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The *period* gene and allochronic reproductive isolation in *Bactrocera cucurbitae*

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Clock genes that pleiotropically control circadian rhythm and the time of mating may cause allochronic reproductive isolation in the melon fly *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae). Flies with a shorter circadian period (*ca.* 22 h of locomotor activity rhythm) mated 5 h earlier in the day than those with a longer circadian period (*ca.* 30 h). Mate-choice tests demonstrated significant pre-mating isolation between populations with short and long circadian periods. Pre-mating isolation did not occur when the mating time was synchronized between the two populations by photoperiodic controls, indicating that reproductive isolation is due to variations in the time of mating and not any unidentified ethological difference between the two populations. We cloned the *period* (*per*) gene of *B. cucurbitae* that is homologous to the *per* gene in *Drosophila*. The relative level of *per* mRNA in the melon fly exhibited a robust daily fluctuation under light : dark conditions. The fluctuation of *per* expression under dark : dark conditions is closely correlated to the locomotor rhythm in *B. cucurbitae*. These results suggest that clock genes can cause reproductive isolation via the pleiotropic effect as a change of mating time.

Keywords: *Bactrocera cucurbitae*; circadian rhythm; mating behaviour; melon fly; *per*; speciation

1. INTRODUCTION

Many evolutionary biologists have focused on sympatric speciation (e.g. Bush 1994; Via 2001; Berlocher & Feder 2002) following Guy Bush's eminent study of speciation taking place without any geographical barriers in a tephritid fly (Bush 1969). One condition that promotes non-allopatric speciation is the reproduction time lag between populations, i.e. allochronic speciation. There are many reports regarding different reproduction timing that prevents gene flow between populations in several different taxonomic organisms (Palumbi 1994; Petit *et al.* 1997). A difference in mating time within a day may cause pre-mating isolation between populations in a species or in closely related species, particularly in insects (e.g. Linsley & MacSwain 1958; Lewontin & Birch 1966; Konno & Tanaka 1996). However, no study has provided experimental evidence for reproductive isolation in such cases, or clarified the gene that causes reproductive isolation via the difference in mating time.

Various behavioural events are controlled by an endogenous clock in insects and exhibit circadian rhythmicity (Saunders 1978). For example, clock genes control mating activity in female *Drosophila melanogaster* (Sakai & Ishida 2001). Many genes implicated in various steps of the circadian clock have been found and studied in *D. melanogaster* (Dunlap 1999; Giebultowicz 2000). These findings in *Drosophila* raise the question of whether the function of homologous genes is also involved in other insects and has any link to pre-mating isolation. The time of mating may be controlled by circadian rhythm, and thus a study of the relationships between clock genes and pre-mating isolation via mating time could clarify the evolutionary mechanism of allochronic speciation.

The melon fly *Bactrocera cucurbitae* (Coquillett) provides a good system for examining the link between mating time and pre-mating isolation because it mates only once a day (Suzuki & Koyama 1980). Copulation begins at dusk and terminates at dawn (Kuba & Soemori 1988). Copulation usually continues for more than 10 h and genital contact is maintained during this period (Yamagishi & Tsubaki 1990). Thus, it is easy to verify the time of mating for this fly. Methods for analysing the length of circadian rhythm, i.e. the free-running period, have been described previously (Shimizu *et al.* 1997).

We demonstrate that pre-mating isolation occurs between fly populations in *B. cucurbitae*, each with a short or a long circadian period. The pre-mating isolation disappears when the time of mating is synchronized for the two populations by photoperiodic controls, indicating that pre-mating isolation is caused only by the difference in mating time. Our molecular analyses indicated that the relative level of *per* mRNA exhibits a circadian rhythmicity. These results suggest that a clock gene might be involved in allochronic pre-mating isolation in *B. cucurbitae*. We also discuss the possibility that temporal shifts in mating propensity based on circadian clocks may underlie the pre-mating reproductive isolation observed in many other species.

