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Original Article

Comparison of daily activity rhythms in individual workers of five Japanese ant species

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Abstract

Circadian rhythm is an endogenous 24 h variation in physiological processes and behaviors. It has evolved in the complexity of natural environments and enables organisms to adapt their behavior to the day and night cycle. To better understand the evolution of circadian rhythms, it is important to clarify the relationship between the morphological / ecological characteristics and phenotypes of daily rhythms. Daily rhythms in social insects are affected by social factors, such as tasks and interactions with individuals. However, data on daily rhythms of ants are available for a relatively small number of ant species. The purpose of this study was to understand how three morphological / ecological features affect the social rhythms of ants: 1) presence-absence / extent of morphological diversity, 2) nest types (underground, arboreal, or nomadic), and 3) feeding habits. We measured locomotor activity rhythms of isolated ants in five Japanese ant species under both light-dark and constant dark conditions. Under the constant dark conditions, workers did not show clear daily rhythms. No significant differences in the strength of daily rhythms were observed between the morphological castes of *Camponotus vitiosus*. The nomadic ants *Pristomyrmex punctatus* displayed clear daily rhythms with bimodal activity peaks under light-dark condition. Feeding habitats did not explain the activity patterns of foragers. In addition, we found that *Formica japonica* callow workers showed clear daily rhythms under light-dark conditions, suggesting the early development of circadian rhythms. This study provides data on individual daily activity rhythms in five new ant species, which further sheds light on the evolution of daily rhythms in ants.

Key words: Circadian rhythm, arboreal ant, nomadic ant, crepuscular activity, Hymenoptera, Formicidae, *Camponotus*, *Formica*, *Nylanderia*, *Lasius*, *Pristomyrmex*.

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Introduction

Circadian rhythm is a periodicity of approximately 24 h in various physiological processes and behaviors (Saunders 2002, Numata & Tomioka 2023). Circadian rhythms are generated by an endogenous program called a circadian clock, which can run even under constant environmental conditions. It has evolved in the complexity of natural environments and enables organisms to adapt their behavior to varying environmental conditions, such as the day and night cycle (Helm & al. 2017). To better understand the evolution of biological rhythms, it is important to clarify the relationship between the morphological / ecological characteristics and phenotypes of daily rhythms.

In social insects, daily rhythms in behavior are highly related to social activities, such as foraging, nursing (Moore & al. 1998), and reproduction (McCluskey 1965, 1967). For example, foragers exhibit clear daily rhythms, whereas nurses lose their daily rhythms in locomotion and task performance by interacting with the brood (Moore & al. 1998, Bloch & Robinson 2001, Shemesh & al. 2007, Bloch 2010, Fujioka & al. 2017). In addition, mating events can drastically change the daily locomotor activity of males and queens. Males and virgin queens of ants and bees exhibit daily locomotor activity rhythms (McCluskey 1965, 1992). After mating, the mated queens lose their daily locomotor activity rhythms during egg laying and brood care (McCluskey 1967, Sharma & al. 2004a, Eban-Rothschild & al. 2011, Fuchikawa & al. 2014). Plasticity and variability in reproduction and brood care are common among bees and ants.

Tab. 1: Summary of ant species and colony information. Collection sites and number of collected colonies or queens for the five Japanese ant species used to investigate daily activity rhythms.

Species	Collection Sites	Number of collected colonies	Collection methods	Nest types	Worker caste	Feeding habit
Camponotus vitiosus	Tsushimanaka, Okayama	2	colony collection	arboreal	triphasic allometry	herbivorous
Formica japonica	Komaba, Meguro, Tokyo	3	queen collection	underground	monomorphism	omnivorous
Lasius japonicus	Minami-osawa, Hachioji, Tokyo	2	queen collection	underground	monomorphism	omnivorous
Nylanderia flavipes	Sapporo, Hokkaido	4	colony collection	soil surface/ underground	monomorphism	herbivorous
Pristomyrmex punctatus	Naha, Okinawa/ Tsushimanaka, Okayama	2/1	colony collection	nomad	monomorphism	omnivorous

Daily rhythms are ubiquitous in insects, but in ants, around-the-clock locomotor activities at individual and colony levels are reported. Even under solitary conditions, some workers do not show circadian activity rhythms on locomotion like *Solenopsis invicta* (see Ingram & al. 2009) and *Camponotus compressus* (see Sharma & al. 2004b). A previous study revealed that specific worker-worker interactions induce around-the-clock locomotor activity at the individual level in a small worker group (Fujioka & al. 2019a). Under colony condition, individual workers do not show circadian activity rhythms of locomotion in *Diacamma* cf. *indicum* (see Fujioka & al. 2021). However, *Temnothorax longispinosus* exhibits daily rhythms of colony-level activity, where colony-level activity was measured as the proportion of active ants over time (Libbrecht & al. 2020).

