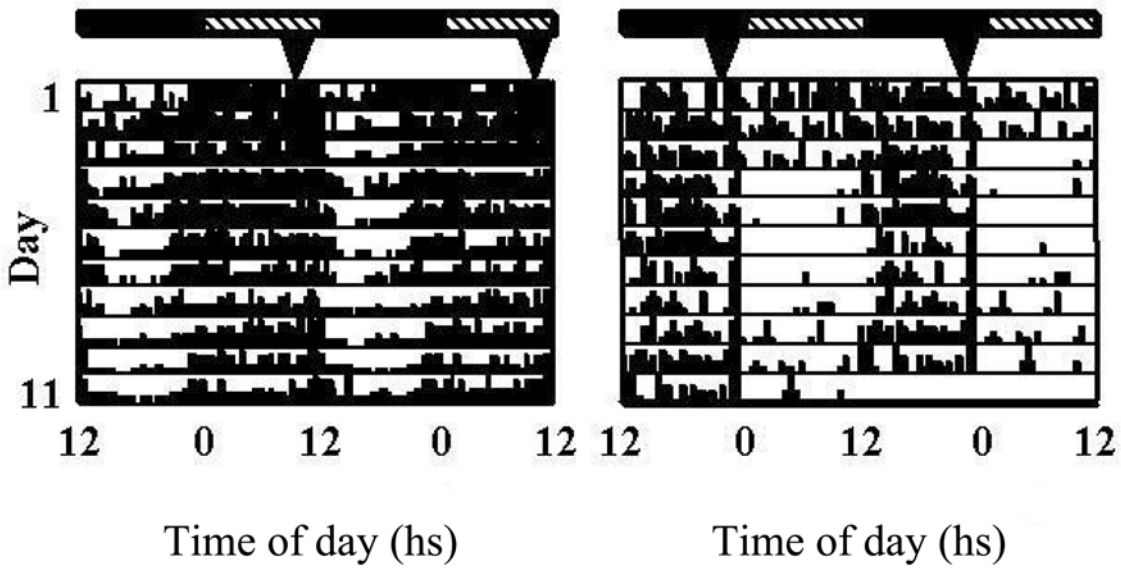
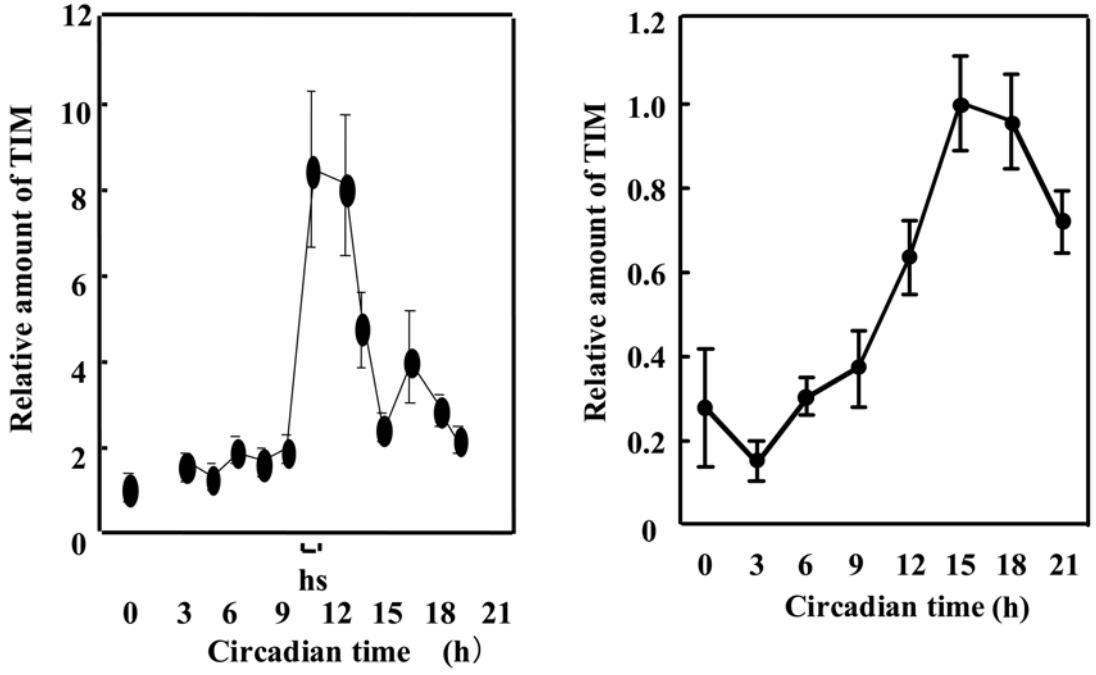


