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# Formosan Subterranean Termite (*Isoptera: Rhinotermitidae*) Soldiers Regulate Juvenile Hormone Levels and Caste Differentiation in Workers

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**ABSTRACT** A caste structure is maintained in termite societies and juvenile hormone (JH) is generally regarded as the most important regulator in these termite colonies. Here, we demonstrate that the soldier caste regulates JH in workers of *Coptotermes formosanus* Shiraki. Worker termites (80–100 individuals) were placed in petri dishes with 0, 5, 10, or 20% soldiers. JH III titers of groups of these workers were monitored at 14, 28, 42, and 56 d. Any changes in soldier caste proportions also were noted at each sample date. On the first sample date, the JH levels in workers were similar among treatments with different initial soldier proportions, and no new soldiers were formed. Over the next three sample dates, the worker JH levels were higher for low initial soldier proportion treatments and vice versa. Concurrently, soldier formation increased with lower initial soldier proportions. JH titers in workers showed a positive and statistically significant relationship to soldier numbers until a certain soldier proportion was reached. These results provide evidence that soldier caste proportions regulate JH levels and thereby caste differentiation in workers. The means by which this regulatory mechanism may proceed is discussed.

**KEY WORDS** *Coptotermes formosanus*, juvenile hormone, soldier formation, caste regulation

A CASTE STRUCTURE IS ESSENTIAL to the survival of eusocial insects. The caste system of termites consists of reproductives, soldiers, and, in higher termites, workers. In lower termites, such as *Coptotermes formosanus* Shiraki, workers are immature insects and do not represent a developmental end point. These workers are numerically the largest group in a colony and may undergo differentiation to become soldiers through a presoldier stage or develop into reproductives with appropriate stimuli. The caste composition of a termite colony at any given time is in a delicate balance. Caste regulation involves both intrinsic and extrinsic factors, such as pheromones, endocrine activity, genetic factors, temperature, and nutrition (Henderson 1998). Within a colony, self-regulation among the various castes occurs once a specific caste proportion is reached, e.g., high levels of soldiers inhibit new soldier formation (Lenz 1976, Renoux 1976, Haverty 1979, Haverty and Howard 1981, Park and Raina 2003), and nymphs inhibit new nymphal development (Miller 1969, Henderson and Delaplane 1994).

Juvenile hormone (JH) plays a key role in caste expression in the lower termites (reviewed and supplemented to by Henderson 1998). A species-specific soldier proportion (Haverty 1977) has been suggested

to be the result of variations in JH thresholds among species (Lenz 1976). Park and Raina (2004) documented that worker JH titers fluctuated (first increase and then decrease) in the process of reaching a certain soldier proportion for a population of workers (6,000 individuals) with no initial soldiers in *C. formosanus*. In addition, laboratory studies showed that the application of JH or JH analogues (JHAs) induced the transformation of workers to soldiers in many termite species, including *C. formosanus* (Howard and Haverty 1979, Howard 1983, Hrdý 1985, Okot-Kotber et al. 1991, Hrdý et al. 2001, Park and Raina 2003, Scharf et al. 2003). These results indicate that workers with high JH titers may transform into soldiers. However, Park and Raina (2004) reported that the JH titer of presoldiers produced from workers treated with the JHA methoprene was significantly lower than that of normally produced presoldiers suggesting that the biological mechanism of JHA may differ from natural JH; thus, caste differentiation studies with JHAs may not represent natural conditions (Scharf et al. 2003).

Intracolony JH regulation by soldiers has been suggested as a mechanism for the maintenance of caste proportions in the lower termites (Henderson 1998). Our objective was to determine whether soldier proportions regulate the endogenous JH titers of workers and the transformation of worker to soldier, and, if so, whether a relationship between changes in the JH titer

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of workers occurs when the proportion of soldiers changes in *C. formosanus*.