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2. MATERIAL AND METHODS

(a) *Insects*

Miyatake (1995) selected and established two lines of *B. cucurbitae* with short (S-line) and long (L-line) development times. The base population for the selection was a mass-reared strain that had been maintained for 41 generations in the Okinawa Prefectural Fruit Fly Eradication Project Office, Okinawa, Japan, according to the method described by Nakamori *et al.* (1992). The time of mating in a day differed between the two lines; the S-flies always mated at an earlier time than did the L-flies (Miyatake 1997). The range of mating time was greater in L-flies than in S-flies (Miyatake 1997), probably because both lines were selected for development time, not for mating time, under mass rearing, and thus the genes controlling circadian rhythm did not fix in the L-flies. We conducted sib mating within each S- and L-line for four generations to obtain fly populations with a more restricted mating-time range. We first randomly selected more than 48 flies from the S- and L-lines and measured their free-running period using the actograph, as described in Shimizu *et al.* (1997). Sib mating was then conducted between flies with a free-running period of less than 22 h in the S-line (hereafter referred to as the S-population) and between flies with a free-running period of greater than 29 h in the L-line (hereafter referred to as the L-population). Individual rearing of flies during the sib mating was conducted according to the method described in Miyatake (1998). The flies in the fifth generation of sib mating were used for the following experiments.

(b) *Measurement of mating time*

The time of mating (the time when copulation begins) was examined in S- and L-populations. Adult flies that emerged on the same day were sexed within three days of emergence. Males and females were kept separately in cages (20 cm × 20 cm × 30 cm) in a laboratory at 25 ± 1 °C under a photoperiod of 14 L : 10 D (hereafter referred to as LD). The time of mating was examined 25 days after adult emergence. A male and a female fly were released into a transparent plastic cup (80 mm diameter, 40 mm height) in which food and water were provided. One hundred pairs were set up for S- and L-populations. The flies were released into the cup from 8 h before 'lights off', and mating pairs were counted for 14 h at 30 min intervals from 4 h before lights off. Observations were performed under a red light during dark periods.

(c) *Pre-mating isolation*

Multiple mate-choice tests and male mate-choice tests were performed for reciprocal combinations of S- and L-populations. Adults in each population that emerged on the same day were sexed within three days of emergence. Males and females were held separately in cages (20 cm × 20 cm × 30 cm) at 25 ± 1 °C. The mating time was synchronized between S- and L-populations by photoperiod control to examine whether pre-mating isolation is due only to variations in the mating time. Two groups, designated as 'synchronized' and 'control', were prepared for S- and L-populations. The protocols of the photoperiod controls for the two groups are given in figure 1. Both groups were reared under the same LD photoperiod, but each had a different lights-off time. Both populations in the control were reared under the same photoperiod. However, the time of lights-off for the synchronized group was set 5 h earlier for the L-population than the S-population due to the 5 h difference in mating time between the two populations (see figure 2).

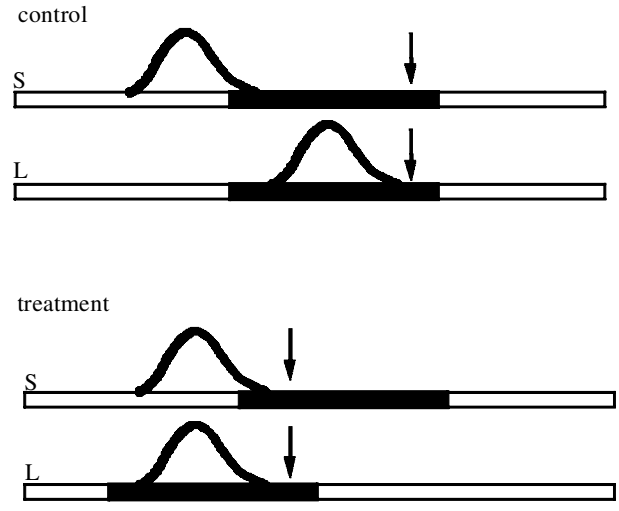

Figure 1. Protocols of photoperiodic controls for the mate-choice tests. Horizontal open bar indicates the photophase; closed bar indicates the scotophase.

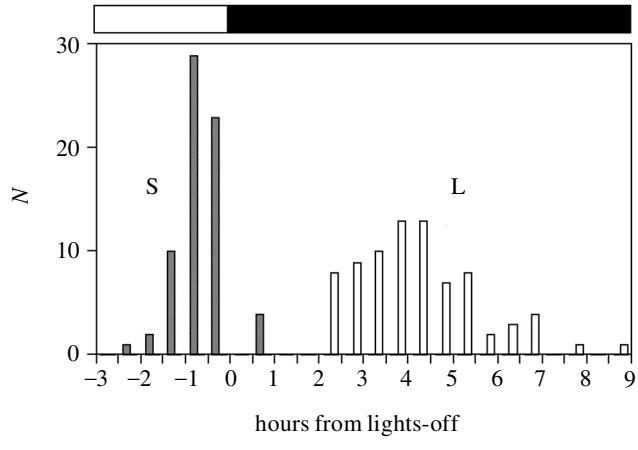

Figure 2. Frequency distributions of mating times for S- and L-populations.

