Molting in workers of the Formosan subterranean termite

*Coptotermes formosanus*

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Abstract

The Formosan subterranean termite, *Coptotermes formosanus*, with its huge colonies, is a major urban pest in several southern states and Hawaii as well as in South Asia. Because of their cryptic nature (underground habitat) and very long life cycle, not much is known about molting in termite workers. In *C. formosanus*, the workers stop foraging and lose their gut fauna, respectively, approximately 10 and 5 days prior to ecdysis. In any given colony an average of 1.01% (range 0.6–1.8) of the workers were found to molt each day under laboratory conditions. Workers destined to molt become sluggish and their head capsules develop a mottled texture one day prior to ecdysis. Ecdysis was generally accomplished with the assistance of other workers, which also fed on the exuviae. Immediately after molting worker mandibles were light pink in color and became fully melanized approximately two days later. Gut fauna were acquired on the fourth day after molting. Flagellates were transferred as small encysted cells from other workers through proctodeal feeding. Juvenile hormone III titer ranged between 30–41 pg/mg bodyweight in all stages except in workers sampled 6 days prior to ecdysis. In these workers the titer was 80.5 pg/mg. The high juvenile hormones (JH) titer may also be involved in causing defaunation. Ecdysteroid titer increased from 2.1 pg/mg in non-molting workers to 359.5 and 332.4 pg/mg one and two days following defaunation, respectively. There was a second smaller peak two days prior to ecdysis.

Keywords: Formosan subterranean termite; *Coptotermes formosanus*; Molting; Gut fauna; Juvenile hormone; Ecdysteroids

1. Introduction

Insect development involves the integration and coordination of growth, metamorphosis and shedding of the hard exoskeleton (Laufer, 1983). Hemimetabolous insects, which include the order Isoptera, undergo an incomplete metamorphosis, with the immature stages resembling the adult. The social insects including termites have a highly developed caste system, with each caste exhibiting marked differentiation in specialized structures to suit a particular function. Apart from molting associated with normal growth, the appearance of a particular caste is preceded by a molt. In general the process of molting involves apolysis, new cuticle formation and ecdysis. Unlike most other insects, very little is known about molting in termites, particularly in the subterranean group. Reasons for the lack of information include the absence of synchronization in the timing of the molt, the cryptic nature of subterranean termites and their long life-cycle. To the best of our knowledge, molting in termites has been studied only in *Kalotermes flavicollis*, (Soltani-Mazouni and Bordereau, 1987), where developmental changes in cuticle, ovaries and colleretal glands between worker–worker and worker–neotenics were compared.

The wood-feeding, lower termites essentially depend on symbiotic gut microflora, comprised of protozoa and bacteria, for the digestion of cellulose (Breznak, 1982). The Formosan subterranean termite *Coptotermes formosanus*, harbors three types of flagellates; *Pseudotrichonympha grassii*, *Holomastigotoides hartmanni* and *Spirotrichonympha leidyi*, as well as spirochetes (Fig. 1) (Breznak and...
Several researchers have reported that termites preparing to molt stop feeding and empty their gut contents (Cleveland, 1925; Buchli, 1958; Soltani-Mazouni and Bordereau, 1987). However, only in *K. flavicollis*, has the time of gut voiding been precisely determined. It occurs seven days before ecdysis (Soltani-Mazouni and Bordereau, 1987). It has also been suggested that the newly molted individuals reacquire gut fauna either by feeding on excrement or anal fluids (proctodeal prophylaxis) of other workers. A time frame for such activity has not been provided.

The most important developmental hormones in insects are ecdysones and juvenile hormones (JHs). In general, 20-hydroxyecdysone is the molting hormone, periodic surges of which cause events involved in molting (Riddiford and Truman, 1978). In contrast JHs are status-quo hormones that maintain the insect in its current form (Truman and Riddiford, 2002). Changes in the levels of these hormones not only regulate the molting process, but also determine the type of molt. In termites the ontogeny of each organ depends primarily on the relative concentrations of JH and ecdysone with intermediate levels of JH resulting in stationary molts (Lebrun, 1991). Although hormonal regulation of molting has been extensively studied among insects, particularly lepidopterans, there are very few reports concerning hormonal changes associated with molting in termites. Okot-Kotber (1983) reported ecdysteroid levels associated with epidermal events during worker and soldier differentiation in *Macrotermes michaelseni*. Later, Okot-Kotber et al. (1993) studied changes in the levels of the two hormones in isolated workers of *Reticulitermes flavipes* as they molted into pre-soldiers.

