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Plausible link between circabidian activity rhythms and circadian-clock systems in the large black chafer *Holotrichia parallela*

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Summary Statement

The large black chafer *Holotrichia parallela* has an endogenous circa”bi”dian rhythm, which is seemingly produced by the circadian clock system. The two-day periodicity appears both in the laboratory and field.

Abstract

Two-day rhythms referred to as circabidian rhythms were reported in humans and mosquitos. However, these rhythms only appear under constant conditions, and functional mechanisms of two-day rhythms have not been known. Here, we report clear circabidian rhythms of large black chafers (*Holotrichia parallela*, Coleoptera: Scarabaeidae) in both the laboratory and field. Under 12 h light and 12 h dark conditions (LD 12:12) at 25°C, *H. parallela* appeared on the ground at the beginning of the dark phase every two days. Under constant darkness, *H. parallela* exhibited free-running with a period of 47.9 ± 0.3 h, suggesting the existence of a clear circabidian rhythm entrained to two LD 12:12 cycles. Phase responses of the circabidian rhythm to light pulses occurred under constant darkness in a phase dependent manner. Phase responses suggest that there are two circadian cycles, each consisted of a less responsive and high responsive period, in a circabidian oscillation, and the circabidian rhythm is driven by the circadian clock. A mark and recapture study showed that beetles repeatedly appeared on the same tree approximately every two days. However, the periodicity was not as rigid as that observed under laboratory conditions in that individuals often switched appearance days. For instance, large precipitation made the two-day rhythm shift the phase by a half cycle of the rhythm at a time. Here, we propose a novel function of the circadian clock characterized by the release of an output

signal every two cycles to produce the two-day rhythm.

Introduction

Various environmental factors that are associated with physical and biological features change periodically on the Earth, and organisms have evolved endogenous rhythms with periods that approximate environmental cycles. Many organisms have circadian rhythms driven by the circadian clock with a period of about a day (Dunlap et al., 1999). The suppression of activities during an appropriate time of a day by the circadian clock is an adaptive behavior associated with escape from predation and other environmental dangers (DeCoursey et al., 1997, 2000). Significant deviation from cyclical periods of activity for 24 h decreases both survivorship and reproduction rates (Spoelstra et al., 2016). Endogenous rhythms with periods similar to other environmental cycles such as tidal, lunar, and yearly cycles have also been progressively studied (Numata and Helm, 2014; Kaiser et al., 2016). Therefore, the use of an endogenous rhythm with a cycle that corresponds to environmental changes likely allows organisms to appropriately anticipate and prepare their physiological states.

However, rhythmicity associated with cycles that inconsistently approximate cyclical environmental changes has been reported. Two-day rhythms referred to as circa“bi”dian rhythms were recorded in humans and mosquitos. For instance, Kleitman (1963) successfully developed 48-h oral temperature rhythms by forcing 48-h sleep-wakefulness routines. In an underground bunker, ~50-h activity cycles were observed in an isolated male subject exposed to constant light conditions, but a cyclical period of a body temperature rhythm was ~25 h (Aschoff et al., 1967). In the mosquito *Culiseta incidens*, a ~46-h flight rhythm was observed in constant dark conditions (Clopton,

1984, 1985). However, occurrences of circabidian rhythms in these two species were infrequent and observed only when organisms were kept for long periods under constant conditions without time cues, or when they were forced to entrain to two-day cycles (Czeisler et al., 1980; Clopton, 1985). Therefore, it is not known whether circabidian rhythms actually occur in nature.

In the large black chafer *Holotrichia parallela* (Coleoptera: Scarabaeidae; previously called *Lachnosterna morosa*), a serious agriculture pest in East Asia, adult populations appear on the ground every two days (Yoshioka and Yamazaki, 1983). Measurements of pheromone titers in the pheromone glands of field-collected females showed that a peak occurred every two days (Leal et al., 1993). However, this periodicity has only been described in groups, and individual periodicity and the mechanisms behind the rhythm are not known. In contrast to humans and mosquitos, *H. parallela* populations seem to have two-day periodicity in the field. If the two-day periodicity of this species is formed by an endogenous free-running circabidian rhythm under constant conditions, *H. parallela* represents a good model that could aid our understanding of how an enigmatic rhythm with a period different from an environmental cycle is assembled by a clock mechanism and how it actually works in the field.

In this study, we chronobiologically examined the individual ground emergence rhythms in field-collected *H. parallela*, and found that this species has a very stable circabidian rhythm under constant darkness. Furthermore, the rhythm was perfectly entrained to every two cycles of 12 h light and 12 h darkness. We also examined phase responses to light pulses to suggest that the circabidian rhythm is determined by a circadian clock. Finally, we did mark and recapture studies to demonstrate that the two-

day periodicity was not as rigid as that observed under solitary laboratory conditions, and the beetles switched the day of appearance in the field. The circadian clock-driven mechanisms might contribute to a half-cycle temporal shift of the circabidian rhythm.

Materials and Methods

Insects. Adult large black chafers, *H. parallela*, were collected on the riverbeds of the Yamato River (34° 35′ 14″ N, 135° 30′ 16″ E) in July of 2012 (Fig. 1) and the riverbed of the Yodo River (34° 43′ 41″ N, 135° 31′ 31″ E) June to August of 2012–2015 (Fig. 2 and 3).

Recording of activity rhythm. The beetles were individually kept in a polystyrene cylindrical container (11.5 cm height, 7 cm diameter). Two-thirds of the container was filled with culture soil (Sakata Seed Corporation, Yokohama City), and the side wall of each container was covered with aluminum foil to prevent light exposure to beetles under the soil surface. Activities of the beetles on the ground were individually recorded under 12 h light and 12 h darkness (LD 12:12) for 10 days and subsequent DD for 10 days at 25 ± 1°C. The light source was fluorescent light and the intensity was 1.75 W/m^2. During the recording, a leaf of the Japanese cherry *Prunus yedoensis* 'Somei-yoshino' was provided as food every five days or once at the beginning of the experiment, and approximately 25 mL of water was sprayed on the soil surface when it dried. The top of the container was covered with a transparent glass plate. A color image of the surface of the soil was taken every 6 min with a web camera (DC-NCR13U, HANWHA Japan, Tokyo). According to differences in the total pixel values between 2 serial photos, beetle movements were detected and plotted on a double-plotted

actogram, and the free-running period was determined using a chi-square periodogram (Enright, 1965; Sokolove and Bushell, 1978).

Total pixel values were calculated per photo, and the value difference, $w\,(x, y)$, was calculated as

$$w(x,y) = \sqrt{\sum_x \sum_y \{(R_n(x,y) - R_{n-1}(x,y))^2 + (G_n(x,y) - G_{n-1}(x,y))^2 + (B_n(x,y) - B_{n-1}(x,y))^2\}}$$

where x, y represents pixel coordinates, $R_n(x,y)$ represents red values per pixel, $G_n(x,y)$ represents green values per pixel, and $B_n(x,y)$ represents blue values per pixel. We also calculated the threshold value of w based on the noise amounts from all recorded photos for each beetle. When a w value was above the threshold, the value indicated that the beetle had moved since the previous photo was taken, and a score of "1" was plotted for that time. When a w value was below the threshold, a score "0" was given. Activities occurring over a 48-h period were plotted on a double-plotted actogram.

To examine the biological clock cycle driving the circabidian rhythm, the phase responses of the rhythm to light pulses were examined under constant darkness (DD). We used light-penetrated sponges instead of soil. Water was added to the sponge through a cotton thread from a water tank set below the container. A cherry leaf was given as food, and the leaf was replaced two times during the recording period in most cases. In other cases, the leaf was only provided at the beginning. After activity recordings were conducted for 9 to 10 days under DD, a 3 h-light pulse was provided two times every 48 h at different phases of the circabidian time (CbT), and activities were recorded for eight to 10 days under DD. The light intensity measured inside of the sponge pile was 0.626 W/m^2.

