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Research article

A case for a free-running circannual rhythm in soldier developmental time of Formosan subterranean termites

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Abstract. The success of all insect societies relies on their ability to maintain optimal levels of different castes. Here we report on an apparent free-running circannual rhythm that optimizes the developmental time of the soldier caste of *Coptotermes formosanus* Shiraki. Over a 3 year period, bioassays were conducted each month (except June) with groups of 100 termite workers in a 28°C incubator in total darkness. The number of days needed for *C. formosanus* soldiers to develop varied depending on the time of the year (month). In March, just prior to the major swarming exodus for alates (April to June), 9 days were required before a worker molted to a presoldier. Longer times were required for such a molt in all other months, with an increasing trend from April to December (from 13 to 30 days) and a decreasing trend from January to February (from 25 to 12 days). Colony origin or the length of time that termites were kept in the laboratory under constant conditions (26 – 28°C, 70 – 80 % RH) before testing (7 days – 1 year) did not affect this rhythm. This is the first demonstrated evidence of a free-running circannual rhythm in a social insect.

Keywords: Coptotermes formosanus, biological clock, soldier formation, caste regulation.

Introduction

Biological rhythms are ubiquitous in various activities of all major groups of living organisms (Koukkari and Sothern, 2006). These rhythms can be daily (circadian), weekly, seasonal, and annual (circannual). Biological rhythms often are synchronized with external environmental cycles (light cycle, temperature, humidity, food quantity and quality). Some of these rhythms become endogenously generated (i.e. become genetically programmed) and can persist in the absence of environmental signals (free-running). It is not surprising that many long-lived organisms of different taxa have evolved mechanisms to optimize their activities to the annual environmental fluctuations that are most significant to the species' survival and reproduction. This synchronization of physiological activities to environmental cues indicates the highly adaptive value of the behavior.

Free-running circannual rhythms have been identified in mammals, birds, lower vertebrates, invertebrates, and plants (Gwinner, 1986). The first evidence of a circannual rhythm among invertebrates was found in the carpet beetle, *Anthrenus verbasci* (L.). The diapause and pupation of this long-lived beetle occurs with a cycle between 10 and 11 months, even when under constant light, temperature and humidity (Blake, 1959).

Caste regulation of termites involves both intrinsic and extrinsic factors and has been studied by a number of investigators with different approaches including hormone, pheromone and molecular technologies (Lenz, 1976; Lefeuve and Bordereau, 1984; Park and Raina, 2004; Mao et al., 2005a; Scharf et al., 2005). In Formosan subterranean termites, Coptotermes formosanus Shiraki, soldier formation is readily observable in the laboratory and thus provides a good model for research on caste regulation. For the past several years, we have been conducting laboratory experiments trying to elucidate the chemical and physiological mechanism(s) that regulates soldier developmental time and numbers in C. formosanus (Mao et al., 2005b, unpubl.). In the process of these experiments we noted that the time required for soldier formation of C. formosanus in the control groups was quite

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Table 1. Total number of colonies, their collection date and number of replications performed in each month.

Month of testing	Number of colonies tested	Colony collection date (mm/dd/yy) [1]	Presoldier development time (day) [2]	Soldier development time (day) [2]	Total replications
January	3	11/07/03 [04] 12/08/03 [04] 05/24/04 [05]*	24.6 ± 1.3	33.8 ± 1.0	16
February	2	05/05/03 [04]* 01/12/05 [05]	11.9 ± 1.2	21.6 ± 1.9	9
March	3	05/05/03 [04]* 08/15/03 [04]* 01/12/05 [05]	8.6 ± 1.0	21.1 ± 1.3	13
April	1	01/12/05 [05]	13.7 ± 2.1	24.5 ± 0.8	6
May	1	05/24/04 [05]*	12.7 ± 0.9	24.7 ± 1.8	3
July	3	05/20/03 [03] 05/14/05 [05] 06/21/05 [05]	13.9 ± 1.6	28.0 ± 1.8	9
August	3	03/07/03 [03]* 05/05/03 [03]* 08/15/03 [03]	22.5 ± 1.9	35.6 ± 2.0	15
September	2	05/12/05 [05]* 06/21/05 [05]	16.3 ± 0.8	28.5 ± 0.8	6
October	2	05/05/03 [03]* 10/14/03 [03]	24.6 ± 2.0	37.8 ± 1.8	24
November	3	10/14/03 [03] 08/12/05 [05]* 11/09/05 [05]	29.5 ± 1.7	40.2 ± 2.1	13
December	1	11/07/03 [03]	30.3 ± 0.3	37.3 ± 5.2	3

[1] The numbers in brackets indicate the year that the tests were performed; * indicates the colonies that were kept in the laboratory for more than 3 months before testing.