Multiple and male mate-choice tests were conducted *ca.* 25 days after adult emergence. A total of 20 flies consisting of 5 virgin females and 5 virgin males from both S- and L-populations for multiple mate-choice tests, and a total of 15 flies consisting of 5 virgin females from both S- and L-populations and 5 virgin males from S- or L-populations for male mate-choice tests were released into a cage (30 cm × 30 cm × 45 cm) with water and food for 9 h before lights-off time. Mating pairs were counted from 9 h after the time of lights-off using a red flashlight. Eight replicates (cages) were prepared for each combination. Flies from each population in four replicates were marked with a spot of quick-drying paint (white: Magic Opaque colour, Teranishi-kagaku-kogyo, Osaka, Japan) on their pronotum to identify the population. Flies from the other populations in the other four replicates were marked in a similar way. Thus, the effect of marking on mating could be accounted for in each test.

The pre-mating isolation for multiple mate-choice tests was estimated by calculating Stalker's joint isolation index (Stalker 1942):

$$I = [(n_{11} + n_{22}) - (n_{12} + n_{21})]/N, \quad (2.1)$$

where n_{11} and n_{22} are the number of homogamic matings, n_{12}

and n_{21} are the number of heterogamic matings between females of the first population and males of the second population and vice versa, and N is the total number of observed copulations. The index was calculated as follows for male mate-choice tests:

$$I = [(n_{11}) - (n_{12})]/N. \quad (2.2)$$

Malagolowkin-Cohen *et al.* (1965) provided the standard error of I:

$$\text{s.e.}\ (I) = [(1 - I^2)/N]^{1/2}, \quad (2.3)$$

where I ranges from -1 to 1, a value of zero indicates random mating, $I > 0$ indicates positive assortative mating, and $I < 0$ indicates negative assortative mating. Contingency χ^2-tests were performed to check for deviations from random mating. Eight replicates were pooled for the tests.

(d) *Locomotor activity rhythm*

Locomotor activities of the flies were measured as follows. Adults were kept singly in containers (36 mm diameter, 66 mm height) and provided with water and sugar. Their locomotor activities were monitored by interruptions from an infrared beam and a photoelectric switch (OMRON, Tokyo). The interruption signals were sent to a computer (NEC, Tokyo), and the numbers recorded at 6 min intervals (see Shimizu *et al.* (1997) for details of the monitoring system). The free-running period was calculated by the least-square spectrum and the χ^2-periodogram softwares (Chiba & Takahashi 1991) from free-running data for 10 days in continuous darkness (DD) after five days of entrainment to LD. The adult age at the transfer from LD to DD varied from two to three weeks. The means were only used for values where the difference between measures calculated by a χ^2-periodogram and the least-square spectrum was within 30 min, to minimize measuring errors. In addition, only samples with highly significant ($p < 0.01$) values from the χ^2-periodogram were used for the analysis.

We examined the parental flies and the first (F_1) and second (F_2) generations after crossing between the S- and L-populations. The free-running rhythms were also recorded in the reciprocal crosses S × L (female × male) and L × S. S × L and L × S flies in the F_2 generation were inbred and designated as SL × SL and LS × LS.

A total of 348 out of 648 flies examined (42 F_0, 138 F_1 and 164 F_2 flies) exhibited free-running rhythms. The remaining 304 flies died during the experiments or did not show a statistically significant free-running period.

(e) *Partial cloning of* per *cDNA*

Total RNAs of the heads from the S-population were extracted by a Fast RNA kit (Bio101), and the first-strand cDNAs were synthesized by a Ready-to-Go T-primed first-strand kit (Amersham Bioscience). A putative partial cDNA fragment of *per* in *B. cucurbitae* was amplified using degenerate primers 5′-TCAGAATTCTGYGTNATHKSNATGCAYGA-3′ and 5′-TTCAAGCTTRTTRTARTTNARYTGRTTRTA-3′, which were used to amplify the *per* gene in *Cecropia* moth (Reppert *et al.* 1994). The fragment was cloned into the pCRII vector (Invitrogen) and sequenced by a PRISM 3400 sequencer (Applied Biosystems). The 3′RACE was performed with the newly synthesized complete matching primer 5′-CCGTTCTGTGTGATGTTACGTCG-3′ and the *NotI*-$d(T)_{18}$ primer (Amersham Bioscience). The 3 kb fragment thus obtained was cloned into pCRII and partially sequenced.