Fig. 1. Rescue of locomotor activity rhythms in *Drosophila melanogaster tim*^{01} mutants by *D. ananassae tim* cDNA. Left and right panels, representative double-plot actgrams of locomotor activities of transgenic flies that were heat- shocked at CT 10.5-11.0 (mimicking the wild-type) and CT 22.5-23.0, respectively. Black and shaded bars show the subjective night and day under DD (constant dark) conditions. Heat shock was applied for 30 min at 37°C at the indicated time every day. Arrowheads indicate the start point of heat shock on the first day.

levels of TIM protein, which decreased thereafter. Heat shock applied at CT 10.5-11.0 and CT 22.5-23.0 induced high TIM levels during the respective subjective night and day, respectively (Fig. 2a; data not shown). These findings indicate that the locomotor behavior of *D. melanogaster tim*^{01} flies harboring hs-*D. ananassae timeless* became nocturnal from diurnal to the timing of *D. annassae* TIM induction.

The mating activities of *D. melanogaster* circadian clock mutants are arrhythmic, indicating that circadian clock genes control the rhythm of mating activity (Sakai and Ishida, 2001). To understand the mating rhythm of *D. ananassae*, we determined the mating frequency at different times of the day. The mating rhythm of *D. ananassae* under DD conditions differed from the reported rhythms of *D. melanogaster* and *D. simulans* (Fig. 3; Sakai and Ishida, 2001). Most *D. ananassae* flies mated during the subjective day rather than during the subjective night (Fig. 3). We then measured mating activity in transgenic *tim*^{01} flies carrying *D. ananassae tim* cDNA to determine whether mating activity could be rescued and whether it is related to levels of the circadian gene product, TIMELESS, as is locomotor activity. The TIM protein levels in *D. ananassae* wild-type flies were initially higher during the subjective night than during the subjective day like those of *D. melanogaster*, although their mating activity peaks clearly differed (Figs. 2b, 3a). Thus, we exposed the transgenic flies to heat shock at CT 10.5-11.0 every day to imitate the TIM cycle of *D. ananassae* wild-type flies and then measured their mating activities. The mating activities of control, heat-shocked, and non-shocked



(a) (b)

Fig. 2. TIM protein levels in flies under DD (constant dark) conditions. (a) *Drosophila ananassae* TIM induced by heat shock at CT 10.5-11.0. in *w*; *tim*^{01}; hs-*D. ananassae tim* flies. Heat shock at 37°C for 30 min rapidly induced TIM. hs appearing under the graph indicates the time when heat shock was applied. (b) *Drosophila ananassae* TIM protein profile during constant dark in wild-type flies.

D. melanogaster wild-type Canton-S flies were similarly rhythmic (data not shown). This result indicated that heat shock itself did not significantly affect the mating activity rhythms of *D. melanogaster* wild-type Canton-S. The mating activities of heat-shocked transgenic *tim*^{01} flies were rhythmic and higher during the subjective night than during the subjective day. The profile of this rhythm differed from both *D. ananassae,* which was the source of the *tim* cDNA, and the background *D. melanogaster* (Fig. 3b). These data suggest that recovery of species-specific mating rhythms requires more-complicated pathways from that observed for locomotor rhythms.