An actogram was drawn as the soil activity recordings were conducted. We set the activity onset as a phase reference point of CbT = 36. A computer program was made to determine the activity onset phase as follows: 1) We first searched for the most active 18-h time zone over 48 h, starting at 10:00 Japan standard time (JST). Total activity scores were calculated, and ranged from 0–180 for each 18-h time zone. The starting time of each 18-h time zone was delayed by 1 h, and the first zone started at 10:00 Japan standard time (JST) and ended at 18:00 JST, the second one was 11:00–19:00 JST, and so on. Time zones with the highest scores were selected. If two or more high zones were present, the earlier zone was selected. 2) Of the high zone, the first appearance of 15 continuous-activities was selected, and the starting time (JST) of the appearance was designated as the activity onset point. The activity onset was determined every 48 h starting at 10:00 JST. 3) We then evaluated the activity onset point. There were five activity onset points at maximum before and after the light pulse. Median of activity onsets before and after the light pulses were calculated for each beetle, and activity onset times that differed from the median by 6 h or more were regarded as outliers. Of the 48-h cycle with outlier points, the first appearance of 15 continuous activities was searched again during a period of the median ± 6 h, and the starting time was set as the revised activity onset points. 4) To determine the CbT of the activity onset, a line with inclination of τ from the chi-square periodogram was drawn at the position where the sum of the squared deviations of the activity onset from the line was lowest. Based on τ and the position of the line, we calculated the CbT of the activity onset, and the difference between the activity onset phase was calculated in CbT between the period before and after the light pulse.

Study sites. Field observations were conducted at the riverbed of Yamato River in Osaka (supprelemtaFig. S1). Temperature ranges of the field during the year were -2.4 to 36.7°C in 2011 and -3.1 to 37.9°C in 2012.

Mark and recapture. The mark and recapture study was conducted on tree E (*Ulmus parvifolia*, 2.7 m) in 2011, and trees A (*U. parvifolia*, 3.4 m) and E in 2012. The distance between trees A and E was 87 m (Fig. S1). We checked the presence of beetles on the trees from May 3 to Sep 8 in 2011 and from May 30 to Sept 26 in 2012. During the observation period, the observer went to the trees at sunset every day with a lantern, and *H. parallela* aggregating on the tree were collected using an insect net and a stepladder. An individual three-digit number was engraved on the elytra of each beetle, and the number of newly captured (unmarked) beetles and marked beetles were recorded separately every night. Beetles aggregating on 10 trees, designated as A–J in Fig. S1, were counted at sunset July 28–29 in 2011 with the help of five observers. Beetles on the tree were collected, and were immediately released after the marked number on the elytra was check and counting of unmarked beetles was conducted.

Beetle collection with pheromone traps. The sex pheromone of *H. parallela,* which is composed of two components, including a major component of L-isoleucine methyl ester and a minor component, linalool, was prepared (Leal et al., 1992). Ammonia was added to L-isoleucine methyl ester hydrochloride ($C_7H_{15}NO_2 \cdot HCl$; CAS no. 18598-74-8, Wako Pure Chemical Industries, Ltd., Osaka, Japan) to obtain L-isoleucine methyl ester, and L-isoleucine methyl ester and linalool ($C_{10}H_{18}O$; CAS no. 78-70-6, Nacalai Tesque, Inc., Kyoto, Japan) were mixed at a 4:1 ratio. The pheromone solution (100 µL)

was placed in a 500-μL plastic tube, and a piece of filter paper was used as the pheromone source. A 1.5-L plastic bottle was set up under the pheromone tube as a nonlethal trap to collect male adults. The pheromone trap was hung on tree A. From June 17 to October 8 in 2010, the number of *H. parallela* captured in the trap and the number of beetles aggregating on the tree A were examined every eight days except for August 27-28. The pheromone for this trap was renewed at 12:00 JST every day. The number was counted every hour from 6:00 JST until 6:00 JST of the following day, and collected beetles in the trap were immediately released after counting was completed. Most beetles were alive, and the number of beetles aggregated on tree A was counted by eyesight and categorized into the following three groups: –, n = 0; +, n = 1–9; ++, n = 10 or more.

Statistical tests. *t*-test (one-sided test), Man-Whitney U-test, chi square test, chi-square goodness of fit test, two-way ANOVA were conducted by using R software (Ihaka and Gentleman, 1996; http://cran.r-project.org) with an additional package ”twoway.anova” (http://aoki2.si.gunma-u.ac.jp/R/src/twoway_anova.R). For detection of the rhythmicity the chi-square periodogram analysis was performed ($P < 0.05$), and a period with the peak value of the variance in the analysis was determined as the rhythm period (Enright, 1965; Sokolove and Bushell, 1978).

Results

Activity rhythm. Under LD 12:12 conditions, male and female beetles appeared on the ground at the beginning of the dark phase every two days (Fig. 1). We occasionally observed that the beetles appeared just below the soil surface and remained there until

the light was turned off. After the light was turned off, the beetles then emerged onto the soil, and were observed feeding and walking. The duration of the appearance on the ground was 6.3 ± 1.6 h (mean ± S.D., n = 10), and the beetles mostly stayed underground for the rest of the day and all of the following day. In the container, the beetles usually stayed about 5 cm below the soil surface during the rest phase. Even under DD, their emergence rhythm continued, and the free-running period was 47.9 ± 0.3 h (n = 7) in males and 47.6 ± 0.3 h (n = 3) in females. *H. parallela* exhibited a clear endogenous rhythm with a period of approximately 48 h under DD, and it entrained to two cycles of LD 12:12 (Fig. 1).

Phase responses of the circabidian rhythm to light pulses. Because the free-running period before the light pulse did not differ significantly between females (47.6 ± 0.3 h, n = 28) and males (47.8 ± 0.5 h, n = 36, *t*-test, $P > 0.05$), we plotted the phase shift values of females and males together (Fig. 2). In 61 of the 65 beetles (1 individual unsexed), the circabidian rhythm continued after the light pulses, and the free-running period was 47.7 ± 0.4 h and 47.7 ± 0.6 h before and after the light pulse, respectively (paired sample *t*-test, $P > 0.05$, n = 61). However, the activity onset phase was advanced, delayed, or unchanged depending on the pulse phase (Fig. 2A–F). We calculated each phase shift value in circabidian time (CbT) and plotted the shift value for the phase at which the light pulse was given (Fig. 3A). The CbT is a time scale covering one full circabidian period (~48 h) during an oscillation under DD, and we set the activity onset phase at 36 h in CbT. To examine the dependency of shift values on circabidian phases, we divided a 48-h cycle in CbT into eight periods (t_1–t_8) that were 6 h each to compare phase shift values between consecutive 2 periods (Fig. 3A). Significant differences were