(f) *Measurements of circadian fluctuation of* per *mRNA abundance*

S- and L-flies were obtained every 4 h in LD followed by two successive days of DD. Two series of total RNA samples, each of which was extracted by a Fast RNA kit (Bio101) from five fly heads, were obtained in both S- and L-populations. The abundance of *per* mRNA was measured by Q-PCR with a PRISM 7700 (Applied Biosystems), as described by Ueda *et al.* (2002) with a minor modification. We used the primer set for the *per* gene of *B. cucurbitae*, 5′-GTCGGGACATTAAG TATGCCAAAA-3′ and 5′-CCTAGACCATCGACGAAAC CAG-3′. A relative abundance of *per* mRNA to *Gpdh* mRNA in each sample was thus obtained.

3. RESULTS

(a) *Mating time*

A majority of flies from the S-population mated before lights-off, whereas L-flies mated after dark (figure 2). Each population had a complete and discrete frequency distribution in mating time. The difference in the time mean of mating was *ca.* 5 h; the mean ± s.d. hours of mating from lights-off were -0.29 ± 0.32 ($n = 70$) for the S-population and $+4.21 \pm 1.29$ ($n = 81$) for the L-population.

(b) *Pre-mating isolation*

Results of the multiple and male mate-choice tests are shown in table 1. Significant positive assortative mating between S- and L-populations was observed when they were reared under the same light conditions (controls). This was reproduced in two replications of the two types of mate-choice test. We observed random matings between the two populations when the flies were synchronized to the same regime of LD cycles in two replications of both types of mate-choice test. These results indicate that pre-mating isolation is only due to the time of mating.

(c) *Locomotor activity rhythm*

The distributions of the free-running periods of flies obtained from crosses between S- and L-populations are shown in figure 3. The period of the S-population was significantly shorter than that of the L-population, and there was no difference between sexes (figure 3*a*; the means and s.d. of free-running period (tau) were 22.55 ± 0.34 in males and 22.10 ± 0.59 in females of the S-population, and 30.04 ± 1.14 in males and 29.62 ± 1.40 in females of the L-population; two-way ANOVA, $F_{1,38} = 664.08$, $p < 0.0001$ for the populations, and $F_{1,38} = 2.28$, $p = 0.1391$ for the sexes).

Free-running periods in F_1 populations exhibited a unimodal distribution in reciprocal crosses (figure 3*b*,*c*). There was no difference in the free-running periods of F_1 populations between reciprocal crosses (two-way ANOVA, $F_{1,134} = 0.06$, $p = 0.8026$). There was no difference in the males between the reciprocal crosses ($t_{75} = -1.056$, $p = 0.2942$), indicating that the gene responsible is not on the sex chromosome. No free-running periods longer than 29 h or shorter than 22 h were observed in F_1 hybrids, whereas they were observed in F_2 (figure 3*d*). These results suggest the presence of a major gene that controls the length of the locomotor activity rhythm.

Table 1. Results of mate-choice tests and isolation indices *I*.

type of mate-choice test	replicate[a]	experiment	number of matings[b] SS	LS	SL	LL	$I \pm$ s.e.	χ^2
multiple	1	synchronized	16	17	7	14	0.111 ± 0.135	0.667
		control	25	3	1	6	$0.771 \pm 0.108^{***}$	20.829
	2	synchronized	13	19	8	12	-0.038 ± 0.139	0.077
		control	17	5	0	7	$0.655 \pm 0.140^{***}$	12.448
male	3	synchronized	19	15	—	—	0.118 ± 0.170	0.471
		control	23	9	—	—	$0.438 \pm 0.159^{*}$	6.125
	4	synchronized	—	—	13	16	0.103 ± 0.185	0.310
		control	—	—	3	18	$0.714 \pm 0.153^{**}$	10.714

[a] Marked on S-flies for replicate 1, marked on L-flies for replicate 2, marked on S-males for replicate 3, and marked on L-males for replicate 4.
[b] Female : male.
$^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