Ants have diverse morphological / ecological traits such as the presence-absence / extent of morphological diversity, colony size, habitat type, nest type, diurnal / nocturnal, feeding type, and foraging strategies (Hölldobler & Wilson 1990, Burchill & Moreau 2016, Trible & Kronauer 2017, Robinson & Jandt 2021). We expect that the around-the-clock activity of ants might be related to the following three morphological or ecological characteristics. First, the presence-absence / extent of morphological diversity in workers can affect daily rhythms. Some ant species vary in their size and shape among workers (Fjerdingstad & Crozier 2006, Wills & al. 2018). *Camponotus compressus* has worker polymorphism (intraspecific variation in worker body size), with minor, media, and major workers, showing increasing body size. The middle-size media workers display nocturnal or diurnal patterns under a light-dark cycle (Sharma & al. 2004b). Although the presence of two chronotypes in the colony is an interesting phenomenon, to the best of our knowledge, there is only one report.

The second trait is nest type. In this study, we focused on three types of nest habitats: underground, arboreal, and nomadic (ephemeral resources). We predicted that nomadic ants may change their activity to react to light cycles because they do not have a nest at a fixed place. We expected arboreal ants to be more resistant to light fluctuations because light and temperature fluctuations inside of an arboreal nest are more significant than those in underground nests where temperature, humidity, and light conditions are more stable. In underground-nesting ants, workers inside the nest may react to light as an unusual condition.

Lastly, we expected that feeding habits might be related to the around-the-clock activity of ants. To forage on some floral resources (Stadler & Dixon 2005), foraging during flowering time is required. By contrast, for generalists, specialist predators, scavengers, or omnivores (Reeves & Moreau 2019), there might be no suitable fixed times during the day because the resource availability temporarily changes. Many previous studies investigated colony-level foraging activities, which vary among species (Das & Gordon 2023). The foraging rhythms respond to climate, season, and colony needs. However, little is known about individual daily rhythms of foragers.

Although many studies have addressed daily rhythms in ants, data on the daily rhythms of individual workers are available in a limited number of ant species. For comparative studies, the accumulation of basic information on individual daily activity rhythms is needed. In this study, we measured daily locomotor activity using five Japanese ant species: *Camponotus vitiosus*, *Formica japonica*, *Lasius japonicus*, *Nylanderia flavipes*, and *Pristomyrmex punctatus*. Characteristics of each ant species are shown in Table 1. These species were selected because they are common in Japan and vary in worker morphological diversity, nest types, and feeding habits. The monitoring of daily activity rhythms in the field is challenging. This study used the Drosophila Activity Monitoring (DAM2) system to monitor daily locomotor activity patterns of individuals under constant dark and light-dark light conditions without temperature oscillation. Locomotor activity is a fundamental trait for understanding daily rhythms even under such artificial conditions.

Tab. 2: Summary of circadian activity rhythms. mean ± SD of maximum autocorrelation coefficient, period, and ratio of daytime activity per total activity. Different letters on maximum autocorrelation coefficient indicate significant differences among worker types / castes based on the Tukey-Kramer tests ($p < 0.05$). Asterisk in daytime activity ratio indicates whether the average daytime activity ratio differs significantly from 0.5 ($p < 0.05$, one sample t-test).

Ant species	Worker type/caste	Number of ants		Mean of maximum autocorrelation coefficient		Period (h)		Ratio of daytime activity per total activity	
		LD	DD	LD	DD	LD	DD	LD	DD
Camponotus vitiosus (Formicinae)	Minor	74	65	0.046 ±0.02	0.053 ±0.033	24.1 ±1.19	24.2 ±1.22	0.629 ±0.12*	0.558 ±0.109*
	Media	31	33	0.048 ±0.018	0.047 ±0.021	24.0 ±1.23	23.7 ±1.1	0.586 ±0.084*	0.521 ±0.067*
	Major	78	62	0.051 ±0.022	0.050 ±0.021	23.8 ±1.21	23.7 ±1.23	0.597 ± 0.12*	0.591 ±0.11*
Formica japonica (Formicinae)	Callow	30	69	0.086 ±0.030 a	0.048 ±0.024 c	24.0 ±0.756	23.7 ±1.04	0.798 ±0.1*	0.588 ±0.092 *
	Nurse	102	26	0.061 ±0.025 b	0.065 ±0.023 bc	23.8 ±1.14	23.7 ±1.02	0.60 ±0.097*	0.581 ±0.091*
	Forager	120	16	0.060 ±0.026 b	0.055 ±0.02 bc	23.9 ±1.06	23.4 ±0.956	0.597 ±0.141*	0.591 ±0.087*
Lasius japonicus (Formicinae)	Nurse	32	57	0.046 ±0.03	0.047 ±0.014	23.9 ±1.13	23.7 ±1.16	0.556 ±0.075*	0.549 ±0.064*
	Forager	45	47	0.050 ±0.02	0.050 ±0.021	24.0 ±1.19	24.0 ±1.08	0.621 ±0.062*	0.517 ±0.063
Nylanderia flavipes (Formicinae)	Nurse	86	141	0.054 ±0.02	0.053 ±0.02	24.1 ±1.04	24.1 ±1.14	0.64 ±0.1*	0.621 ±0.13 *
	Forager	20	39	0.058 ±0.019	0.057 ±0.019	24.2 ±0.951	23.7 ±1.07	0.655 ±0.11*	0.60 ±0.13*
Pristomyrmex punctatus (Myrmicinae)	Nurse	70	114	0.098 ±0.053 a	0.048 ±0.017 b	23.8 ±0.72	23.8 ±1.20	0.50 ±0.13*	0.526 ±0.089*
	Forager	76	95	0.099 ±0.053 a	0.058 ±0.024 b	24.0 ±0.665	24.1 ±1.17	0.453 ±0.12	0.52.8± 0.11*