detected between the phase shift values of t_3 (median = -1.6 h) and t_4 (median = 1.25 h, Man-Whitney U-test, $P = 0.004$), between t_6 (median = 0.4 h) and t_7 (median = -2.6 h, U-test, $P = 0.002$), and between t_7 and t_8 (median = -0.4 h, U-test, $P = 0.046$). At the beginning of t_8, it appeared that the phase shift direction changed from delayed to advanced. In contrast, shift values were small, and a significant difference was not observed between t_1 (median = 0.3 h) and t_2 (median = -1.9 h, U-test, $P = 0.112$), between t_2 and t_3 (U-test, $P = 1.000$), between t_4 and t_5 (median = 0.4 h, U-test, $P = 0.262$), and between t_5 and t_6 (median = 0.4 h, U-test, $P = 0.800$). If the circadian clock cycles twice in a 48 h CbT, change of phase shift values hypothetically occurs between t_3 and t_4, and between t_7 and t_8. Our data agree to this. Less responsive periods to light theoretically occur $t_{1\text{-}2}$ and $t_{5\text{-}6}$, and significant differences were not detected in these periods. No significant difference between t_2 and t_3 and between t_4 and t_5 suggests that phase shift values in $t_{3\text{-}4}$ is weaker than in $t_{7\text{-}8}$ because during $t_{3\text{-}4}$ *H. parallela* do not receive light under soil and their sensitivity might be weakened. When phase shift values in the first and second 24 h CbT period was superimposed, phase shift direction appeared similar between the first and second cycles (Fig. 3B). When the 24-h time frame of the superimposed plot was divided into 4 periods (T_1-T_4), significant differences were not detected between T_1 (median = 0.2 h) and T_2 (median = -0.7 h, U-test, $P = 0.413$), but detected between T_2 and T_3 (median = -1.8 h, U-test, $P = 0.006$), and between T_3 and T_4 (median = 0.4 h, U-test, $P = 0.002$).

Circadian-like activity rhythms were observed after the light pulse in four of the 65 beetles (Fig. 2G, Fig. S2). This suggests that *H. parallela* exhibits an oscillator with a cycle of about 24 h. Because the circadian-like rhythm was observed in response to light pulses given at a variety of phases (5.0, 8.0, 15.5, or 43.0 h in CbT), no specific phase

seemed to change from a circabidian to a circadian rhythm.

Appearance times in the field. To understand the emergence times in the field, we counted the number of male *H. parallela* collected using a pheromone trap set on tree A (Chinese elm *Ulmus parvifolia*) every hour for 24 h (Fig. S1). Male beetles were mostly trapped within a few hours after sunset from June to October (Fig. 4). A large number of *H. parallela* were trapped from June 25–26 to August 12–13, and the appearances continued until October 7–8. With the exception of trapped males, *H. parallela* (males and females) appeared on the tree on which the trap was set. These beetles stayed on the tree throughout the night, and mating was often observed within 1 h of sunset. *H. parallela* stayed on the tree throughout the night, and their appearance and disappearance was mostly synchronized with sunset and sunrise, respectively (Fig. 4).

Individual appearance rhythms on the tree. To examine the individual rhythms in the field, we conducted a mark and recapture study on tree E in 2011 and on trees A and E in 2012 (Fig. S1). The first appearance dates of *H. parallela* were June 3 in 2011 and June 4 in 2012. We set June 3 as a reference date, and named it "the first day" in both 2011 and 2012. Days of emergence were distinguished between odd days and even days. The 1st (June 3), 3rd (June 5), 5th (June 7), 7th (June 9), and following days were designated as "odd days" and the 2nd (June 4), 4th (June 6), 6th (June 8), 8th (June 10), and following days were the "even days" for both years. Beetles were marked at the first appearance. Beetles marked on the odd day were categorized into "the odd-day marked group," and those marked on the even day were placed into "the even-day marked group."

In 2011 on tree E, the numbers of the even- and odd-day marked group did not differ significantly (Table 1). The rate of beetles recaptured on the marked tree two times or more was 26.1%, and the number was 37. Twenty-four of 37 beetles appeared only on an even number of days, counted from the first appearance (the marked day), during the observation period. For example, male specimen no. 67 appeared 2, 4, 8, 10, 14, 16, 18, and 22 days after the marked day (Fig. 5). The other 13 beetles reappeared on an odd number of days, counted from the first appearance. Female specimen no. 2 reappeared on 2, 4, 6, 8, 9, 13, 15, 17, and additional days after the first appearance (Fig. 5). We named the latter behavior "temporal switching", and the occurrence of temporal switching between the odd-day marked and even-day marked groups did not differ significantly in 2011 (Table 2). Of the 13 beetles, nine, three, and one beetles switched the appearance once, twice, and four times, respectively (Fig. S3). The last appearance day in 2011 was September 1.

In 2012, the last appearance day was September 19 on trees A and E. More beetles were found on the larger tree A than tree E, but the rate of beetles recaptured two times or more did not differ significantly between trees A and E (Table 1, chi square test, $\chi^2_{(2, n = 269)} = 0.178$, $P > 0.05$). In contrast to the 2011 population, the population size in 2012 was significantly larger in the odd-day marked group than the even-day marked group on tree E, but it was not different on tree A (Table 1 and Fig. 6). In 2012, 33 beetles from two trees exhibited temporal switching, and 29, 3, and 1 beetles switched appearance days once, twice, and three times, respectively. For beetles marked on trees A and E, the temporal switching rate was significantly larger in the even-day marked group (Table 2). Most beetles were observed repeatedly only on the tree where the beetle was marked, but some reappeared on different trees. For instance, female specimen no. 330 appeared on

tree A at the first appearance, but it appeared on tree E the second time and tree A at a later time (Fig. 5). This phenomenon was referred to as spatial switching. The spatial switching rate was about 10%, and it did not differ significantly between beetles marked on trees A and E (Table 2, Fig. S3).

Figure 6 shows seasonal variation in the emergence of beetles. We counted the number of male and female beetles separately each day, and a significant difference in the number of beetles was detected between days but not between sexes (two-way ANOVA, $P > 0.05$ in sexes, $P < 0.01$ in days, Table S1). In 2011 at tree E, the odd-day marked and even-day marked groups mostly reappeared on different days in June and July. However, in August and September, when very few beetles appeared, synchronization of appearance days occurred between the two groups (Fig. 6A). In 2012, differences in population size were observed between the odd-day and even-day marked groups on tree E (Table 1). Two-day periodic appearances were only obvious in the large population of the odd-day marked group (Fig. 6B). On tree A, an abrupt disappearance of unmarked (newly captured) beetles occurred in the even-day marked group after June 20 (Fig. 6B). There was heavy rain from Typhoon No. 4 and a tropical cyclone that occurred June 19–22 (Japan Meteorological Agency, 2012). The heavy rains led to the flooding of the ground at the observation site on June 22 (an even day), and no beetles appeared on that day on trees A and E (see insert in Fig. 6B). The beetles appeared again on June 23. After this submersion, beetles from the even-day marked group exhibited temporal switching (appearing on the odd days), and complete synchronization occurred for nine days from June 23 to July 1 between the odd-day and even-day marked groups on both trees, with the exception of one specimen on June 28 on tree E (Fig. 6B). This explains the high percentage of temporal switching in the

even-day marked group in 2012 (Table 2). After the heavy rain, more beetles were newly captured on the odd days than the even days, suggesting that many of the unmarked beetles also exhibited temporal switching after the water submersion.

Distribution range of *H. parallela.* Because the percentages of recaptured beetles on trees A and E were not very high, we suspected that the beetles might appear on neighboring trees. We examined the occurrence of beetles marked on tree E and unmarked beetles on 10 trees (including tree E) on July 28 and 29 of 2011 (Fig. S1, Fig. 7). The total number of appearances for two days at the 10 trees was 111, but no beetles were captured at trees B, F, and J. Beetles originally marked on tree E were mostly found on tree E (n = 14). Regarding other trees, only a single marked beetle was found on trees A (sex, unknown) and G (female), but all other beetles were unmarked.

We chased seven beetles that flew away from tree A around sunrise in June and July of 2012, and found that they dug into the soil located within a 15-m diameter from tree A (Fig. 8). These observations suggested that most *H. parallela* individuals repeatedly visited the same tree that was close to their daytime resting place.