### Materials and Methods

Termites from two *C. formosanus* colonies were collected from infested trees on the Calcasieu River in Lake Charles, LA, by using wood pieces (southern yellow pine, *Pinus* sp.) in a polyvinylchloride tube inserted into the trunks of the trees. The experimental units were 100 by 15-mm petri dishes (VWR, West Chester, PA). Each dish contained two filter papers (7.5 cm in diameter, Ahlstrom Co., Mt. Holly Springs, PA) moistened with 3 ml of distilled deionized water and a moistened 37 by 37 by 2-mm balsa, *Ochroma lagopus* Swartz, wood piece. Groups of workers (80–100 individuals) were introduced into the dishes with 0, 5, 10, and 20% of soldier as treatments (total termites per dish, 100). The dishes were sealed with Parafilm (Pechiney Plastic Packaging, Menasha, WI) to retain moisture and placed into a 28°C incubator in constant darkness. In total, eight dishes were prepared for each treatment and colony combination. At 14, 28, 42, and 56 d after treatment (DAT), two samples of 50 workers were taken from two dishes from each treatment and colony combination to determine JH titers. The number of presoldiers, soldiers, and workers in each dish also was noted at each sample date. Final soldier proportions were calculated based on the number of presoldiers and soldiers.

For *C. formosanus*, the only JH homologue is JH III (Liu et al. 2004, Park and Raina 2004). JH titers were analyzed on a Trace 2000 series gas chromatograph (GC) coupled to a Finnigan Polaroid mass spectrometer detector (MS). An internal standard, JH III ethyl ester, was made from synthetic JH III (Sigma, St. Louis, MO) by using a mild transesterification method (Mori et al. 1973, Liu et al. 2004) and quantified on the same GC-MS system (Liu et al. 2004). The GC was equipped with a DB-5MS column (30 m by 0.25 mm by 0.25  $\mu$ m, J & W Scientific, Folsom, CA). The carrier gas was helium with a flow of 1.5 ml/min. The initial GC temperature was maintained at 60°C for 1 min and then increased at a rate of 40°C/min to 240°C and held for 10 min. The injection port and transfer line temperatures were set at 220 and 250°C, respectively. The ion source temperature was at 200°C. Termite samples were prepared according to Liu et al. (2004) and Park and Raina (2004). Fifty termite workers were homogenized in a mixture of 1 ml of hexane, 0.5 ml of acetonitrile, and 0.5 ml of 2% NaCl with 200 pg of internal standard by using a W-385 sonicator (Heat Systems Ultrasonics Inc., Farmingdale, NY). The homogenized samples were centrifuged at 4,000  $\times$  g for 10 min (IEC, Needham Heights, MA), and the supernatant was collected. Two additional extractions with 1 ml of hexane each were performed. The supernatants were combined and dried under nitrogen and resuspended in 200  $\mu$ l of hexane. The samples were cleaned by eluted through an activated Al<sub>2</sub>O<sub>3</sub> column (6 cm, 1 g of Al<sub>2</sub>O<sub>3</sub>) with 1.8 ml of hexane and 1.8 ml of 10% ethyl ether in hexane (E/H). JH III fraction was collected

by eluting 2.7 ml of 30% E/H hexane and dried under nitrogen. The residue was derivatized in a 60°C oven for 20 min after suspended in 75  $\mu$ l of *d*<sub>4</sub>-MeOH and 75  $\mu$ l of 5% trifluoroacetic acid in *d*<sub>4</sub>-MeOH. The derivatized samples were further cleaned by eluted through another activated Al<sub>2</sub>O<sub>3</sub> column with 1.8 ml of hexane and 1.8 ml of 30% E/H. The *d*<sub>3</sub>-methoxyhydrin derivative JH III was collected by eluting 2.7 ml of 50% E/H and then dried under nitrogen and resuspended in 6  $\mu$ l of hexane for analysis. Injections of 2  $\mu$ l were made and detected in a selected ion mode at *m/z* 76. JH titers were quantified based on the *m/z* 76 peak at a specific retention time determined by another injection of the same sample in a full scan mode (*m/z* 50–300). The specific mass fragments that we used for identifying *d*<sub>3</sub>-methoxyhydrin derivative JH III were *m/z* 76 and 225. For the internal standard they were *m/z* 76 and 239.

Data were analyzed using SAS version 9.0 (SAS Institute 1990). PROC MIXED with treatment or sample date as fix effects and colony as a random effect was performed followed by Tukey's procedure for mean separation at  $\alpha = 0.05$ . Linear regressions were performed with the changes in soldier proportion between 14 DAT and the next two or three sample dates (28, 42, and 56 DAT) as a dependent variable and the corresponding changes in JH titers as an independent variable.