[2] Mean ± SEM.

different depending on the time of the year. To validate these observations, we compiled all data from control groups of experiments conducted between July 2003 and November 2005. A case for a free-running circannual rhythm in C. formosanus soldier developmental time was discovered.

Materials and methods

Termites tested in this study were collected from 12 distinct field colonies in New Orleans and Lake Charles, Louisiana. Nine of them were collected using the milk crate-trapping technique (Smith et al., 2004) and kept in 140-L trash cans with lids in the laboratory under constant conditions (26–28°C, 70–80 % RH). The other three were collected using PVC tubes containing wood sticks (*Pinus* spp.) inserted into infested trees (see Henderson and Forschler, 1997). These collected termites were placed in 20.3 (diameter) by 7.6-cm clear plastic containers (Pioneer Plastics Inc., Dixon, KY) before closing them in a laboratory drawer with the above temperature and RH conditions. All collections were composed of workers and soldiers (nymphs were present occasionally). No reproductives were observed in these collections.

The experimental units were 100 by 15-mm Petri dishes, each containing two filter papers (7.5 cm in diameter) moistened with 3 ml of distilled deionized water and a moistened 37 by 37 by 2-mm balsa (*Ochroma lagopus* Swartz) wood piece as a food source. One hundred termite workers (at least 3rd instar) were introduced into the dishes and the dishes were sealed with Parafilm® and placed in a 28°C incubator in total darkness. These set-ups were used for a variety of experiments not reported here; this resulted in 117 replicates of control groups over a 3 year period from which we gathered the data for this report (Table 1). Observations were made daily and the days required for the first appearance of presoldiers and soldiers were recorded. At 40 days after treatment 92 of the replicates were dismantled and final termite presoldier, soldier and worker numbers were recorded. The remaining 25 replicates were evaluated beyond 40 days and were not dismantled until the first soldier appeared. Data were analyzed using SAS PROC MIXED followed by Tukey HSD for mean comparisons and PROC REG for regression analysis.

Results and discussion

The number of days needed for presoldier and soldier formation was significantly different among months ($P < 0.0001$) and showed a distinct pattern that was related to the time of the year (month). On average only 9 days were required before a worker molted to presoldier in March. Longer times were required for such a molt in all other months, with an increasing trend from April to December except September (from 13 to 30 days average) and a decreasing trend from January to February (from 25 to 12 days average). The number of days required for soldier formation was closely aligned with the formation time of presoldiers (Table 1, Fig. 1).

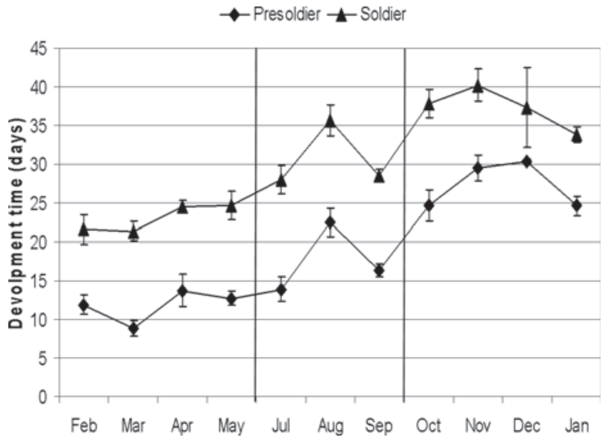

Fig. 1. Number of days required for the first presoldier or soldier formation in different months of the year. Two vertical lines divide the year into three stages (Feb.-May, July-Sept. and Oct.-Jan.) relevant to swarm activity with significantly different development time for presoldiers and soldiers and significantly different soldier proportions at 40 days after treatment (see Table 2).

Table 2. Presoldier and soldier development time and proportions in different stages relevant to swarm activity.