Discussion

Endogenous two-day rhythm in *H. parallela.* The present study revealed that *H. parallela* exhibited clear endogenous circabidian rhythms with regard to its appearance on the ground under laboratory conditions. Individual appearances in the field also fit an approximate two-day cycle, but individuals often switched appearance days, presumably based on environmental conditions.

Circabidian rhythms were observed as ~50-h periodicity of sleep-wakefulness in humans under conditions free of time cues (Czeisler et al., 1980) and ~46 h of flight activity rhythms in the mosquito *C. incidens* under constant conditions (Clopton, 1984). These circabidian rhythms were labile and subsequently returned to circadian or became obscure. Because circabidian rhythms in human subjects are associated with sleep-wakefulness activities that were observed during desynchronization from body temperature rhythms that oscillate in a circadian manner, researchers concluded that circabidian rhythms resulted from the uncoupling of multi-oscillators under constant conditions (Aschoff et al., 1967). Clopton (1984) basically followed this idea to interpret the rhythms of mosquitos. In these reports, the circabidian rhythm was thought to be expressed only under non-natural conditions as one unique character of the coupled circadian clock system. However, the circabidian rhythm observed in *H. parallela* clearly differed from labile rhythms. In *H. parallela*, the two-day rhythm appeared stable under both LD and DD experimental conditions, and the rhythm was also observed in the field. Their circabidian appearance or activity on the ground might contribute for reduction of a prey risk and saving energy for reproduction. Less appearance seems disadvantageous because the opportunity to find a mate is diminished by half. *H. parallela* might cope with less mating opportunity by increasing mating efficiency. In fact, mating period in *H. parallela* is short compared with other congeneric species (Matsumoto, 2010). They might increase mating frequency to copulate with more partners than other congeneric species.

Entrainment of circabidian rhythm to light and dark cycles. The circabidian rhythm in *H. parallela* entrained to two cycles of LD 12:12, with activities every other night. Because beetles stay underground during the day, they are barely exposed to light. Beetles with a free-running period shorter than 48 h likely came up to the surface of the ground before the lights were turned off, thus, following an internal clock. Actually, we occasionally observed movement of the ground surface before the lights were turned off on the appearance day, suggesting that beetles came up just below (not on) the soil surface to sample the light. On non-appearance day, light sampling behavior was not observed. Light sampling behavior has been shown in some cavern dwelling bats and the flying squirrel *Glaucomys volans* (Twente, 1955; DeCoursey, 1986), which leaves the den after arousal to sample light through the sampling porthole. If light was seen, the squirrel returned to the den to take a small nap before venturing out again (DeCoursey, 1986). When the light was off during sampling, the squirrel left the den. This unique entrainment process is similar in *H. parallela*.

H. parallela individuals with a free-running period longer than 48 h, which allowed it to come up to the surface of the ground after the light was off. This allowed them to sample light at dawn to entrain LD cycles. In the field, the beetles actually stayed on the tree throughout the night until dawn (Fig. 4). Under laboratory conditions, *H. parallela* only stayed on the ground for 6 h after the light was turned off. We think that this resulted from confined solitary conditions without a mate and the inability to fly (Fig. 1).

H. parallela likely monitor light intensity to determine the timing of emergence on the ground. In a congeneric species, *Holotrichia loochooana*, adult emergence and mating behaviors were observed in the laboratory under natural light conditions. Emergence on the ground and female calling behaviors occurred at a certain range of light intensity (Kawamura et al., 2001). Since the *H. parallela* mating time is limited (Yoshioka and Yamasaki, 1983), it seems important for *H. parallela* individuals to wait for the start of darkness just below the surface of the ground and to appear quickly on the ground to facilitate population synchronization.

Biological clocks driving the circabidian rhythm. To determine a cycle of the clock that drives the circabidian rhythm, we examined the phase responses of the rhythm to the zeitgeber light. Researchers demonstrated that phase advances or delays in the rhythm in response to a zeitgeber depend on clock phases, and this is a unique characteristic of oscillator-type clocks (Pittendrigh, 1960). Phase response curves have been constructed for different kinds of biological rhythms, and the curve periods of approximately 24 h, 12.4 h, and one year have been revealed in respective circadian, circatidal, and circannual rhythms (Pittendrigh and Minis, 1964; Akiyama, 1997; Miyazaki et al., 2005; Satoh et al, 2008). In the circadian clock, clock phases are divided into the subjective day and night periods. In the cricket *Gryllus bimaculatus*, circadian activity rhythms exhibit little response to light pulses during the subjective day, but a delay in the first half and an advance in the last half of the subjective night were observed (Okada et al., 1991). In *H. parallela*, we observed two sets of the less responsive period and responsive (delay or advance) period in one circabidian cycle.

This result suggested that a circabidian cycle is composed of two cycles of the circadian oscillator. However, phase responses were rather obscure to draw a fine curve. To obtain clear phase response curves more number of beetles might be necessary. Meanwhile, four beetles showed circadian-like activity rhythms after light pulses, suggesting that *H. parallela* are capable of driving activity in a circadian manner. Phase responses to light pulses by the circabidian rhythm and change of the circabidian rhythm to circadian-like activity rhythm support the idea that the circadian clock generates the circabidian rhythm in *H. parallela*.

Clopton (1984) proposed a mechanism for the circabidian rhythm in mosquitos, based on a coupled oscillator model. When mosquitos or humans are kept free from timing cues, oscillator coupling between circadian clocks becomes weakened, and a labile oscillator produces a two-day rhythm that is uncoupled from the rigid 24-h oscillator. Uncoupled labile oscillator lengthens its period and recoupled to rigid 24-h oscillator when its period becomes twice. Another mechanism for making longer rhythm than intrinsic oscillators has been hypothesized as beats (Bünning, 1962). Beats appear originating from a difference in period length between oscillators that are not coupled. Semi lunar periodic phenomena may originate from a cooperation of a diurnal (about 24 h) and a tidal rhythm (about 12.4 h). However, we think clock mechanisms of *H. parallela* rhythms are different from oscillator coupling or beats, because appearance of the circabidian rhythm are rigid, not labile, both under LD and constant conditions, and no oscillator components other than the 24-h oscillator were detected in phase response experiments. Further rhythmic components with different cycles producing a beat every 48 h are hardly imagined.

We suppose the most parsimonious interpretation that the circabidian output is produced every two oscillations of the circadian clock entraining with 24 h light-dark cycles. The frequency demultiplication hypothesis, in which biological rhythms with a long period are derived from rhythm with a short period through a process of frequency demultiplication, has been supported in the circasemilunar rhythm in *Pontomya oceana* (Soong and Chang, 2012). The results indicate presence of the counter mechanisms in which cycle numbers of the circadian oscillations are counted to make circasemilunar emergence rhythm. To make circabidian rhythm also 2 circadian cycles might be counted in *H. parallela*.

Spoelstra et al. (2016) suggested that biological clocks with cycles that do not coincide with environmental cycles are eliminated by natural selection. With regard to biological clocks, the evolution of a cycle that is not associated with an environmental cycle may be difficult. If there are adaptive values associated with a two-day rhythm, it might be easy to produce circabidian rhythms by modifying the output systems of the circadian clock, which releases a signal every two cycles of the clock.

Circabidian rhythms in the field. The two-day periodicity of individuals was observed in the field. However, its periodicity was not constant, and the absence of a period was often observed. Since beetles marked on tree E were barely found on other trees (Fig. 7), we think that *H. parallela* individuals rarely go to other trees, but they frequented bushes around the tree on which they were marked. Actually, we observed eating Poaceae grasses by *H. parallela*.