### Results

Worker JH titers were not significantly different among treatments at 14 DAT ( $F = 0.90$ ;  $df = 3, 10$ ;  $P = 0.4820$ ), and no presoldier formation was observed in any of the treatments (Fig. 1; Table 1). At 28 DAT, worker JH titers in 0% soldier treatments were significantly higher than that of the other three treatments ( $F = 7.70$ ;  $df = 3, 10$ ;  $P = 0.0053$ ), indicating that the JH titers in workers increased more rapidly when soldiers were absent (Fig. 1). At 42 DAT, worker JH titers rose to a similar level in 0, 5, and 10% soldier treatments but stayed lower in the 20% soldier treatments ( $F = 9.70$ ;  $df = 3, 10$ ;  $P = 0.0026$ ). As JH titers increased in workers, more presoldiers were formed in 0, 5, and 10% treatments (Table 1). Worker JH titers dropped sharply in 0, 5, and 10% soldier treatments to a level similar to that of 20% soldier treatment ( $F_{3, 10} = 0.21$ ;  $P = 0.8851$ ) at 56 DAT. Concurrently, all treatments except the 20% soldier proportion treatment had increased their soldier ratio to  $\approx 12\%$  (Table 1).

When the data were analyzed within treatments over the sample dates, significant differences in worker JH titers were found in 0% ( $F = 24.05$ ;  $df = 3, 10$ ;  $P < 0.0001$ ), 5% ( $F = 20.34$ ;  $df = 3, 10$ ;  $P = 0.0001$ ), and 10% ( $F = 30.25$ ;  $df = 3, 10$ ;  $P < 0.0001$ ) soldier treatments but not in the 20% soldier treatment ( $F = 1.59$ ;  $df = 3, 10$ ;  $P = 0.2531$ ). Compared with that of 14 DAT, worker JH titers increased in 0, 5, and 10% soldier treatments at 28 and 42 DAT then dropped to a similar level at 56 DAT, whereas in 20% soldier treatment worker JH titers stayed the same through

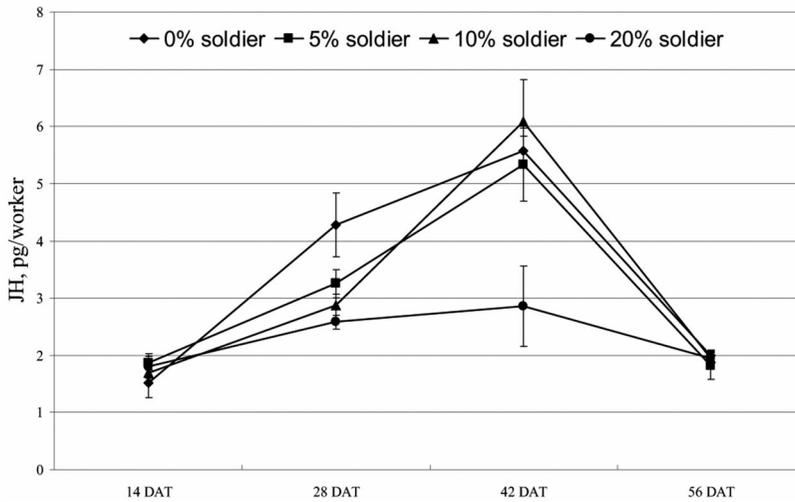


Fig. 1. Worker JH titers ( $\pm$ SE) at four sample dates with different initial soldier proportions.

out the experiment. A significant linear relationship was present between changes in worker JH titers and changes in soldier proportion up to 42 DAT (Fig. 2B) but not up to 54 DAT (Fig. 2A).

Discussion

Previously, the role of JH in soldier caste formation was mainly defined by the application of exogenous JH or JHA. We have shown here that the formation of soldiers is directly associated with higher endogenous worker JH levels in *C. formosanus*. More soldiers were formed with increasing worker JH titers. The results are in agreement with Park and Raina (2004) who showed that JH titers for a population of workers with no initial soldier presence were affected by changes in soldier production over time. In addition, the current study shows that the number of soldiers already present influenced the worker JH titers in a quantitatively and timely manner until a set soldier proportion was achieved, i.e., the change in worker JH titers was positively related to the change in soldier proportions up to 42 DAT before reaching a certain soldier proportion (12% in this study) at 56 DAT (Fig. 2B). This relationship disappeared when 56 DAT data were included in the regression analysis (Fig. 2A) and suggests that a threshold of soldier proportion may be required for JH suppression in workers, presumably to