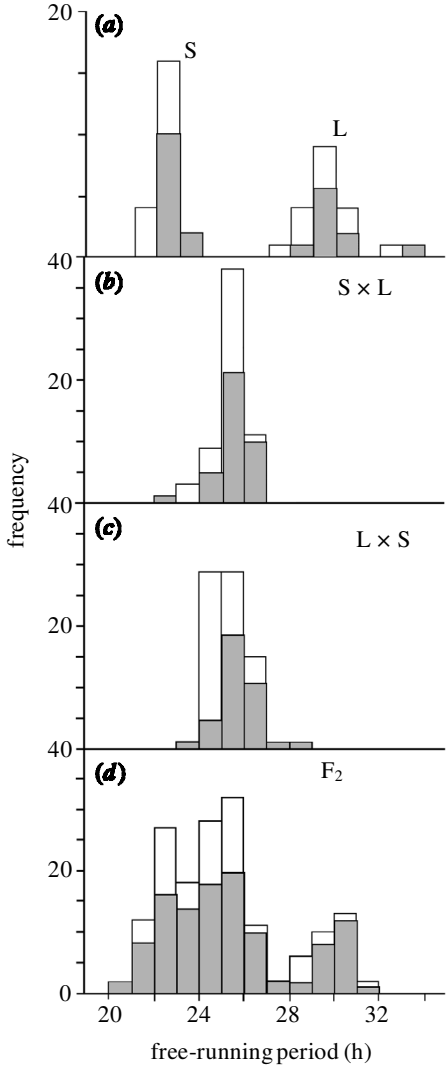

Figure 3. Periods of the free-running rhythm in (*a*) S- and L-populations and (*b*,*c*) their hybrids. (*b*,*c*) Males (shaded bars) and females (open bars) are shown separately in the F_1 hybrids. (*d*) SL × SL (open bars); LS × LS (shaded bars).

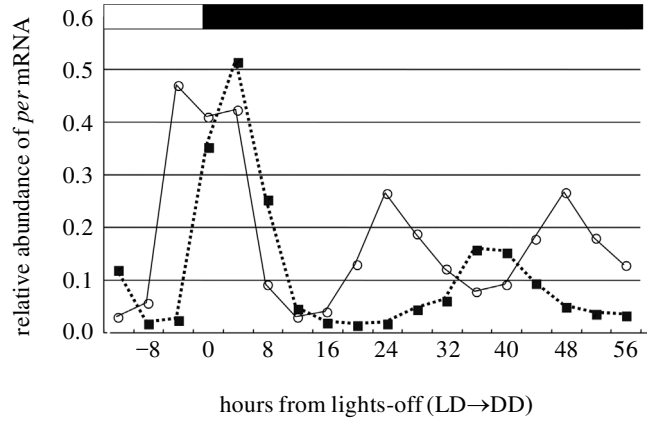

Figure 4. Relative *per* mRNA levels in S-population (solid line: 22 h) and L-population (dotted line: 30 h) examined at 4 h intervals under DD after LD conditions. The mean values of two measurements are shown for the relative abundance of mRNA. Horizontal open bar indicates the photophase; closed bar indicates the scotophase.

(d) *Circadian rhythm of per mRNA level*

The relative level of *per* mRNA in both S- and L-populations exhibited a robust daily fluctuation under LD (figure 4). The peak phases were at dusk in both populations, although the level of the former began to elevate at an earlier phase than the latter. A difference in the phase of *per* mRNA cycling was also observed among *per* mutants of *Drosophila* (Hardin *et al.* 1990). The fluctuation of *per* mRNA was free running in both populations in DD, whereas the peak levels became lower than in LD (figure 4). The S-flies exhibited two peaks of *per* abundance in 48 h under DD. The calculated period of the peak duration in DD was 22 h. Only one peak was observed in the L-flies in the first two days under DD. The period was calculated as 30 h based on the duration between the peaks in LD to DD. These periods correspond well to those of the locomotor activity rhythm in both populations.

4. DISCUSSION

Mate-choice tests between the two populations with short and long periods indicated significant pre-mating isolation. The pre-mating isolation disappeared when the time of mating was synchronized between the populations by photoperiodic controls. This indicates that the pre-mating isolation in *B. cucurbitae* is due to a variation in the time of mating and not due to any unidentified ethological difference between the two populations, at least in laboratories. Males of *B. cucurbitae* form a lek under natural conditions, and females visit the lek to copulate (Iwahashi & Majima 1986). Further pre-mating isolation may occur, if the time when females visit the lek differs from the time that males form the lek. Therefore, pre-mating isolation between populations is probably stronger in the field than in the laboratory, if flies of populations that differ in the time of female acceptance visit leks at different times.