In the field, *H. parallela* occasionally showed temporal switching of the two-day rhythm. June 22–23, 2012 represented a period when beetles were not able to come out on the ground because of heavy precipitation, which resulted in the synchronization of appearances in the odd-day marked and even-day marked groups. For this synchronization, the even-day marked group was altered to appear on the odd days. In 2012, the majority of beetles started to appear on tree E and were marked from late June after the heavy precipitation, thus, resulting in population size differences between odd- and even-day marked groups. Such a clear synchronization was not found in 2011, although there was heavy rain in September. Emergence synchronization seems to depend on environmental changes, and rain is only one factor.

In 2011, some even-day marked beetles showed temporal switching that resulted in a synchronized occurrence with the odd-day marked group in August and September when population size was smaller. It is known in *H. loochooana* and *Dasylepida ishigakiensis,* which belong to the same Melolonthinae subfamily as *H. parallela*, exhibit calling and mating behaviors that occur in the population at a fixed period of the day (Kawamura et al., 2001; Arakaki et al., 2004; Yasui et al., 2007; Fukaya et al., 2009; Tokuda et al., 2010). Yoshioka and Yamasaki (1983) reported that *H. parallela* mate soon after sunset, and we also confirmed this in the field. Males were caught by pheromone traps a few hours after sunset, indicating that males quickly fly toward pheromones, and mating subsequently occurs. If copulation occurs during a restricted time of day in these species, sympatric and synchronized population occurrences are needed to increase copulation efficiency. Temporal switching in *H. parallela* may play a role in the synchronization of sympatric populations. Considering the fact that

individual activities recorded in the laboratory never switched to the next day, temporal switching was likely induced by environmental and social cues. *H. parallela* uses circabidian rhythms in a flexible manner to change emergence days in order to adjust to the changing environment.

How does the temporal switch occur? If the circabidian rhythm was made by a hypothetical circabidian clock, a phase shift of a half cycle (~24 h) of the clock had to occur to switch the appearance days. For an oscillator-type clock, it is hard to make a half-period phase shift at one time (Benstaali et al., 2001), and it usually takes a transient period to complete a full shift. If temporal switching was adaptive for the beetle (e.g., facilitated avoidance of aversive conditions or increased population size), the beetles had to develop some mechanism to make a shift of appearance occur one day at a time. The circabidian rhythm driven by the circadian clock system might facilitate an appearance day switch at once. Circabidian output might be activated or suppressed after counting two circadian oscillations. If so, some unknown environmental stimuli may modulate the day-counting system to give an output after one or three circadian oscillations, thus resulting in temporal switching. Here, we propose a novel functional circabidian rhythm that is affected by the circadian clock. In the future experiment we clarify an involvement of the circadian clock in the circabidian rhythm in molecular and neuronal bases.

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Figures

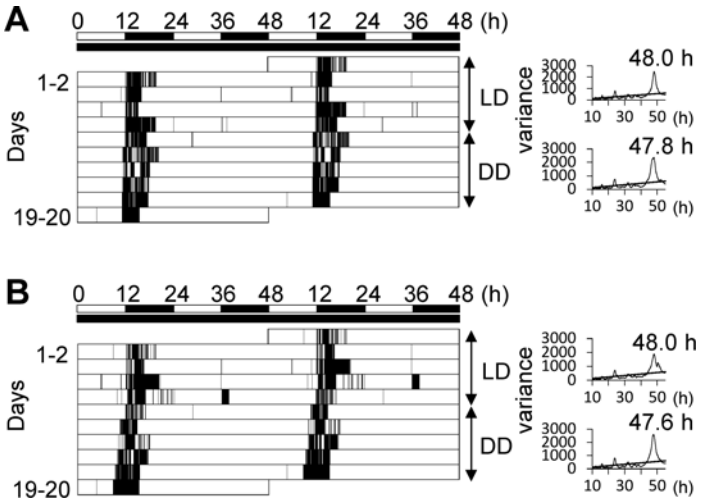

Fig. 1. Activity rhythms of an adult *Holotrichia parallela* male (A) and female (B) on the ground. Activities recorded over a 48-h period are shown in the double-plotted actogram for 20 days. Black bins on the horizontal line represent activities. White and black bars on the actogram indicate light and dark periods, respectively. The beetles were recorded under 12 h light and 12 h dark conditions for 10 days and under constant dark conditions for 10 days. Two graphs on the right side show a chi-square periodogram analysis during light and dark (LD, *upper*) and constant dark conditions (DD, *below*). Hours on the graph shows the period of the rhythm analyzed by the chi-square periodogram