reduce the frequency of worker transformation. This would explain why at 56 DAT, worker JH titers were at a similar level for all the treatments (a threshold of  $\approx$ 12% in this study was reached). The data also indicate that a certain amount of time (at least 42 d in this study) may be needed for soldiers to influence worker JH titers when the soldier proportion is below the threshold. When the number of soldiers is sufficient (above the threshold, 20% in this study), JH titers in workers remained at a constant level. It is possible that this time-lag effect of soldiers on changes in worker JH titers can sometimes spiral out of control. For example, in some of our laboratory colonies, supernumerary soldier populations have been observed, possibly the result of the combination of disturbance to the colony during collection and the need to produce nymphs and more soldiers in the colony.

*C. formosanus* soldier proportions are normally maintained at  $\approx$ 10% (Haverty 1977) with seasonal increases occurring especially near the time of alate flight (Waller and La Fage 1987, Delaplane et al. 1991). The main regulator seems to be the soldiers themselves because soldiers inhibit new soldier formation once an optimal soldier percentage is reached while nymphal (alate) development increases (Haverty 1979, Henderson 1998, Park and Raina 2003). Because soldiers develop in response to increasing amounts of JH and alates develop in response to decreasing

Table 1. Effect of initial soldier proportions on presoldier formation and final soldier proportions at different sample dates

Sample date	Initial soldier proportion							
	0%		5%		10%		20%	
	Presoldiers	% soldiers	Presoldiers	% soldiers	Presoldiers	% soldiers	Presoldiers	% soldiers
14 DAT	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	5.1 $\pm$ 0.0	0 $\pm$ 0	10.3 $\pm$ 0.1	0 $\pm$ 0	20.0 $\pm$ 0.3
28 DAT	3.0 $\pm$ 0	3.3 $\pm$ 0.2	0.5 $\pm$ 0.5	5.7 $\pm$ 0.5	0.3 $\pm$ 0.0	10.6 $\pm$ 0.4	0 $\pm$ 0	19.9 $\pm$ 0.3
42 DAT	5.5 $\pm$ 0.6	7.3 $\pm$ 0.8	2.3 $\pm$ 0.6	8.7 $\pm$ 0.9	1.8 $\pm$ 0.3	12.1 $\pm$ 0.7	0.5 $\pm$ 0.3	20.8 $\pm$ 0.5
56 DAT	4.8 $\pm$ 1.5	12.4 $\pm$ 1.0	2.5 $\pm$ 0.3	11.3 $\pm$ 0.8	1.0 $\pm$ 0.7	13.0 $\pm$ 0.4	0.5 $\pm$ 0.3	20.7 $\pm$ 0.7

Data are mean  $\pm$  SE ( $n = 4$ ). Percentages of soldiers were calculated based on the sum of number of presoldiers and soldiers.