In the current study of *C. formosanus* workers, we developed a staging system to identify physiologically synchronous individuals for the period prior to, during and after the molt. We identified the time when gut fauna are lost and reacquired, and monitored the fluctuations of JH and ecdysteroid titers.

### 2. Materials and methods

#### 2.1. Collection of termites

For frequency of molting and JH titer determination, termites from four separate colonies were collected during June–July 2004 in bucket traps set up in four different areas in New Orleans, LA. Soldier proportions in all four colonies were <5% at the time of collection. The termites were placed on moist spruce slats in plastic boxes and held in an incubator maintained at 28 ± 1°C, 70 ± 5% RH and constant darkness. To study the loss and reacquisition of gut fauna and determine ecdysteroid titers, termites were
collected during May 2006 from a single colony in City Park area of New Orleans.

2.2. Molting frequency and sequence

Since workers collected in bucket traps do not molt for approximately 10 days following their collection, we used termites that had been in captivity for more than 15 days to determine the frequency of molting. These termites were examined daily for nine days and the number of newly molted individuals recorded. In another experiment, termites (either 1000 workers alone or with 100 soldiers) from three colonies were placed in plastic boxes and provided with moist spruce slats. The containers were examined every other day for 1 month for incidence of molting. Data were analyzed by a two-way ANOVA using the GLM procedure (SAS, 8.2). To determine the sequence of molting, 100 termite workers from one of the colonies provided with moist spruce slats. The containers were examined every other day for 1 month for incidence of molting. Data were analyzed by a two-way ANOVA using the GLM procedure (SAS, 8.2). To determine the sequence of molting, 100 termite workers from one of the colonies were placed on 1 cm² pieces of blue diet (agar 750 mg, cellulose 4 g, Nile blue A 4.5 mg, water 25 ml) in 90 × 15 mm² Petri dishes. After 48 h, workers that did not pick up the blue color and had thus voided their guts in preparation to molt, were removed and placed in Petri dishes on moist filter paper and examined daily for ecdysis. This experiment was repeated five times. A few of the pre-molt workers were placed under a time-lapse video camera (Panasonic AG-6740 recorder, and Mycroflex™ video imaging system, EmCal Scientific, San Diego, CA) to obtain a visual record of the process of ecdysis.

2.3. Loss and reacquisition of gut fauna

As in the previous experiment, workers were placed on blue-diet and those that did not pick up the blue color within 48 h were transferred to Petri dishes with moist filter paper. Worker abdomens were gently squeezed into a drop of saline, which was examined for the presence of gut flagellates and spirochetes to confirm gut voiding. In the experiment to determine the time of reacquisition of gut fauna, three Petri dishes each with 10 newly molted workers and 90 blue-colored workers were set up. Three molted workers, one from each dish, were examined each day for 9 days for gut fauna as described previously (Raina et al., 2004). Briefly, the alimentary canal of each worker was dissected in 100 μl phosphate-buffered saline (PBS), and the flagellates and spirochetes were counted from two fields under phase contrast using a Bright-Line® hemocytometer (Reichert, Buffalo, NY) and Olympus BX 60 microscope. In the third experiment, to determine the exact time of reacquisition, six Petri dishes each with five newly molted and 45 blue workers were set up. Blue workers were removed from one of the dishes after one day and the process repeated with the remaining dishes on days 2, 3, 4 and 5. On the sixth day molted workers in all the dishes were examined for gut fauna.