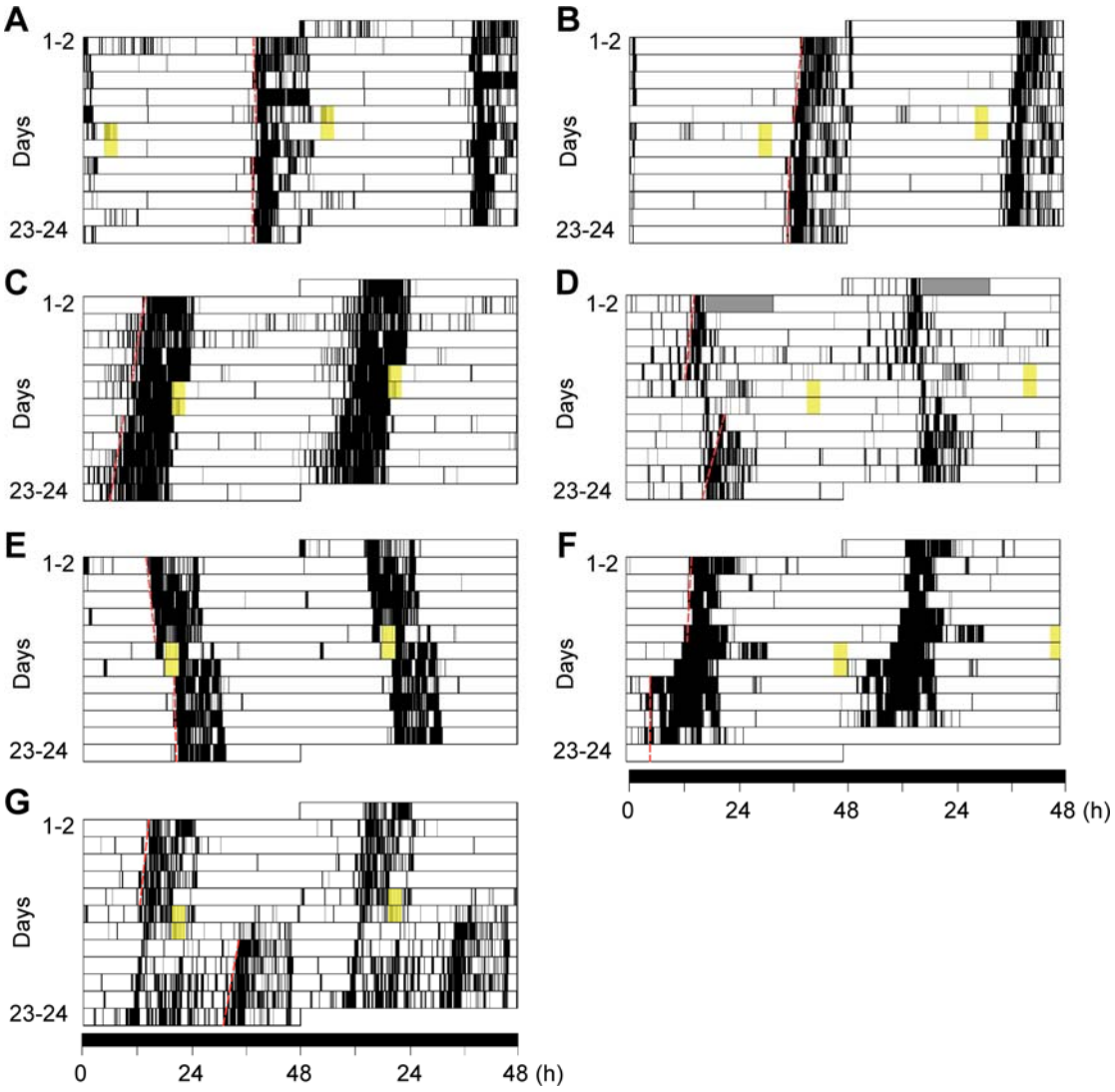

Fig. 2. Activity rhythms of *Holotrichia parallela* under constant dark conditions with light pulses. Activities recorded over a 48-h period are shown in the double plotted actogram for 24 days. Black bins on the horizontal line represent activities on the ground. A gray bar indicates a period with no data, and a yellow bar indicates a light pulse. Red broken lines indicate the approximate lines of an onset phase of activity. Light pulses were emitted at circabidian times (CbT) = 4.0 h (A), CbT = 34.5 h (B), CbT = 47.0 h (C), CbT = 17.5 h (D), CbT = 39.5 h (E), CbT = 22.5 h (F), and CbT = 8.0 h (G). Some beetles exhibited circadian-like activity after the light pulses as seen in (G).

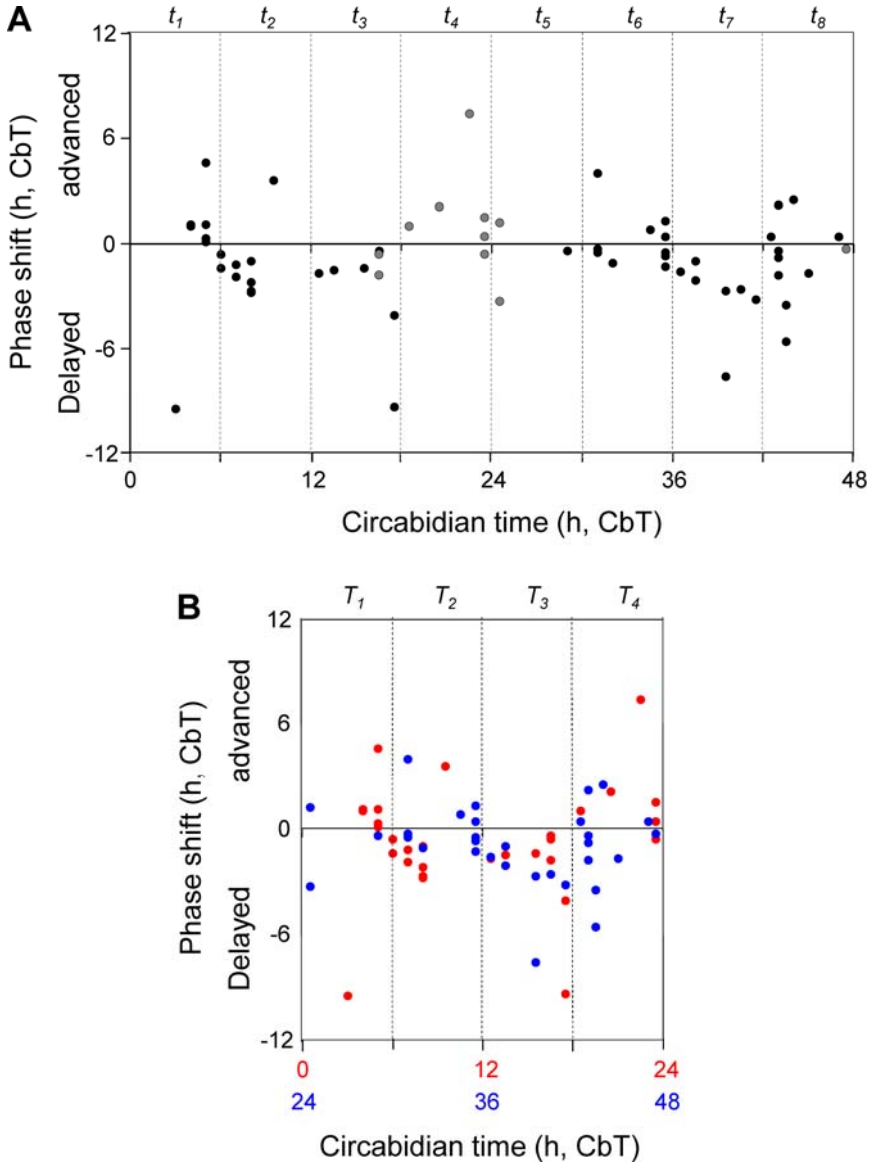

Fig. 3. Phase responses of *Holotrichia parallela* to 3-h light pulses emitted at different circadian times under constant darkness. Advanced and delayed phase shift values are plotted as positive and negative values on the ordinate. The activity onset is set as a phase reference point of CbT = 36 h. **A**. One oscillation of a circadian cycle is divided into eight terms (6 h each) from t_1 to t_8 to examine the dependency of shift values on circadian phases (see text). Cycles of less responsive and responsive phases occur twice in a circadian oscillation. Gray dots indicate individuals provided with less food

compared to individuals denoted with black dots, but activities were not affected. **B**. The same data in **A,** the first half (CbT = 0-24 h, red) and last half (CbT = 24-48 h, blue) of a circabidian cycle are superimposed in a 24-h time frame (N = 61).