Material and methods

Materials

Five ant species were collected in Japan and identified using Terayama & al. (2014). The collection sites and number of collected colonies are shown in Table 1. In *Formica japonica* and *Lasius japonicus*, mated queen ants were collected during their mating season (from May to August) and kept in the laboratory for one year. After the colony foundation, workers emerging from the colony in the laboratory were used for locomotor activity measurement on the year of queen collection. Colonies of *Camponotus vitiosus*, *Nylanderia flavipes*, and *Pristomyrmex punctatus* were collected from the field. The focal five ant species have two types of workers (Tab. 1). Monomorphism, on the one hand, is represented as an isometric variation over a limited body size range. A triphasic allometry, on the other hand, occurs when the allometric line breaks at two points and consists of three groups of workers: major, media, and minor workers.

Definition of task-group and morphologically distinct workers

Nurses and foragers were defined as workers inside and outside the nest, respectively. For workers in *Camponotus vitiosus*, major (> 2.2 mm), media (2.2-1.5 mm), and minor (< 1.5 mm) were defined based on head width (Fig. S1a, as digital supplementary material to this article, at the journal's web pages). The callows of *Formica japonica* were distinguished based on their body color (Fig. S1bc). Thus, callows were defined as one to three days old workers.

Locomotor activity measurement

All colonies were reared inside an incubator at 25 °C ± 1 °C in 12 h:12 h light:dark conditions (light was turned on at 8 a.m. and turned off at 8 p.m.). Individual locomotor activity was recorded using the Drosophila Activity Monitoring System (DAM2) (TriKinetics, Waltham, MA) in 10 s bins for more than 5 days. Two light conditions, 12 h:12 h light:dark (LD) or constant dark (DD), were used. LD and DD experiments were performed in parallel for a given ant species, where a single ant was only observed in either LD or DD. The ants for experiments in both LD and DD were obtained from colonies that were always kept under 12 h light-12 h dark. DAM2 is a single-beam monitor. Individual ants were loaded into glass tubes (65 × 5 mm). The tubes were covered with rubber caps containing food (10% sucrose and 1% agar) and a small cotton plug at the other end. The time-series data per sec, from 0:00 on Day 1 to 24:00 on Day 5, was converted to the total number of line crossings per minute (data points = 7200). In addition, ratio of daytime activity per total