2.4. JH and ecdysteroid titers

Workers from one of the colonies were examined under transmitted light of a dissecting microscope and all those having a clear gut were removed. Dissection of these workers confirmed that all of them had voided their guts. Subsequently, each day, additional workers with clear guts were removed and placed in separate Petri dishes with moist filter paper. For JH titer determination, we used workers 6 and 1 d prior to ecdysis (based on the observation that gut was voided 6 d before ecdysis), during ecdysis and 1 d after ecdysis in addition to control workers that were not destined to molt within 6–8 days. Because it was extremely difficult to obtain sufficient amounts of hemolymph from Formosan subterranean termites for JH determination, we instead used whole body extracts of 10 workers/sample for our assays. All termite samples were weighed before processing. An internal standard containing 2 ng of JH III ethyl ester in acetonitrile was added to all samples. Samples were analyzed for JH titers by gas chromatography-mass spectrometry (GC-MS) according to Bergot et al. (1981) with modifications as previously described (Park and Raina, 2004). Samples were collected and analyzed in triplicate.

For the determination of ecdysteroid titers we used a new colony collected from the City Park area in New Orleans, 1 year after hurricane Katrina. About 1000 workers were examined every day and those that appeared to have voided their guts were removed. Gut voiding was further confirmed by gently squeezing the abdomen with forceps over a drop of saline. Presence of any flagellates indicated that the gut had not been fully purged. Such workers were discarded. The selected workers were marked as 6 d. Hence, for these determinations we used workers 5, 4, 3, 2, and 1 d before ecdysis, during ecdysis, 3–4 h and 1 d after ecdysis. Five workers of each group were weighed and placed singly in 0.5 ml of 75% methanol in 1.5 ml microcentrifuge tubes. After homogenization and centrifugation at 14,000 g for 5 min, the supernatant was removed and the pellet was redissolved in 0.5 ml methanol by vortexing. Tubes were centrifuged as before and the two supernatants were combined in 0.5 × 50 mm² borosilicate culture tubes. After drying in a Speed Vac, the tubes were shipped to Beltsville for ecdysteroid determination. Ecdysteroid titers were measured using an enzyme immunoassay developed by T. Kingan (Kingan, 1989) as described in Gelman et al. (2002). The data were analyzed using one-way ANOVA followed by Dunnett’s post test.

3. Results

3.1. Molting frequency and sequence

Workers collected from the bucket traps and brought into the laboratory did not molt for first 10–15 d. In the experiment to determine frequency of molting under laboratory conditions and performed 15 d after trapping,
an average of 0.6% of the workers molted on the first day of observation (data not presented). The frequency gradually increased and reached 1.8% on day 6, thereafter leveling at about 1.2% per day. In the experiment with workers from three colonies, with and without the presence of soldiers and observed for a period of 1 month, the average daily incidence of molting was 1.14% (Table 1). Although more workers molted in the presence of soldiers the difference was not statistically significant (data not presented). Two small peaks in the incidence of molting were observed, first on day 6 and the second on day 24. By day 28 the incidence of molting dropped to 0.5%.

When workers were placed on blue diet, most of them picked up the blue color within 48 h. Only the ones that were preparing to molt did not acquire any blue color and had lost or were in the process of losing their gut fauna. Through careful monitoring of these individuals, it was determined that gut voiding took place approximately 6 d before ecdysis. This could also signal the initiation of apolysis. The day before ecdysis, workers became inactive and their head capsules developed a mottled appearance (Fig. 2A). Within the next 12 h, the workers assumed a coma-like posture (Fig. 2B) and the old cuticle had visibly separated. Since an individual termite undergoing ecdysis was not able to complete ecdysis by itself, removal of the old cuticle was accomplished with the help of other workers that quickly consumed the discarded exoskeleton, except for the mandibles. Soon after ecdysis the workers were white in color and had light-pink mandibles (Fig. 2C). After 24 h, the mandibles turned orange (Fig. 2D) and within the next 24 h, they were fully melanized. The visual observations of these events were further confirmed with the help of time lapse video recordings.

