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Trophic Path of Marked Exuviae Within Colonies of *Coptotermes gestroi* (Blattodea: Rhinotermitidae)

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Abstract

Nitrogen, a limiting growth factor in wood-feeding insects, was hypothesized to play a role in the recently discovered behavior of subterranean termites returning to the nest to molt. *Coptotermes gestroi* (Wasmann) exuviae is approximately 11% N by dry weight, and therefore a potentially rich source of recyclable nitrogen. Exuviae from a *C. gestroi* colony were marked with immunoglobulin G (IgG) and were fed to two-year-old *C. gestroi* colonies. IgG-marked exuviae were detected with an enzyme-linked immunosorbent assay. The IgG marker was later detected in every caste and life stage except first-instar larvae (L_1). The proportion of individuals positive for the marker varied by caste, with the queens always being positive for the marker. The queens and second-or-higher-instar workers (W_{2+}) had significantly higher concentrations of the marker than the eggs and L_1. The trophic path of exuviae includes individuals that directly fed on marked exuviae (workers and possibly second-instar larvae) and individuals that secondarily received marked exuviae through trophallaxis (queens, kings, and soldiers). This study described the trophic path of consumed exuviae and demonstrated its role in the recycling of nitrogen in a subterranean termite. Molting at the central nest may be an efficient means to transfer nitrogen from shed exuviae to recipients and may be a nitrogen recycling behavior conserved from a termite ancestor.

Key words: exoskeleton, nitrogen conservation, molting

As nitrogen is limited in woody tissues (~0.03 to 0.15% N) (Cowling and Merrill 1966), the ancestor of wood-feeding termites had to evolve several nitrogen conservation strategies to enable colony growth (Nalepa 1994, 2011). Nitrogen is partially obtained through associations with nitrogen-fixing symbionts and their products, and also trophically acquired through feeding on eggs, egg cases, exuviae (shed exoskeletons), dead nestmates, fluids originating from nestmates, feces, or nitrogen-rich soils (Mauldin and Smythe 1973, La Fage and Nutting 1978, Nalepa 1994, Mullins et al. 2021). Proctodeal trophallaxis, i.e., the exchange of alimentary fluids from the anus of the donor to the mouth of the recipient (McMahan 1969), can be used by the colony to transfer nitrogenous compounds and other nutrients resulting from the digestion of wood between individuals (Leach and Granovsky 1938, Machida et al. 2001, Nalepa 2015). This allows for the dynamic allocation of nutritional resources within termite colonies (Nalepa 1994, 2015; Machida et al. 2001). Termite workforce is comprised of individuals maintained in a permanent juvenile state (small body size, thinner cuticle), a trait that originated during the evolution of eusociality in termites (Nalepa 1994, 2011, 2015) which minimizes nitrogen use, since insect cuticle is composed mainly of protein and chitin (e.g., amino acids and N-acetylglucosamine, Kramer et al. 1991). The fact that hemimetabolous insects must molt several times during their development impacts the nitrogen demands of the colony, as *Coptotermes gestroi* (Wasmann) exuviae contain approximately 11% N by dry weight (Tong et al. 2021). Mira (2000) estimated that over ~58% of the nitrogen in exuviae may be recycled through post-molt feeding in the American cockroach, *Periplaneta americana* (L.) (Blattodea: Blattidae). *Coptotermes* termites also engage in post-molt feeding on exuviae – nestmates quickly consume the exuviae of newly-molted termites (Raina et al. 2008, Xing et al. 2013, Kakkar et al. 2016), a trait conserved from their wood-feeding ancestors (Nalepa 1994).