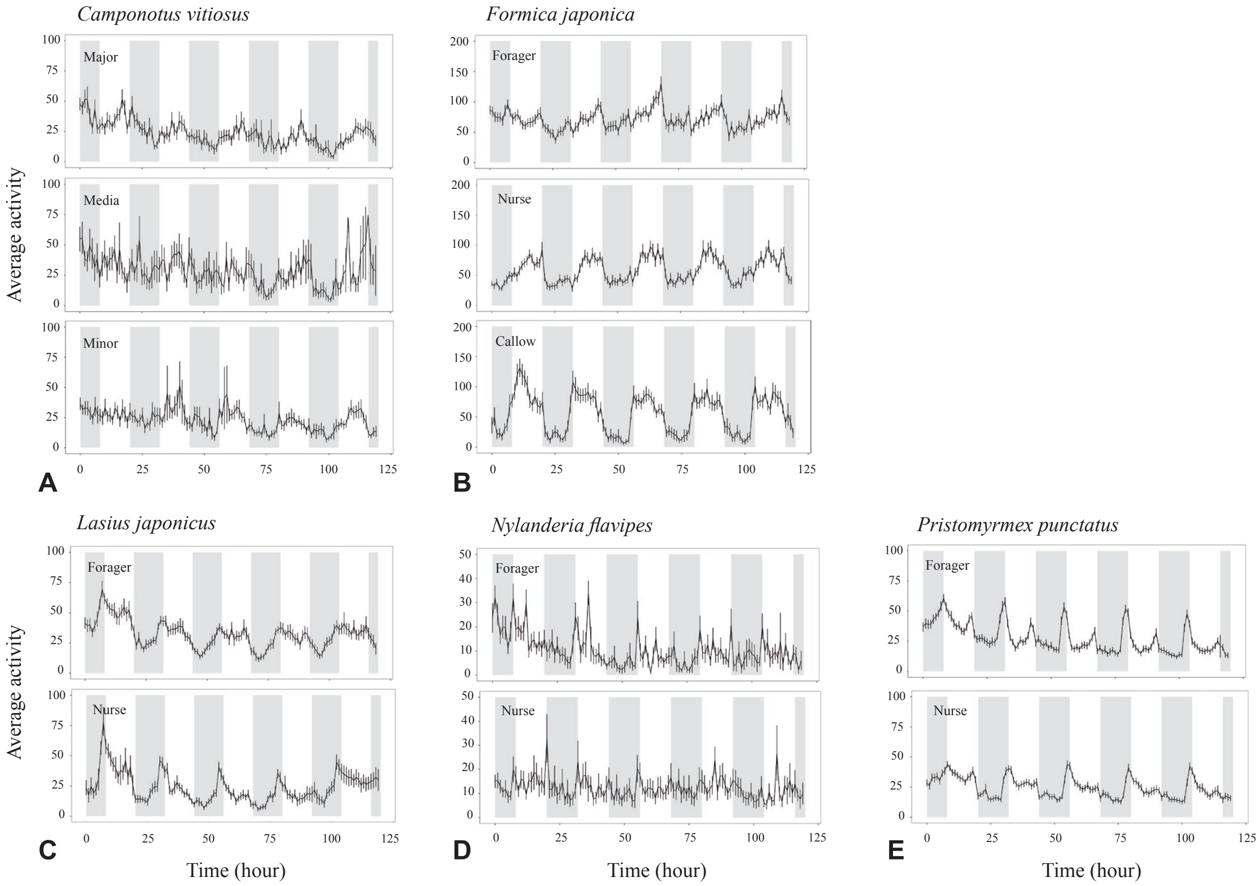

Fig. 1: Actograms under light-dark cycles for five consecutive days at 25 °C. The average activity is the average crossing activity of the ants in a one-hour bin, as a group profile for the worker type / caste. The grey shadows indicate the light-off phase.

activity was calculated for each day. The average ratio of 5 days was used as an indicator of preferred time-of-day activity for each individual. The activity between 8 a.m. and 8 p.m. was referred to as "daytime activity" regarding the lighting regime. Actograms were made with an average activity level of one-hour bin to show group-level profiles.

Statistics

The strength of daily rhythms for each individual was defined using the maximum autocorrelation coefficient, based on previous studies (Mormont & al. 2000, Nishihara & al. 2002, Casey & al. 2020, Kikuchi & al. 2020). Autocorrelation coefficients were calculated using 1 min lag from 0 to 1560 min (26 h). The maximum autocorrelation coefficient is a continuous variable, ranges between -1 and 1 and is independent of periodicity. The periodicity (min) was the period between 1320 min (22 h) to 1560 min (26 h) corresponding to maximum autocorrelation coefficient.

To test the effect of light cycles on maximum autocorrelation coefficient, linear mixed models (LMMs, $p < 0.05$) were performed using the R package "lmerTest", for each species separately. Maximum autocorrelation coefficient was the response variable (y), and the light conditions (DD or LD) were explanatory variable (x). Colony ID and worker types (callow, nurse, or forager) / worker castes (major, media, or minor) were treated as random effects. The maximum autocorrelation coefficient was compared among species using the Tukey-Kramer test ($p < 0.05$) for LD and DD conditions separately. The maximum autocorrelation coefficient was compared among worker types / castes within species using the Tukey-Kramer test. One-sample t-tests ($p < 0.05$) were used to assess whether the daytime activity ratio differed significantly from 0.5 for each worker type / caste separately. All statistical analyses were performed using the R Studio software.

Results

We measured locomotor activity rhythms of 1543 ants across five ant species. To obtain group-level profiles, we plotted the average crossings for each worker type / caste in one-hour bins (Figs. 1 and 2). There were no statistically significant differences in periodicity among the worker types and morphological castes under both LD and DD conditions (Fig. S2).