3.2. Loss and reacquisition of gut fauna

As mentioned above, the guts of C. formosanus workers contain three species of flagellates as well as spirochetes (Fig. 1). Most of the workers preparing to molt lost their gut fauna approximately 6 d prior to ecdysis. This was confirmed by monitoring the time when feeding ceased and the gut was visibly clear. Removal and examination of the guts of a few randomly selected individuals enabled us to further confirm the loss of gut fauna. When newly molted workers were placed in the presence of blue-colored workers, the former did not acquire any of the flagellates or spirochetes for the first 3 d following ecdysis (Table 2). Only in one case were a few H. hartmanni found 3 d after molting. Although the numbers were relatively low, all three species of flagellates and spirochetes appeared for the first time 4 d after ecdysis. Apart from the few well-defined

Table 1

<table>
<thead>
<tr>
<th>Colony</th>
<th>Percent molting among Workers + soldiers</th>
<th>Workers only</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-18</td>
<td>1.45 ± 0.15</td>
<td>0.95 ± 0.13</td>
</tr>
<tr>
<td>U-48</td>
<td>1.47 ± 0.18</td>
<td>0.84 ± 0.13</td>
</tr>
<tr>
<td>CP-1956</td>
<td>0.86 ± 0.17</td>
<td>1.19 ± 0.15</td>
</tr>
<tr>
<td>Mean</td>
<td>1.26 ± 0.35</td>
<td>0.99 ± 0.18</td>
</tr>
</tbody>
</table>

Colonies contained either 1000 workers with 100 soldiers or 1100 workers. Averages are of three replicates for each colony ± 5D.
There were large numbers of very small cells with cilia. After 7–8 d, the numbers of both flagellates and spirochetes increased significantly but were still low compared to those in healthy field-collected workers (Table 2). In another experiment, blue workers were sequentially removed after keeping them with newly molted workers for 1–5 d, and examining the latter on the sixth day. We did not find any flagellates or spirochetes if the blue workers were removed during the first 3 d (data not shown). However, when blue workers were removed on day 4 and 5, flagellates and spirochetes were always present in the guts of the molted workers.

### 3.3. JH and ecdysteroid titers

The titer of JH III in control workers (collected approximately 1 month before extraction) was 41.3 pg/mg wet body weight (Fig. 3A). The titer increased significantly to 80.5 pg at the time of gut voiding which occurs approximately 6 d before ecdysis, and dropped to control level(s) in workers one day before and after ecdysis as well as during ecdysis. Ecdysteroid titers, which were extremely low (2.1 pg/mg wet body weight) in control workers, showed a 180 fold increase 5 days before ecdysis (Fig. 3B). Titers remained high for another day, and by day 3 prior to ecdysis, levels dropped and approximated that of control workers. There was a second smaller peak that occurred 2 days before ecdysis followed by yet another decrease to about 12–15 pg that was observed during and 1 day after ecdysis.

### 4. Discussion

In most insect species, separation of the epidermis from the overlying cuticle, also known as apolysis, is considered to be the first step in the molting process that culminates in ecdysis (Wigglesworth, 1973). In the case of *C. formosanus* and possibly other termite species, cessation of foraging followed by the loss of their gut fauna is loosely associated with the initiation of molting. Most of the details related to the cascade of events leading to ecdysis in a termite species are described here for the first time. The fact that workers, freshly collected from bucket traps, did not molt for 10–15 days after collection indicated that, in a field colony, workers destined to molt may stop foraging at least 10 days prior to ecdysis. Buchli (1958) had reported that just as in other insects, termites preparing to molt stop eating and almost completely empty their gut. In our study of *C. formosanus*, we found that gut voiding took place 6 d prior to ecdysis. The next observable stage, 1 d prior to ecdysis, was when workers became sluggish and their head capsules acquired a mottled appearance. Shedding of the cuticle required the assistance from additional workers, which would in all instances consume the discarded tissue. According to Grassé (1949) quoted in Lainé and Wright (2003) in *Reticulitermes* spp., the exuviae could be eaten by the individual itself or by other workers.

There have been no previous estimates of the percentage of workers in a termite colony undergoing a molt at any given time. Haverty and Howard (1979) reported that staging among several species of subterranean termites including *C. formosanus* was impossible because of...
asynchronous molting. This can probably be attributed to the presence of a highly fecund queen in a mature colony, and the continuous production of new workers. Under our laboratory conditions approximately 1% of the workers in a colony molted each day. In colonies kept under laboratory conditions for more than a year and those with low moisture, the frequency of molting is generally even lower. Absence of soldiers did not significantly alter the proportion of workers molting each day although, some of them exhibited a worker to pre-soldier molt. However, the frequency in field colonies may vary with the younger workers molting more frequently than the older workers. With that low percentage of workers molting each day, theoretically it would take more than three months for all the workers in a colony to undergo one molt. This has important implications in estimating the time required to eliminate a colony through the use of chitin synthesis inhibitors in baits, as these chemicals are supposed to only disrupt new cuticle formation and thus the molting process.