Discussion

We earlier reported that the mating activity of *D. melanogaster tim*^{01} mutant flies is arrhythmic, like locomotor activity (Sakai and Ishida, 2001). The present study showed that the mating activities of *D. melanogaster tim*^{01} flies harboring *D. ananassae tim* cDNA had mating activity rhythm, but the profile differed from these of both *D. melanogaster* and *D. ananassae*. This rhythm was not bimodal as observed in *D. melanogaster,* and its peak was largely delayed relative to that of *D. ananassae* (Fig. 3). The circadian clock gene, *period*, plays a role in the mating rhythms of flies (Tauber *et al.*, 2003). The mating peak of the *D.*

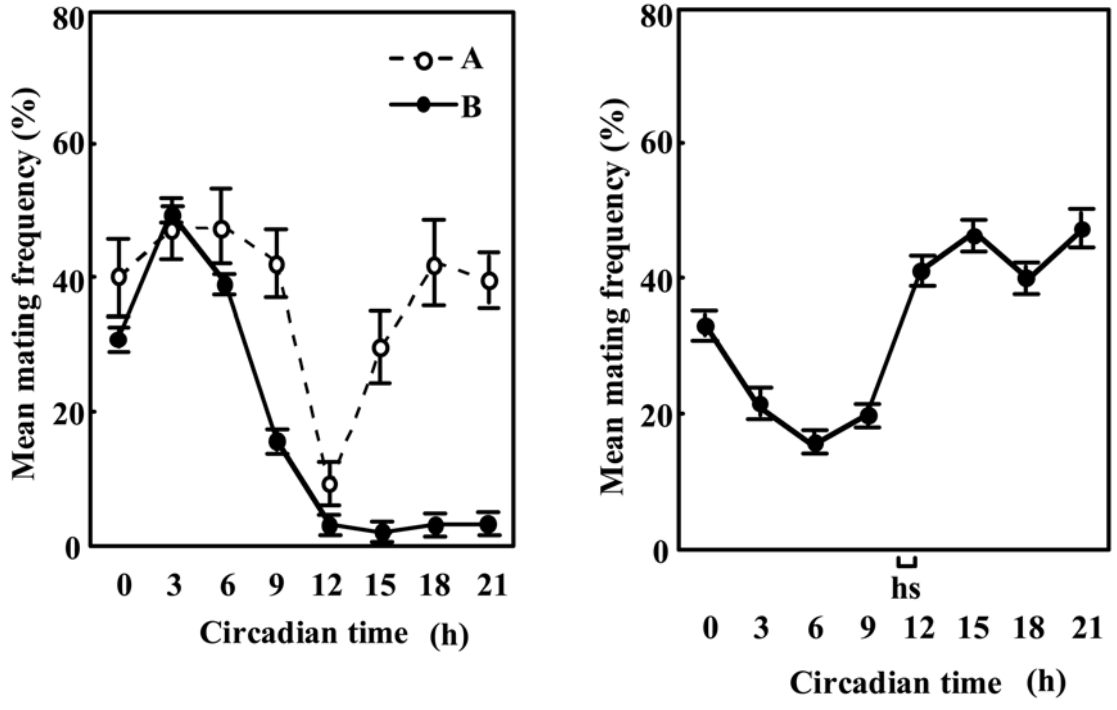


Fig. 3. Mating activities of *Drosophila* flies at different times of the day. Error bars indicate the SEM. Males and females were allowed to mate for 20 min. (a) Mating activities of *D. melanogaster* Canton-S (A) and in *D. ananassae* HW (B) strains. (b) Mating activities in transgenic *w; tim*^{01}*; hs-D. ananassae tim* flies that were heat-shocked every day at CT10.5-11. hs appearing under the graph indicates the time when heat shock was applied.

pseudoobscura transformant line that expresses *D. pseudoobscura per* fused to the *D. melanogaster per* promoter was later than that of *D. pseudoobscura* (Tauber *et al.*, 2003). These results support the notion that *period* genes play a role in temporal reproductive isolation between populations of closely related species (Tauber *et al.*, 2003) or within a species (Miyatake *et al.*, 2002) by changing the timing of mating behavior. The present data suggest that *tim* might also be a putative speciation gene like *period*.