Strength of daily rhythms

The strength of daily rhythms of *Formica japonica* and *Pristomyrmex punctatus* under the LD condition were greater than those under the DD condition (Tab. S1, LMM, $p < 0.05$). Under light-dark conditions, *P. punctatus* had a higher autocorrelation coefficient than the other four species (Tab. S2, Tukey-Kramer test, $p < 0.01$). In

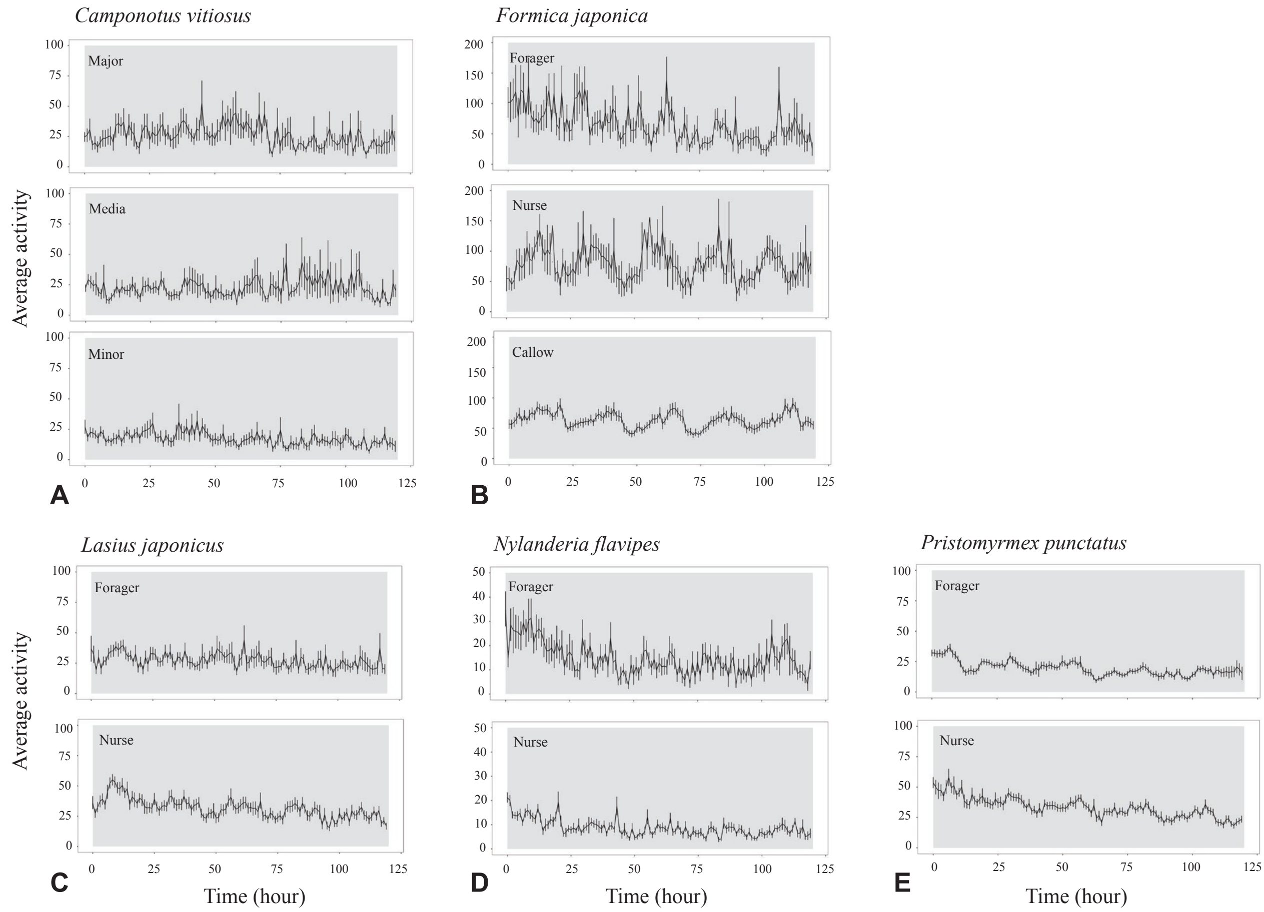

Fig. 2: Actograms under constant-dark conditions for five consecutive days at 25 °C. The average activity is the average crossing activity of the ants in a one-hour bin, as a group profile for the worker type / caste. The grey shadows indicate the light-off phase.

addition, *F. japonica* had a higher autocorrelation coefficient than *Lasius japonicus* and *Camponotus vitiosus* (Tab. S2, Tukey-Kramer test, $p < 0.01$). There were no species differences in the strength of daily rhythms under DD conditions (Tab. S2, Tukey-Kramer test).

Morphological caste, task group, and callow workers

There were no differences in the strength of daily rhythms among the morphological castes of *Camponotus vitiosus* (Tab. 2). Additionally, there were no differences in the strength of daily rhythms between foragers and nurses in *Lasius japonicus* and *Nylanderia flavipes* (Tab. 2). In *Formica japonica*, callow workers had stronger daily activity rhythms than those of nurses and foragers under LD conditions (Tab. 2, Fig. 3B, Tukey's HSD test, $p < 0.05$). In the group-level profile, both callows and nurses had clear peaks, whereas foragers had small peaks (Fig. 1B). In *Pristomyrmex punctatus,* foragers had bimodal peaks under LD conditions (Fig. 1E).