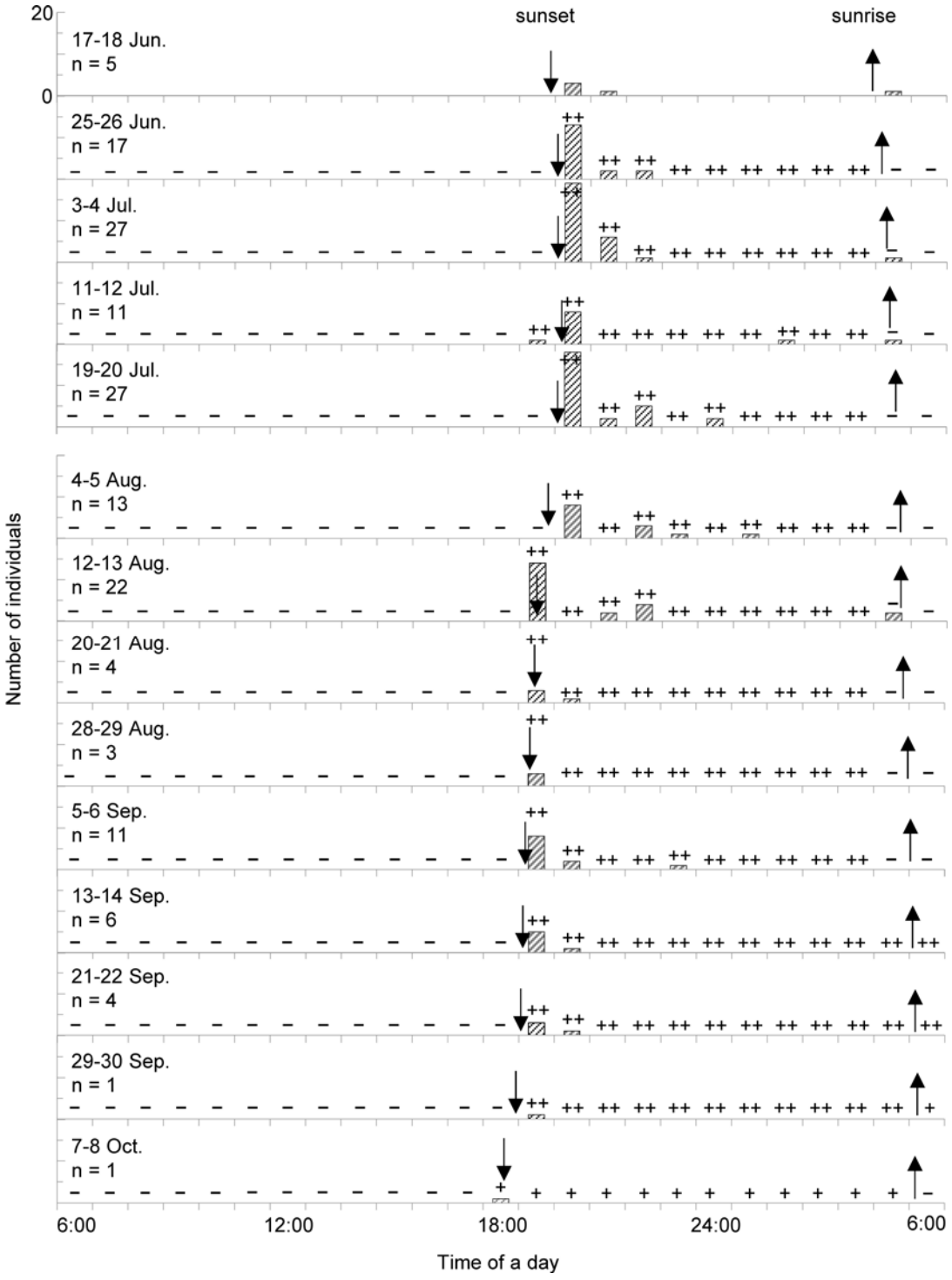

Fig. 4. Appearance time of *H. parallela* on the tree *Ulmus parvifolia*. Columns show the number of male *H. parallela* captured by the pheromone trap. Symbols ++, + and - represent the number of beetles on the tree, 10 or more (++), less than 10 (+) and 0 (-).

Downward arrows show the time of sunset and upward arrows show the time of sunrise. Abscissa indicates time of the day from 6:00 AM to the next 6:00 AM.

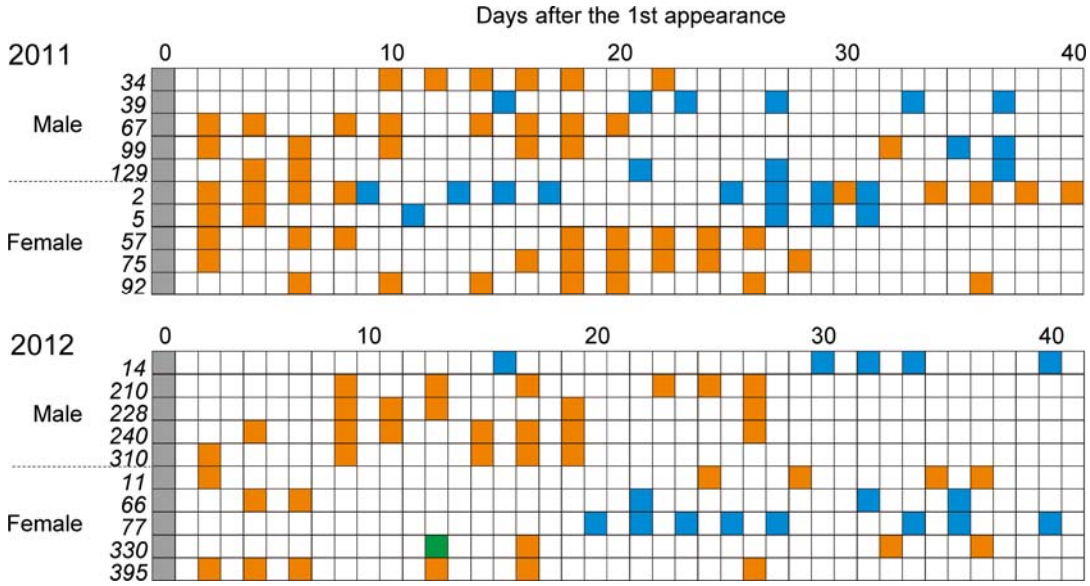

Fig. 5. Representative individual plots of *Holotrichia parallela* appearance in the field. The horizontal axis indicates the number of days from the first appearance. Orange cells and blue cells indicate even and odd numbers of days, respectively, counted from the first appearance. The green cell highlighted for female specimen no. 330 in 2012 shows spatial switching. No. 330 was marked on tree A, appeared at tree E 13 days after the first appearance (green cell), and was back on tree A on other days.

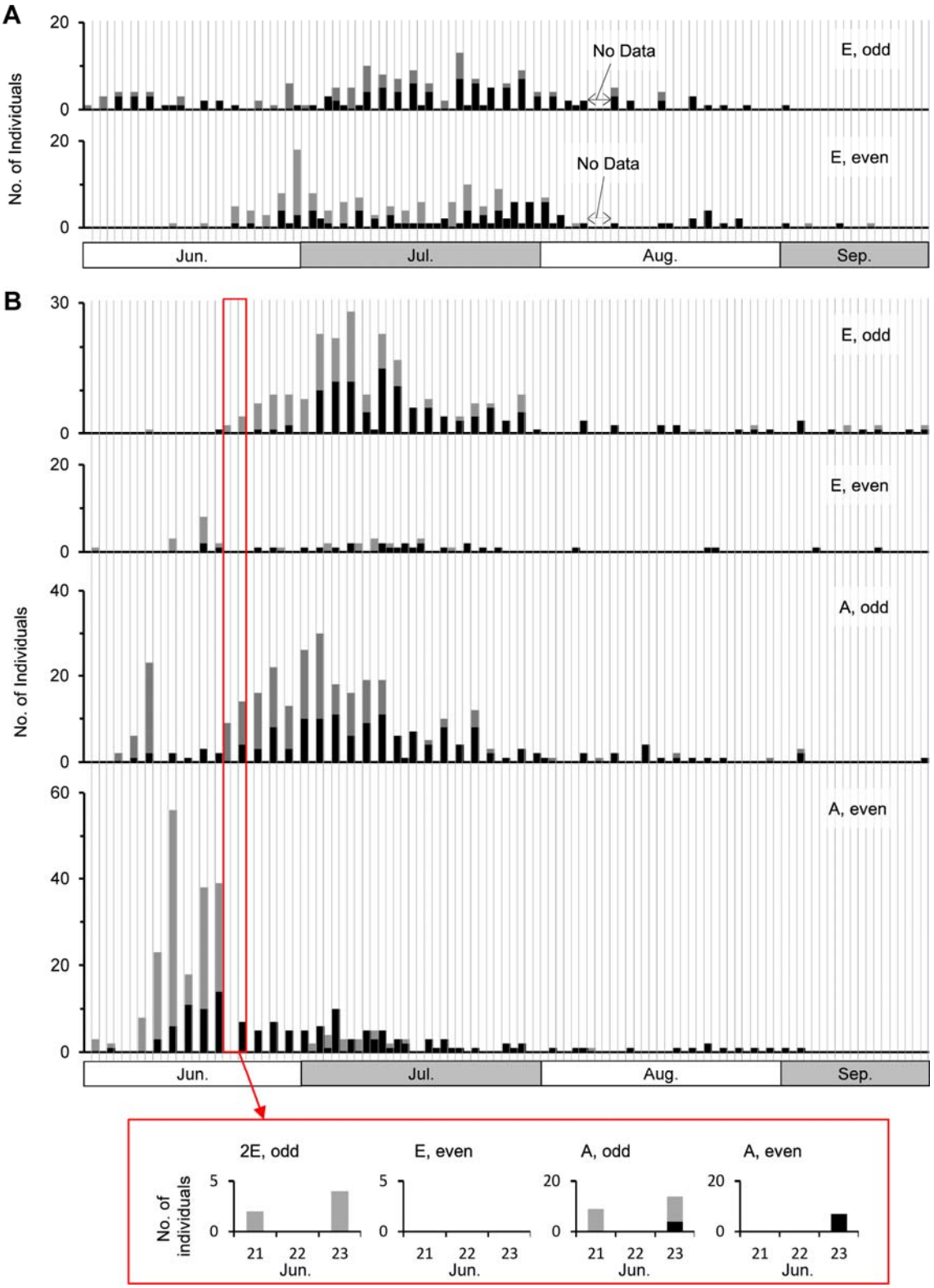

Fig. 6. Seasonal changes in the emergence of *Holotrichia parallela* at the riverbed of Yamato River in 2011 (A) and 2012 (B). The beetles were separately plotted on the