Circadian rhythms are measured primarily by monitoring the locomotor activity in *Drosophila*, but other behavioural processes might also be under circadian control. In addition, life-history traits may be under such control, as reported in the changes in the developmental speed of *per* mutants of *Drosophila* (Kyriacou *et al.* 1990). Many other physiological and behavioural events change in the same way, if we assume that a central circadian clock governs the oscillation of events in an organism (Kyriacou & Hall 1980; Pittendrigh 1981; Matsumoto *et al.* 1994; Wong *et al.* 1995). Sakai & Ishida (2001) recently reported that the *per* gene controls the time of mating, as well as the locomotor activity, in female *D. melanogaster* .

A number of genes implicated in various steps of the circadian clock have been discovered and studied in *D. melanogaster* (Dunlap 1999; Giebultowicz 2000; Panda *et al.* 2002). Timing mechanisms in all living organisms appear to involve molecular feedback loops such that the transcriptions of clock genes are inhibited by their own products. This negative feedback loop involves several genes acting together as part of a clock (Glossop *et al.* 1999). We examined the relative level of *per* mRNA in both S- and L-populations that exhibited robust daily fluctuation under LD. The periods of *per* mRNA cycling under DD correspond well to those of locomotor rhythm in S- and L-populations. This suggests that the fluctuation of *per* expression is closely linked to the locomotor rhythm in *B. cucurbitae*, although further studies are necessary to determine the molecular difference in clock genes between the two populations. The difference may be in the *per* gene itself, or another clock gene may be involved.

Several tephritid species mate at particular times of day (Smith 1989), and the differences in mating times between populations have important ecological consequences. For example, the only known barrier to hybridization between *B. tryoni* and *B. neohumeralis* is the time of mating. The former mates at dusk; the latter mates during the daytime (Lewontin & Birch 1966). The mating time in *Anastrepha* tephritid flies may also be a reproductive barrier among three sympatric species in South America (Malavasi *et al.* 1983). A similar relationship has been reported in other tephritid flies (Selivon & Morgante 1997). The tephritid species are thus suitable animals for studies of how mating time is controlled by a circadian clock and for finding a link between clock genes and speciation.

Reproductive isolation may occur due to the difference in mating time based on the following three scenarios. First, the time of mating could be fixed in a small population by random genetic drift, thus pre-mating isolation may develop among populations with different mating times. Second, the time of mating may become a target for direct natural selection due to predation pressures. For example, most tephritid fruitflies have lek mating systems in which males aggregate for mating at a particular time of day (Shelly & Whittier 1997). If lekking flies are attacked by predators such as wasps (Hendrichs *et al.* 1994) at a particular time, then natural selection would favour a specific mating time. Natural selection acts directly on circadian mechanisms in this case. Third, pleiotropic effects can cause reproductive isolation through adaptive radiation for life-history traits (Miyatake & Shimizu 1999). Lines of the melon fly that were selected for short and long developmental periods produced a shift in mating time during the evening that resulted in significant pre-mating isolation in mate-choice tests (Miyatake & Shimizu 1999). Many tephritid fruitflies have a wide range of host plants (White & Elson-Harris 1992). A life-history trait such as development time can become a target for natural selection if a fly population develops a new host plant because of a change in its environment (Feder 1998). An intriguing possibility is that ecological adaptive radiation could have important ramifications that cause speciation via a by-product of genes with pleiotropic effects.

In addition to tephritid flies, reproductive isolation between intra- or interspecific populations may be caused by the difference in the egg spawning cycle of sea urchins (Lessios 1984; Palumbi 1994), in the flowering time of plants (Petit *et al.* 1997), and in the daily mating time of some moths (Konno *et al.* 1981; Konno & Tanaka 1996) and bees (Koeniger *et al.* 1996; Linsley & MacSwain 1958). Intensive studies of the relationship between clock genes and pre-mating isolation in these organisms may provide intriguing and novel results in speciation fields.

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REFERENCES

Berlocher, S. H. & Feder, J. L. 2002 Sympatric speciation in phytophagous insects: moving beyond controversy? *A. Rev. Entomol.* **47**, 773–815.

Bush, G. L. 1969 Sympatric host race formation and speciation in frugivorous flies of the genus *Rhagoletis*. *Evolution* **23**, 237–251.

Bush, G. L. 1994 Sympatric speciation in animals: new wine in old bottles. *Trends Ecol. Evol.* **9**, 285–288.

Chiba, Y. & Takahashi, K. 1991 *Chronobiology handbook* (in Japanese). Tokyo: Asakura shoten.

Dunlap, J. C. 1999 Molecular bases for circadian clocks. *Cell* **96**, 271–290.

Feder, J. L. 1998 The apple maggot fly, *Rhagoletis pomonella*: flies in the face of conventional wisdom about speciation? In

Endless forms: species and speciation (ed. D. J. Howard & S. H. Berlocher), pp. 130–144. Oxford University Press.