Diurnal activity

All species exhibited diurnal activity patterns. Under LD conditions, the ratio of daytime activity per total activity was significantly higher, except in *Pristomyrmex punctatus* foragers (Tab. 2, Fig. 4A - E). Under DD conditions, the ratios of daytime activity per total activity was significantly higher, except for foragers of *Lasius japonicus* (Fig. 4F - J). In *P. punctatus*, the activity of foragers showed bimodal peaks; the morning peak appeared before the light was turned on (Fig. 1D). This explains why the ratio of daytime activity per total activity of foragers was low, even when the strength of daily rhythms was high (Tab. 2, Fig. 3D). Six workers of *Camponotus vitiosus* had a low value of the ratio of daytime activity per total activity (< 0.35) (Fig. S3). However, these ants did not have a clear nocturnal activity pattern.

Discussion

We investigated the daily rhythms of five ant species distributed in Japan. Circadian activity rhythms of ants can be entrained by light and dark cycles and / or temperature (North 1987, 1993, Roces 1995). However, our data showed no clear entrainment to the light cycle in *Camponotus vitiosus, Lasius japonicus* and *Nylanderia flavipes*. In addition, we found that the focal ants did not have clear circadian rhythms under constant dark condition (Fig. 2). It should be noted that arrhythmic activity rhythms in locomotor activity do not mean an absence of a functioning circadian clock in ants.

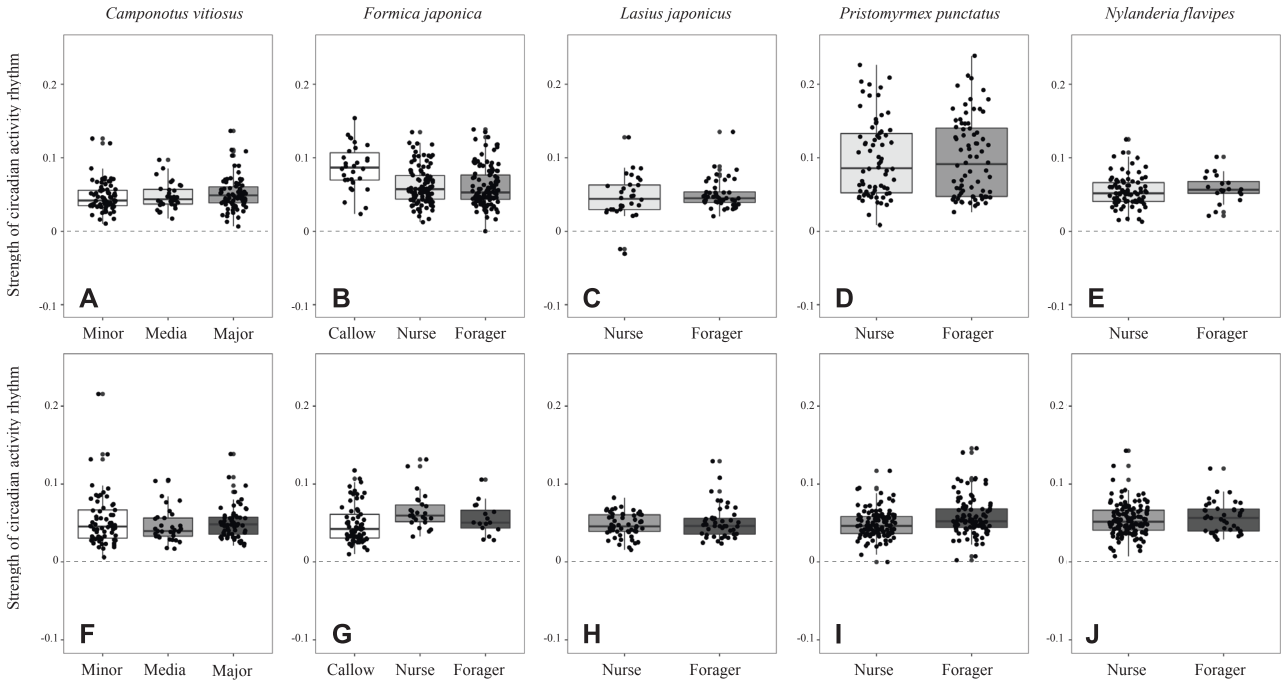

Fig. 3: Strength of circadian activity rhythms in five ant species. Individual activities were measured under light / dark (A - E) and constant dark conditions (F - J). The strength of circadian activity rhythm was calculated using an autocorrelation coefficient.