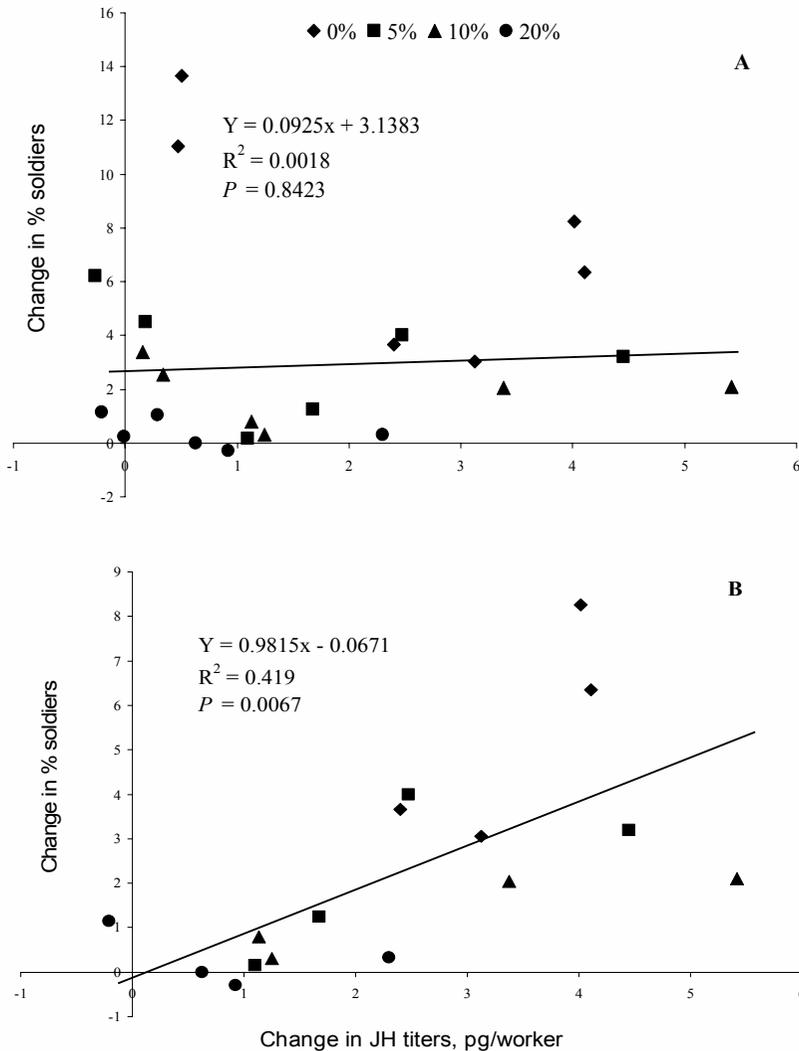


Fig. 2. Relationship between changes in JH titers and changes in soldier proportions up to 56 (A) or 42 (B) days after treatment. Changes were calculated as the values at 28, 42 and 56 d after treatment minus the values at 14 d after treatment.

amounts of JH, Henderson (1998) proposed a hypothesis that presoldiers/soldiers act as JH “sponges,” absorbing or otherwise taking away the pheromone from the system, thus inhibiting soldier formation and stimulating worker-to-alate transformation. The soldier is the only sterile caste in lower termites and gain inclusive fitness by pushing brothers and sisters to reproductive status. The current study supports that hypothesis by showing that existing soldiers lower the JH levels of their worker nestmates. It is interesting to note that although there is evidence that soldiers can stimulate the production of nymphs (Bordereau 1985, Springhetti 1985), the presumed mechanism of this occurring as a response to lower JH levels (Yin and Gillot 1975, Stuart 1979) does not seem to follow such a simple rule for *C. formosanus*. In both *Reticulitermes* spp. and *C. formosanus*, it is very easy to show soldier

production in the laboratory. However, *C. formosanus* nymphal development has never been observed in the laboratory (Raina et al. 2004), whereas it is common place for nymphs to be produced in laboratory groups of *Reticulitermes* spp. (Pawson and Gold 1996). It may be that the lower threshold for soldier production in *C. formosanus* compared with *Reticulitermes* spp. as proposed by Henderson (1998) is similar for nymphal development, i.e., an extremely low JH level is necessary. Small groups of workers in the laboratory may not be able to achieve this dynamic, which is achievable in the field. In addition, there are probably other factors involved in alate caste formation for the species.

The mechanism(s) by which soldiers influence worker JH levels could be physiological, behavioral, or a combination. Pheromones associated with soldiers

have been suggested to act as repressors in soldier formation (Lüscher 1961). Okot-Kotber et al. (1991) reported that soldier head extracts from several termite species could block soldier formation in *Reticulitermes flavipes* (Kollar) and that the inhibitory component(s) was not species specific. Park and Raina (2003) observed that physical contact is required for soldiers to inhibit worker-soldier transformation in *C. formosanus*. An alternative to soldiers acting as JH sponges is the possibility that during physical contact workers pick up some chemical cues from soldiers that trigger a reduction of JH biosynthesis or an increase in the amount and/or activity of JH-metabolizing enzymes. In addition, the hypothesis that soldiers produce an anti-JH agent (Lüscher 1972) cannot be discounted. The identity and/or the mechanisms behind the pheromonal signals in *C. formosanus* remain unknown and are being investigated. Lefeuve and Bordereau (1984) provided some insight by their discovery of a primer pheromone in the soldier's unique frontal gland that regulates soldier formation in *Nasutitermes lujae* (Wasmann).

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