Male courtship songs differ among *Drosophila* species, and this contributes to sympatric speciation (Kyriacou *et al.*, 1989). Females discriminate *D. ananassae* and its sibling species, *D. pallidosa*, according to male courtship songs, and the loci of sexual isolation appear to map near the Delta locus of the second chromosome (Doi *et al.*, 2001). *Drosophila melanogaster* and *D. simulans* are sibling and sympatric species that have not been isolated by pre-mating. However, their mating behavioral rhythms are antiphase. During frequent *D. melanogaster* mating, the ratio of *D. simulans* mating is lower, but this increases when *D. melanogaster* mates infrequently (Sakai and Ishida, 2001). The mating behavioral rhythm of

D. ananassae differs from these of *D. melanogaster* and *D. simulans* (Fig. 3a, Sakai and Ishida, 2001; Tauver *et al.*, 2003). Our data support the notion that the species-specific timing of mating behavior as well as differences in male courtship songs play roles in reproductive isolation for sympatric speciation.

The present study found that the locomotor activity rhythm of *tim* null mutants carrying *D. ananassae tim* cDNA could be entrained by changing the timing of TIM induction by using heat shock (Fig. 1). The locomotor activity level of *D. melanogaster* is higher during the subjective day, while the TIM level is higher during the subjective night (Yang and Sehgal, 2001). Our transformant flies were similarly active during the subjective day when heat shock was applied to increase TIM levels during the subjective night. On the contrary, when flies were heat-shocked to increase TIM levels during the subjective day, their activity levels were higher during the subjective night (Figs. 1, 2a). These results demonstrate a close correlation between the phases of the locomotor activity rhythm and the timing of TIM induction. On the other hand, the phase of the mating activity rhythm does not appear to be closely correlated with that of the timing of TIM induction, because the mating peaks of our transgenic (Fig. 3b) and wild-type (Fig. 3a) flies differed when we imitated the timing of wild type TIM induction (Fig. 2). Our data suggest that the rhythms of locomotor activity and mating behavior have different output pathways underlying the central circadian system. Tauber *et al.* (2003) also suggested that periods of locomotor activity are not causally related to mating behavior, although both rhythms might be manifested from the same central oscillator. Thus, we propose that the pathways of mating activity rhythms are more molecularly complex compared to those of locomotor activity rhythms.

Locomotor activity is related to mating or sexual receptivity in many insects including *Drosophila* (Ringo, 1996). For example, virgin ant queens have a circadian locomotor activity rhythm, whereas mated queens laying eggs do not, and their activity levels are much lower than those of virgin females. Mated queens that have stopped laying eggs resume circadian locomotor rhythm (Sharma *et al.*, 2004). Female German cockroaches that display higher locomotor activity are sexually receptive and such activity is reduced after mating, indicating that the female locomotor activity is primarily associated with finding a mate. Moreover, locomotor activity and sexual receptivity appear to be controlled by the same mechanisms in which the juvenile hormone is a major factor. However, mating can affect the frequency of locomotor activity but does not cause females to exhibit locomotor rhythm (Lin and Lee, 1998). Most investigations that have addressed a relationship between locomotor activity and mating or sexual activity have been conducted after the flies have mated (post-mating). However, considering these findings, further study is required to clarify the pre-mating mechanism, including the mating behavioral rhythm, in order to clarify the molecular mechanism of speciation.

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果蠅的種化：時鐘基因在種的特殊交尾及活動行爲的時機角色

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關鍵詞：日週律、時鐘基因、交尾、種化、*timeless* 基因。