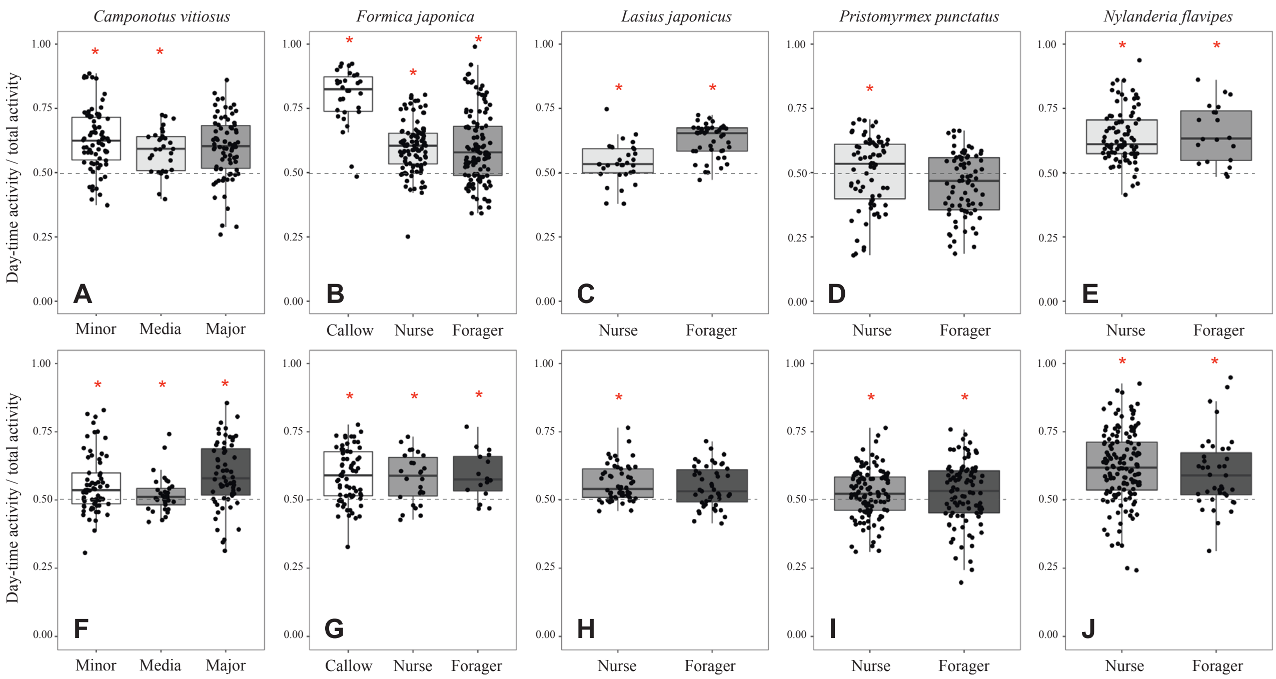

Fig. 4: Diurnal habits in five ant species. Individual activities were measured under light / dark (A - E) and constant dark conditions (F - J). Y-axis is the ratio of daytime activity per total activity. Asterisks indicate whether the average daytime activity ratio differs significantly from 0.5 ($p < 0.05$, one sample t-test).

Our first question was whether morphological castes could affect daily rhythms. Among monomorphic ants, there was significant variation in the strength of daily rhythms (Tab. S2). Thus, the extent of morphological diversity was not the main explanation for arrhythmic activities in ants. Additionally, previous studies suggested the possible evidence of shift work, referring to a multi-chronotype coexistence in an ant colony (Sharma & al. 2004b, Mildner & Roces 2017). However, our results showed that no worker displayed nocturnal activity pattern in any morphological caste in *Camponotus vitiosus* (Fig. 4B and G, Fig. S3). The variation in daily activity rhythms in the colony involved a shift work (i.e., a chronological division of labor), which adapts workers' activity rhythms to the needs of the colony throughout the day. However, shift work in social insects has been rejected by several previous studies (Moore & al. 1998, Charbonneau & Dornhaus 2015, Fujioka & al. 2019b), and supported by two previous studies (Sharma & al. 2004b, Mildner & Roces 2017). Our findings of arrhythmic activities in workers suggest that workers appear to perform an all-day-long colony activity without shift work.

Next, we investigated whether the nest type can affect daily rhythms. The nomadic ant *Pristomyrmex punctatus* exhibited clear daily rhythms under the LD condition. *Pristomyrmex punctatus* does not construct elaborate nests (Tab. 1) but lives on the ground and relocates frequently during the reproductive season (Tsuji 1988). Thus, this nomadic ant might be sensitive to light and active during a suitable time. In addition, we expected that arboreal ants might be more resistant to environmental fluctuations because they live under large fluctuations in light and temperature. The arboreal ant *Camponotus vitiosus* had weaker activity rhythms than the subterranean nesting ant *Formica japonica* (Tab. S1). However, there were no differences in rhythmicity among *C. vitiosus, Nylanderia flavipes*, and *Lasius japonicus* (Tab. S1). Our results showed that the nomadic characteristics can be related to the reaction towards light; however, there were no clear trends observed in arboreal versus subterranean nesting ants.