Stages	Replicates	Presoldier development time (day) [1]	Soldier development time (day) [1]	Presoldier proportion (%) [1]	Soldier proportion (%) [1]
Feb. to May	31	10.9 ± 0.8 a	22.2 ± 0.8 a	2.0 ± 0.4	9.4 ± 0.9 a
Jul. to Sep.	30	18.7 ± 1.3 b	31.9 ± 1.3 b	2.7 ± 0.4	3.4 ± 0.4 b
Oct. to Jan.	56	26.0 ± 1.1 c	37.2 ± 1.0 c	2.6 ± 0.5 [2]	3.2 ± 0.5 b
$F_{2,\,114}$, P		49.14, <0.0001	47.82, <0.0001	0.84, 0.4362	31.46, <0.0001

[1] Mean ± SEM at 40 days after treatment; means followed the same letter were not significantly different (Tukey, $\alpha = 0.05$).
[2] Number of replicate at 40 days after treatment was 31 (not 56); the degrees of freedom for the *F* test were 2 and 89 (not 2 and 114).

Table 3. Presoldier and soldier development time in different month of a colony collected on May 05, 2003.

Month of testing	Replicates	Presoldier development time (day) [1]	Soldier development time (day) [1]
February	6	10.3±1.4 a	18.7±1.9 a
March	3	10.0±2.0 a	23.0±2.1 a
August	6	18.7±1.5 b	31.3±1.5 b
October	12	22.4±2.0 b	35.5±1.7 b
$F_{3,\,23}$, P		8.42, 0.0006	16.8, <0.0001

[1] Mean ± SEM; means followed the same letter were not significantly different (Tukey, $\alpha = 0.05$).

To show the pattern more clearly, the data were grouped into 3 stages related to *C. formosanus* swarming activity periods. The results showed that for both presoldier and soldier the development time was significantly different among stages (Table 2, Fig. 1). Immediately prior to and during swarm (February to May) presoldiers and soldiers developed more quickly followed by the stage immediately post swarm (July to September) and the stage prior to swarm (October to January). The presoldier proportions at 40 days after treatment were not significantly different among stages. However, soldier proportions were significantly different with the highest proportion being immediately prior to and during swarm stage (February to May) indicating that fast development time resulted in a higher number of soldiers (Table 2). Regression analysis showed that the data significantly fit a linear model (P <0.0001) with days required for presoldier or soldier formation as a dependent variable and month as an independent variable (coded as February = 1, March = 2, etc). The model equations are $Y = 7.66 + 1.93X$ and $Y = 20.36 + 1.78X$ for presoldier or soldiers, respectively. The adjusted R^2 were 0.4237 and 0.3657, respectively.

Our results suggest that the pace of *C. formosanus* soldier development shows environmental independence and is controlled by an endogenous circannual mechanism. In most months, multiple colonies were tested, and yet the number of days for presoldier and soldier development remained fairly stable regardless of colony origin or the length of time in captivity under constant conditions (Table 1, Fig. 1). Moreover, a single colony tested in different months showed a great variation in presoldier and soldier development times. One example we can use to show this latter point is a colony collected on May 05, 2003 that was tested in August and October 2003 and February and March 2004. Presoldier and soldier development time of this colony were significantly different among months (Table 3). Furthermore, when data were analyzed based on the length of captivity before testing, termites kept in the laboratory for less than 3 months versus termites kept in the laboratory for more than 3 months, colony effect was significant only for the months of February and March (P ranged from 0.0069 to 0.0409). In February, termites in captivity for less than 3 months required significantly more time to form pre-

Table 4. Presoldier and soldier development time (day) of colonies with different captivity periods.

Month of testing		Colonies in less than 3 months captivity[1]	Colonies in more than 3 months captivity[1]
January	Presoldier	25.7 ± 1.1 (11)	22.0 ± 3.6 (5)
	Soldier	33.9 ± 0.6 (11)	33.4 ± 3.2 (5)
February[2]	Presoldier	15.0 ± 1.0 (3)	10.3 ± 1.4 (9)
	Soldier	27.3 ± 0.7 (3)	18.7 ± 1.8 (9)
March[2]	Presoldier	5.0 ± 0.0 (4)	10.4 ± 1.1 (9)
	Soldier	17.3 ± 0.8 (4)	23.2 ± 1.2 (9)
August	Presoldier	25.8 ± 4.2 (6)	20.3 ± 1.3 (9)
	Soldier	38.8 ± 4.3 (6)	33.4 ± 1.5 (9)
September	Presoldier	17.3 ± 0.3 (3)	15.3 ± 1.3 (3)
	Soldier	30.0 ± 1.0 (3)	27.0 ± 0.6 (3)
October	Presoldier	26.8 ± 3.4 (12)	22.4 ± 2.0 (12)
	Soldier	40.1 ± 3.1 (12)	32.5 ± 1.7 (12)
November	Presoldier	30.7 ± 2.3 (9)	26.8 ± 1.9 (4)
	Soldier	42.3 ± 2.7 (9)	35.5 ± 1.7 (4)