Giebultowicz, J. M. 2000 Molecular mechanism and cellular distribution of insect circadian clocks. *A. Rev. Entomol.* **45**, 769–793.

Glossop, N. R. J., Lyons, L. C. & Hardin, P. E. 1999 Interlocked feedback loops within the *Drosophila* circadian oscillator. *Science* **286**, 766–768.

Hardin, P. E., Hall, J. C. & Rosbash, M. 1990 Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. *Nature* **343**, 536–540.

Hendrichs, J., Katsoyannos, B. I., Wornoayporn, V. & Hendrichs, M. A. 1994 Odour-mediated foraging by yellowjacket wasps (Hymenoptera: Vespidae): predation on leks of pheromone-calling Mediterranean fruit fly males (Diptera: Tephritidae). *Oecologia* **99**, 88–94.

Iwahashi, O. & Majima, T. 1986 Lek formation and male–male competition in the melon fly, *Dacus cucurbitae* Coquillett (Diptera: Tephritidae). *Appl. Entomol. Zool.* **21**, 70–75.

Koeniger, N., Koeniger, G., Gries, M., Tingek, S. & Kelitu, A. 1996 Reproductive isolation of *Apis nuluensis* Tingek, Koeniger and Koeniger, 1996 by species-specific mating time. *Apidologie* **27**, 353–359.

Konno, Y. & Tanaka, F. 1996 Mating time of the rice-feeding and water-oat-feeding strains of the rice stem borer, *Chilo suppressalis* (Walker) (Lepidoptera: Pyralidae). *Jpn. J. Appl. Entomol. Zool.* **40**, 245–247.

Konno, Y., Honda, H. & Matsumoto, Y. 1981 Mechanisms of reproductive isolation between the fruit-feeding and the Pinaceae-feeding types of the yellow peach moth, *Dichocrocis punctiferalis* Guenee (Lepidoptera: Pyralidae). *Jpn. J. Appl. Entomol. Zool.* **25**, 253–258.

Kuba, H. & Soemori, H. 1988 Characteristics of copulation duration, hatchability of eggs and remating intervals in the melon fly, *Dacus cucurbitae* Coquillett (Diptera: Tephritidae). *Jpn. J. Appl. Entomol. Zool.* **32**, 321–324.

Kyriacou, C. P. & Hall, J. C. 1980 Circadian rhythm mutations in *Drosophila melanogaster* affect short-term fluctuations in the male's courtship song. *Proc. Natl Acad. Sci. USA* **77**, 6729–6733.

Kyriacou, C. P., Oldroyd, M., Wood, J., Sharp, M. & Hill, M. 1990 Clock mutations alter developmental timing in *Drosophila*. *Heredity* **64**, 395–401.

Lessios, H. A. 1984 Possible prezygotic reproductive isolation in sea urchins separated by the Isthmus of Panama. *Evolution* **38**, 1122–1148.

Lewontin, R. C. & Birch, L. C. 1966 Hybridization as a source of variation for adaptation to a new environment. *Evolution* **20**, 315–336.

Linsley, E. G. & MacSwain, J. W. 1958 The significance of floral constancy among bees of the genus *Diadasia* (Hymenoptera, Anthophoridae). *Evolution* **12**, 219–223.

Malagolowkin-Cohen, C. H., Simons, A. S. & Levene, H. 1965 A study of sexual isolation between certain strains of *Drosophila paulistrum*. *Evolution* **19**, 95–103.

Malavasi, A., Morgante, J. S. & Prokopy, R. J. 1983 Distribution and activities of *Anastrepha frateculus* (Diptera: Tephritidae) flies on host and nonhost trees. *Ann. Entomol. Soc. Am.* **76**, 286–292.

Matsumoto, A., Motoshige, T., Murata, T., Tomioka, K., Tanimura, T. & Chiba, Y. 1994 Chronobiological analysis of a new clock mutant, *Toki*, in *Drosophila melanogaster*. *J. Neurogenet.* **9**, 141–155.

Miyatake, T. 1995 Two-way artificial selection for developmental period in *Bactrocera cucurbitae* (Diptera: Tephritidae). *Ann. Entomol. Soc. Am.* **88**, 848–855.

Miyatake, T. 1997 Correlated responses to selection for developmental period in *Bactrocera cucurbitae* (Diptera: Tephritidae): time of mating and daily activity rhythms. *Behav. Genet.* **27**, 489–498.