In addition, we investigated whether feeding habits can affect daily activity. Foragers typically exhibit clear circadian rhythmic activities in social insects (Bloch & Robinson 2001, Shemesh & al. 2007). No clear differences between nurses and foragers in activity patterns were observed at the individual level in this study. It is possible that omnivorous ants exhibit all-day-long activity that correspond to their wide range of food resources. *Formica japonica*, *Lasius japonicus*, and *Pristomyrmex punctatus* are omnivorous ants; they hunt insect prey and feed on aphid honeydew (Okamura & Yamane 1994, Katayama & Suzuki 2003, Suzuki & al. 2004, Kaneko 2018, Sunamura & al. 2020, Hyodo & Tanaka 2022). *Nylanderia flavipes* is a herbivore based on low isotope ratios of nitrogen (^{15}N / ^{14}N; $\delta^{15}N$) and carbon (^{13}C / ^{12}C; $\delta^{13}C$) (Hyodo & Tanaka 2022). *Camponotus vitiosus* is considered as an aphid-attending ant (H. Fujioka, unpubl.). Contrary to our expectations, the omnivorous ant *P. punctatus* tended to show clear daily rhythms (Fig. 1E), whereas the omnivorous ants *F. japonica and L. japonicus* and the herbivorous ants *N. flavipes* and *C. vitiosus* had low values of the strength of daily rhythms (Tab. 2). We conclude that feeding habits do not explain their circadian activity rhythms. The reactions of foragers' activity rhythms to food availability and the presence of competitors should be investigated in future studies.

We used laboratory-reared colonies of *Formica japonica* and *Lasius japonicus* and field-collected colonies of *Camponotus vitiosus, Nylanderia flavipes*, and *Pristomyrmex punctatus*. It is possible that the laboratory environment changes activity rhythms. Workers of both laboratory-reared colonies of *F. japonica* and field-collected colonies of *P. punctatus* showed daily rhythms. Thus, arrhythmic activity doesn't appear to be caused by laboratory rearing. Colony age and colony size can be potential factors controlling daily activity rhythms. Species with large colony sizes, *F. japonica* (600 - 46,000, see Kondoh 1968), *L. japonicus* (mature colony: several thousands), and *P. punctatus* (4000 to 320,000, see Tsuji 1988, 1995) had stronger daily rhythms than *C. vitiosus* (max. colony size: 500), and *N. flavipes* (average colony size: 200 - 300, see Masuko 2010) under the light-dark cycles. This study used young colonies (about one year old) of *F. japonica* and *L. japonicus*. In the future, we need to investigate the activity rhythms in mature colonies of these species.

Pristomyrmex punctatus foragers displayed bimodal activity peaks, one in the morning and the other in the evening (Fig. 1). Patterns of crepuscular activity have been described in several insect species (Helfrich-Förster 2000, Nisimura & al. 2005, Medeiros & al. 2014, El Keroumi & al. 2022). This activity pattern is considered as an adaptation to environmental changes (e.g., low latitude) because of avoidance of hot and / or dry daytime (Menegazzi & al. 2017). However, the ecological relevance of bimodal activity in *P. punctatus* remains unknown. Focal ant colonies were collected from a subtropical area (Okinawa) and a relatively warm temperate area (Okayama). One possibility is the adaptation to the environment by avoidance of activity during hot daytime.

Interestingly, *Formica japonica* callow workers entrained their daily activity rhythms to a light cycle. The experimental treatment was artificial because callow workers were not exposed to the light or sun in the wild. Even under DD conditions, half of the *F. japonica* callow workers exhibited daily activity rhythms. A previous study showed that the onset of circadian activity occurs in callows (< 1 week old) of the ponerine ant *Diacama* cf. *indicum* (Fuchikawa & al. 2014). By contrast, callow honeybee workers do not exhibit strong circadian activity rhythms, suggesting the postembryonic development of circadian activity rhythms (Moore 2001, Eban-Rothschild & al. 2012). Thus, the onset of circadian activity occurred earlier in two ant species than in honeybees. Further studies on the developmental process of circadian rhythms in ants are needed to demonstrate the differences in the development of circadian clock systems among social insects.

Our study revealed large variations in daily activity rhythms in locomotion among five ant species. For phylogenetic analyses to understand the evolution of ecological traits and behavior, descriptive papers of basic information on individual daily activity rhythms across species are required. Our findings thus contribute to advancing future study for the evolution of daily rhythms in the future.

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