[1] Mean ± SEM (number of replicates).
[2] Development time were significantly different between colony categories (Tukey, $\alpha = 0.05$) in these months.

soldiers and soldiers than those in captivity for more than 3 months (Table 4). The opposite result was observed in March where termites with less than 3 months in captivity required fewer days than termites with more than 3 months in captivity (Table 4). The length of time in captivity had little effect on overall soldier development time. The soldier developmental time appeared to be independent of the influence of reproductives since collected termites, regardless of time away from reproductives of the natal nest, did not affect the circannual rhythm.

It is important to note that this study did not control for all environmental factors (and rhythms) that might affect soldier development. For example, barometric pressure has a yearly cycle with higher readings in winter months (and near sunrise) and lower readings in summer months (and near sunset). This cycle may play a role in controlling the early evening swarm activity of *C. formosanus* and also may influence soldier development in the laboratory. Geomagnetic field variations also can cycle rhythmically and influence biological processes but are very complex (Dubrov, 1978). Becker and Gerisch (1981) provided experimental evidence that geomagnetic fields can influence termite feeding activity rhythms. Further investigation is required to determine if our argument for a circannual rhythm in *C. formosanus* soldier development time is truly free-running (i.e. independent of environmental cycles).

A termite colony can be viewed as a complete, well-balanced unit where individuals are able to optimally respond to colony needs. The life span of a mature termite colony can range from several years to several decades (or more). The most notable annual rhythm in termites is alate production and swarming activity. Under field conditions, *C. formosanus* stages annual swarm that starts in April and ends in June with peaks in May (Henderson and Delaplane, 1994). Further investigation into termite swarm events proved that the pattern of large swarms and the intervals between large swarms was significantly correlated among years and did not appear to be trigged by any environmental cues examined (Nix, 2005). Nix (2005) argued that this provides support that the swarm event is controlled by intrinsic factors at a colony level, as earlier suggested by Henderson and Delaplane (1994). However, no experiments to demonstrate that swarm events are a free-running rhythm have been conducted.

Field observations show that soldier proportions peak at swarming time (Delaplane et al., 1991). The individuals of termite soldier caste have strongly sclerotized heads and mandibles (in most) specially adapted for defense. Because of this specialty, soldiers must be fed by workers, and thus there are a limited number of soldiers that a colony can support. Soldier proportions are species-specific with seasonal fluctuations (Haverty, 1977) that appear to coincide with defense needs. *C. formosanus* normally maintain soldier proportion at ≈10 % with seasonal increases occurring near the time of alate flight (between April and June) (Delaplane et al., 1991). Soldiers guard alate exit openings during the swarm (Emerson, 1939; Wilson, 1971) and also help to push out alates (Wilson, 1971). Individual soldiers also promote alate formation and increase their inclusive fitness (Henderson, 1998 and references therein). In our experiments workers took less amount of time to develop to presoldiers and then to soldiers immediately prior to and during swarm season which ensures higher soldier presence during the swarm. The reported circannual rhythm agrees with field observations on soldier numbers and indicates a well synchronized colony plasticity to deal with colony needs.

Entrainment of a free-running rhythm is an essential characteristic of biological rhythms and photoperiod is the most frequently identified external environmental pace setter (Koukkari and Sothern, 2006). However, for subterranean termites, photoperiod seems less important since they are living most of their lives in an enclosed dark environment and most individuals (workers and soldiers) are without eyes. The clock mechanism underlying the rhythm reported here is not known. Juvenile hormone (JH) plays an important role in termite soldier formation (Lenz, 1976; Hrdý, 1985). Higher JH levels result in presoldier and soldiers molt, while lower JH levels will result in formation of reporductives (Stuart, 1979; Henderson, 1998; Park and Raina, 2004; Mao et al., 2005a). Seasonal changes in JH titer in individuals of a *C. formosanus* colony do appear to have a pattern that benefits survival and reproduction of the species (Liu et al., 2005). The regulating mechanism of the reported circannual rhythm may include a differential response of protein and/or enzyme activities or gene expression to chemical cues such as JH.

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