Miyatake, T. 1998 Genetic variation in pre-mating period of the mass-reared melon fly, *Bactrocera cucurbitae* (Diptera: Tephritidae). *Appl. Entomol. Zool.* **33**, 29–33.

Miyatake, T. & Shimizu, T. 1999 Genetic correlations between life history and behavioral traits can cause reproductive isolation. *Evolution* **53**, 201–208.

Nakamori, H., Kakinohana, H. & Yamagishi, M. 1992 Automated mass production system for fruit flies based on the melon fly, *Dacus cucurbitae* Coquillett (Diptera: Tephritidae). In *Advances in insect rearing for research and pest management* (ed. T. E. Anderson & N. C. Leppla), pp. 441–454. Boulder, CO: Westview.

Palumbi, S. R. 1994 Genetic divergence, reproductive isolation and marine speciation. *A. Rev. Ecol. Syst.* **25**, 547–572.

Panda, S., Hogenesch, J. B. & Kay, S. A. 2002 Circadian rhythms from flies to human. *Nature* **417**, 329–335.

Petit, C., Lesbros, P., Ge, X. & Thompson, J. D. 1997 Variation in flowering phenology and selfing rate across a contact zone between diploid and tetraploid *Arrhenatherum elatius* (Poaceae). *Heredity* **79**, 31–40.

Pittendrigh, C. S. 1981 Circadian systems: entrainment. In *Handbook of behavioral neurobiology*, vol. 4 (ed. J. Aschoff), pp. 95–124. New York: Plenum.

Reppert, S. M., Tsai, T., Roca, A. L. & Sauman, I. 1994 Cloning of a structural and functional homolog of the circadian clock gene *period* from the giant silkmoth *Antheraea pernyi*. *Neuron* **13**, 1167–1176.

Sakai, T. & Ishida, N. 2001 Circadian rhythms of female mating activity governed by clock genes in *Drosophila*. *Proc. Natl Acad. Sci. USA* **98**, 9221–9225.

Saunders, D. S. 1978 *Insect clocks*, 2nd edn. Oxford: Pergamon.

Selivon, D. & Morgante, J. S. 1997 Reproductive isolation between *Anastrepha bistrigata* and *A. striata* (Diptera, Tephritidae). *Brazil. J. Genet.* **20**, 583–585.

Shelly, T. E. & Whittier, T. S. 1997 Lek behavior of insects. In *The evolution of mating systems in insects and arachnids* (ed. J. C. Choe & B. J. Crespi), pp. 273–293. Cambridge University Press.

Shimizu, T., Miyatake, T., Watari, Y. & Arai, T. 1997 A gene pleiotropically controlling developmental and circadian periods in the melon fly, *Bactrocera cucurbitae* (Diptera: Tephritidae). *Heredity* **79**, 600–605.

Smith, P. H. 1989 Behavioural partitioning of the day and circadian rhythmicity. In *Fruit flies: their biology, natural enemies and control*, vol. 3B (ed. A. S. Robinson & G. Hooper), pp. 325–341. Oxford: Elsevier.

Stalker, H. D. 1942 Sexual isolation studies in the species complex *Drosophila virilis*. *Genetics* **27**, 238–257.

Suzuki, Y. & Koyama, J. 1980 Temporal aspects of mating behavior of the melon fly, *Dacus cucurbitae* Coquillett (Diptera: Tephritidae): a comparison between laboratory and wild strains. *Appl. Entomol. Zool.* **15**, 215–224.

Ueda, H. R., Matsumoto, A., Kawamura, M., Iino, M., Tanimura, T. & Hashimoto, S. 2002 Genome-wide transcriptional orchestration of circadian rhythms in *Drosophila*. *J. Biol. Chem.* **277**, 14 048–14 052.

Yamagishi, M. & Tsubaki, Y. 1990 Copulation duration and sperm transfer in the melon fly, *Dacus cucurbitae* Coquillett (Diptera: Tephritidae). *Appl. Entomol. Zool.* **25**, 517–519.

Via, S. 2001 Sympatric speciation in animals: the ugly duckling grows up. *Trends Ecol. Evol.* **16**, 381–390.

White, I. M. & Elson-Harris, M. M. 1992 *Fruit flies of economic significance: their identification and bionomics*. Oxon: CAB International.

Wong, A., Boutis, P. & Hekimi, S. 1995 Mutations in the *clk-1* gene of *Caenorhabditis elegans* affect developmental and behavioral timing. *Genetics* **139**, 1247–1259.