The primitive nesting type of termites is 'one-piece,' which refers to the behavior of nesting within the food source (Abe 1987), while the more derived 'intermediate' and 'separate-piece' nesting types refer to termites that forage outside of their nests (Higashi et al. 2000). *Coptotermes* termites forage outside of their nesting sites, and it was recently found that *C. formosanus* Shiraki (Blattodea: Rhinotermitidae) workers always return to the central nest (location of the reproductives and brood) to molt, leading to the hypothesis

that, in addition to molting at the safest place possible (Xing et al. 2013), this behavior could be tied to efficiently provision the queen and/or brood with nitrogen recycled from exuviae (Kakkar et al. 2017, Tong et al. 2023). One-piece termites molt at the nest by default, and it is unknown if other separate-piece termites also always return to the central nest to molt. Therefore, the behavior of returning to the central nest to molt may: 1) be a conserved ancestral trait, 2) have resulted from constraints related to molting [e.g., reduced risk of predation, presence of nonforaging nestmates to aid in molting, reduction of nonfeeding termites at foraging sites and tunnels (Kakkar et al. 2017, Xing et al. 2013), and/or centralized reacquisition of gut fauna lost prior to ecdysis (Raina et al. 2008)], and/or 3) have been selectively reinforced by efficiently recycling nitrogen from exuviae to needy individuals in the central nest. Regardless of the nature of the behavior of returning to the central nest to molt, nitrogen conservation via exuviae consumption in subterranean termite colonies can have an effect on colony growth (Tong et al. 2023).

If exuviae are fed to specific castes and instars, especially those that are confined to the central nest, returning to the central nest to molt would be an efficient means to optimize redistribution of nitrogen to individuals that require it the most, such as queens (for egg production) and larvae (for growth) (Dadd 1985). *Coptotermes* kings and queens can invest about half of their nitrogen reserves to their brood during colony foundation in a nitrogen-poor environment (Mullins and Su 2018, Chouvenc 2022), and nitrogen supplementation in *Zootermopsis* spp. Emerson (Blattodea: Archotermopsidae) increased offspring production (Shellman-Reeve 1990, 1994). These examples reinforce the idea that nitrogen is a limiting factor for reproduction in termites and suggest that nitrogen supplementation can have a positive effect on queen fertility and colony growth in subterranean termites as well (Tong et al. 2023, Konishi et al. 2023). However, how termites redistribute recycled nitrogen within the colony remains speculative. Determining the trophic path of exuviae within a subterranean termite colony may lead to insights on how nitrogen resources are distributed within the colony. If there is no pattern to the trophic path, then the hypothesis that termites return to the central nest to efficiently recycle nitrogen from exuviae to the queen and larvae may be rejected. However, if there are differences in which individuals feed on/are fed exuviae, the hypothesis may be further explored. Finding the trophic path of recycled exuviae within a colony subset can also reveal nitrogen allocation processes within a growing colony.

Immunomarking is a highly specific, nontoxic, and relatively inexpensive means to mark and study arthropods (Hagler and Machtley 2016). A unique biomarker, rabbit immunoglobulin G (IgG), can be used to mark food items, and the presence of the IgG can be detected from the bodies of the consumers using an enzyme-linked immunosorbent assay (ELISA) (Hagler and Durand 1994). This method has previously been used to study feeding relationships in *Reticulitermes flavipes* (Kollar) (Blattodea: Rhinotermitidae) (Buczkowski et al. 2007).

The objective of this study is to determine the trophic path of *C. gestroi* exuviae within colony subsets by using an IgG marker and ELISA procedure. We hypothesized that recycled exuviae are fed to the queen or larvae, as they are found exclusively in the central nest (Du et al. 2016) and likely have higher nitrogen requirements than other colony members owing to their reproductive and growth needs, respectively (Mattson 1980, Dadd 1985).

Materials and Methods

Eighty exuviae were collected from a four-year-old *C. gestroi* laboratory colony following Tong et al. (2020). Each exuvia was immersed for 5 s in a rabbit IgG (I5006, Sigma-Aldrich Inc., St. Louis, MO) (5 mg/mL dH_2O) solution and allowed to air dry.