graph between the odd day-marked and even-day marked groups. Males and females are plotted together (see text for detail). June 3 was set as the 1st day in both 2011 and 2012. Beetles marked on the 1st (June 3), 3rd (June 5), 5th (June 7), 7th (June 9), and following days were grouped into "the odd day marked group", and those marked on the 2nd (June 4), 4th (June 6), 6th (June 8), 8th (June 10), and following days were grouped into "the even day marked group". Black columns show recaptured marked individuals, and gray columns show newly captured individuals. The data from June 21 to 23 in 2012, when temporal switching mainly occurred, is extracted in a red inset below. See Table 1. for sample size.

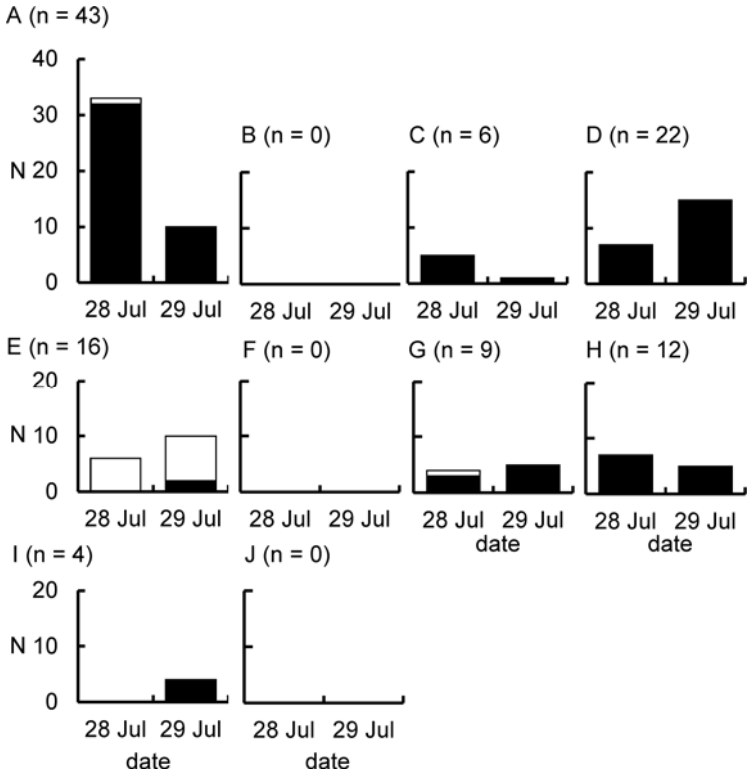

Fig. 7. Number of *Holotrichia parallela* individuals that appeared on trees A–J July 28–29 in 2011. White columns show the number of beetles marked on tree E, and black columns indicated unmarked beetles. One marked beetle was found on trees A and G, and most was on tree E. No beetles were found on trees B, F, and J.

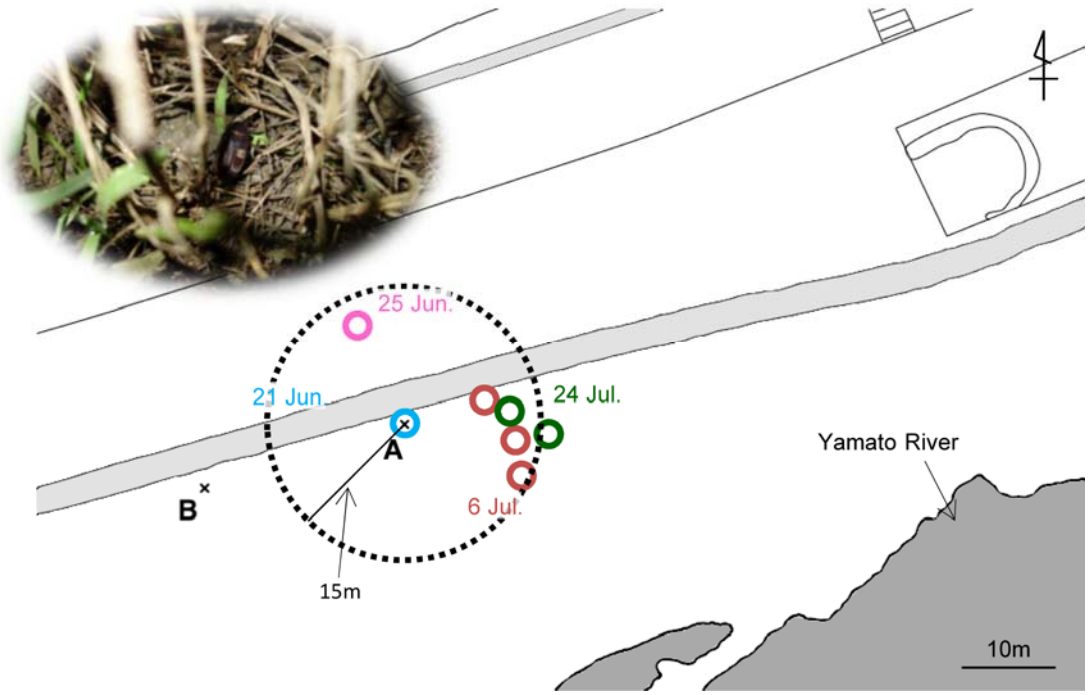

Fig. 8. Flight ranges of *Holotrichia parallela* at sunrise. The cross indicates trees A and B. Colored circles indicate a point where the beetle went underground from tree A. Circles of the same color indicate beetles observed on the same day. The broken line shows an area within a circle (15-m diameter) from tree A.

Tables

Table 1. Marked numbers and rates of recaptured *Holotrichia parallela* on the tree *Ulmus parvifolia*.

Year- tree *[1]	Marked numbers				Recaptured rates (%) *[3]
	Total	Odd-day marked group	Even-day marked group	*P* *[2]	
2011-E	142	80	62	0.131	26.1
2012-A	332	167	155	0.378	22.0
2012-E	137	112	25	3.05E-12	22.6

*1 Year and tree at which *H parallela* were marked. *2 The results of chi-square goodness of fit test between odd- and even-day marked group. *3 Percentage of beetles recaptured 2 times or more at the marked tree.

Table 2. Occurrence of temporal switching and spatial switching in *Holotrichia parallela*.

Year-tree *1	N *2	temporal switching			spatial switching	
		% Odd-day marked group	% Even-day marked group	*P* *3	%	*P* *4
2011-E	37	22.2 (18)	47.3 (19)	0.109	-	-
2012-A	73	12.5 (40)	72.7 (33)	1.66 E-07	13.7	0.571
2012-E	31	0 (26)	80.0 (5)	1.02 E-06	9.7	

*1 Year and tree at which *H parallela* were marked. *2 The number of beetles which reappeared 2 times or more at the marked tree. *3 The results of chi-square test in temporal switching rates between odd- and even-day marked group. *4 The results of chi-square test of spatial switching between tree A and E in 2012.