Staging of workers that had just voided their gut was made possible by feeding workers on blue diet. Individuals that did not pickup the blue color had voided their gut, which was further confirmed by examination of the gut contents of live workers as well as dissection of randomly selected individuals. It was established that workers preparing to molt, void their gut approximately 6 d before ecdysis. Soltani-Mazouni and Bordereau (1987) reported that K. flavicollis workers maintained at 27 °C voided their gut 7 d prior to ecdysis. Cleveland (1925) had speculated that in R. flavipes, protozoa may be destroyed prior to ecdysis through feeding on salivary secretions from other nestmates. Although there is no direct evidence as to the presence of a regulatory factor involved in defeaunation, it is possible that the high JH titer observed 6 d prior to ecdysis and coincident with the loss of gut fauna, is involved in triggering defeaunation. Haverty and Howard (1979) have reported that exposure of R. flavipes workers to methoprene, a JH analog, caused defeaunation. This hypothesis needs further investigation. According to the dogma, the gut fauna is reacquired by a newly molted worker through proctodeal feeding (Cleveland, 1925; Buchli, 1958). Again using blue-dyed workers, we established that no flagellates were acquired by a newly molted worker during the first 3 d after ecdysis. Acquisition was first observed on the 4th day, presumably through proctodeal feeding from non-molting workers. It appears that the usually large protozoa are acquired as small encysted cells that rapidly transform into their normal shape and size. Transfer of encysted cells may have evolved to escape the grinding mechanism present in the foregut that functions to break the wood into small particles. Nalepa (1984) has reported that as the wood roach Cryptocercus punctulatus, prepares to molt, the symbiotic flagellates present in its gut undergo gametogenesis and encystment. After molting, as the newly molted roaches start feeding, the protozoa are transferred as encysted cells by feeding on anal fluids of other individuals.

Two classes of hormones, juvenoids and ecdysteroids, are involved in molting. Whereas, ecdysteroids trigger apolysis and regulate new cuticle formation, juvenoids determine the nature of the molt (Truman and Riddiford, 2002). It is also known that relative titer differences are much more relevant than absolute titer values to trigger stage and tissue-specific hormone cascades. C. formosanus workers have a very low JH titer when collected from the field (< 10 pg/mg wet weight). The titer increases to > 40 pg within 1 month of their being brought into the laboratory leading to increased soldier production (Park and Raina, 2005). Subsequently the titer decreases and remains between 20 and 30 pg/mg. Okot-Kotber et al. (1993) reported that in R. flavipes workers, JH titer increased immediately after their removal from the colony and peaked at 6–8 days before declining. The two fold increase in JH titer of workers 6 days before undergoing ecdysis appears to be linked to the molting process. However, for the 2–5 d period prior to ecdysis, we did not determine the JH titer. In contrast, the ecdysteroid titer in our experiments, increased 170 fold 5 d prior to ecdysis, and persisted at these high levels for 2 d before the sharp decline. Okot-Kotber et al. (1993) also reported a sharp rise in ecdysteroid titers on day 8 followed by a sharp decline on day 9 in isolated workers (from which soldiers had been removed) of R. flavipes (the isolated workers generally have a tendency to molt into pre-soldiers). In C. formosanus, we observed another, much smaller peak, 2 d before ecdysis. Okot-Kotber (1983) citing Riddiford and Curtis (1978) suggests that the first peak of ecdysteroids may be important for apolysis whereas the second peak that occurs when JH levels are low may result in a worker-worker molt.

We have for the first time provided a description of the events that precede ecdysis in a subterranean termite and have developed a staging system to identify physiologically synchronous workers. We also report the time that flagellates and spirochetes are eliminated from the gut and then reacquired following molting. Measurement of JH and ecdysteroid titers at times prior to, during and following the molt provides an insight into the role of these developmental hormones in termite molting.

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