Two-year-old *C. gestroi* incipient colonies (n = 4) were used for the assay. Following Chouvenc and Su (2014) for caste and instar determination, a subset (=replicate) from each colony consisted of one queen, one king, five first-instar larvae (L_1), 10 second-instar larvae (L_2), 20 first-instar workers (W_1), 35 second-or higher instar workers (W_{2+}), and five soldiers (S). The bottoms of plastic Petri dishes (60.0 mm in diameter) were lined with moistened media pads (47.0 mm in diameter). Moistened media pads were also taped to the inside of each Petri dish lid to provide additional moisture. Direct observation during preliminary studies confirmed *C. gestroi* workers feed on exuviae from other colonies. After colony subsets were added to the Petri dishes, 20 IgG-marked exuviae were added to the center of each Petri dish. The Petri dishes were then placed into covered plastic containers (17.5 × 12.5 × 7.0 cm^3) lined with wet paper towels and kept in the laboratory at 28 ± 1°C for 2 d. Within the 2 d of observation, the queens had laid eggs in all replicates. To ensure the same number of samples for analysis per caste per subset (owing to inherent mortality), samples consisted of the queen, king, two eggs, two L_1, four L_2, nine W_1, 20 W_{2+}, and three S. Two days after the initial introduction of exuviae, the subset samples were frozen for 24 h at −20°C. Negative controls were six eggs, three L_1, three L_2, three W_1, three W_{2+}, and three S from each colony that were not exposed to IgG.

Each sample (including negative controls) was placed into a 1.5 ml centrifuge tube with 85 µl of tris buffered saline (TBS), such that each centrifuge tube contained one individual termite or two eggs. Samples were homogenized with micropestles and centrifuged for 1 min at 8,000 rcf. Seven known concentrations of IgG were prepared (7.8 ng/ml to 500 ng/ml) to serve as positive controls.

The ELISA protocol followed Hagler and Machtley (2016). Each well of a 96-well microplate was coated with 50 µl anti-rabbit IgG primary antibody (R2004, Sigma-Aldrich Inc., St. Louis, MO) diluted 1:500 in TBS. The plate was kept at 4°C overnight. The antibody was discarded, and each well was blocked with 100 µl of 1.0% non-fat milk (M7409, Sigma-Aldrich Inc., St. Louis, MO) for 30 min. The milk was discarded, and 75 µl of the supernatant of each sample (including negative controls) and 75 µl of each positive control were added to their corresponding wells. Blanks were 75 µl of TBS. The microplates were then kept at room temperature for 1 h.

After each well was triple-rinsed with 100 µl TBS-Tween (20%), 50 µl of rabbit IgG antibody conjugated with horseradish peroxidase (A6154, Sigma-Aldrich, Inc., St. Louis, MO) diluted 1:10,000 in 1.0% nonfat milk was added and left at room temperature for 1 h. Then, each well was triple-rinsed with 100 µl TBS-Tween (20%), and 50 µl of tetramethylbenzidine (TMB One Component HRP Microwell Substrate, Surmodics, Eden Prairie, MN) was added to each well. After 10 min, the plates were read with a microplate spectrophotometer (BioTek Synergy 4, Winooski, VT) set at a wavelength of 650 nm.

The mean and standard deviation of the optical density (OD) readings of the negative controls in each plate were calculated. A sample was considered positive if its OD was greater than or equal to the mean OD of the negative controls plus six times the standard deviation OD of all the negative controls (Hagler and Machtley 2016). The proportions of individuals per caste/instar positive for the marker were arcsine transformed and subjected to a one-way an analysis of variance (ANOVA) with caste/instar as the explanatory variable. Means were separated using Tukey's honestly significant difference (HSD) test (α = 0.05).

For each plate, the lowest OD of the blanks (TBS) was subtracted from all wells. The ODs of the positive controls (x-axis) were plotted

against their known concentrations (y-axis; 0 to 62.5 ng/ml) to form a linear standard curve (R^2 values ranged from 0.992 to 0.998) to which each sample OD was fit. Samples that had OD values less than three times the standard deviation plus the means of the blanks were given a concentration of zero (Classen et al. 1987). For each colony (n = 4), the total concentrations of IgG per caste/instar were summed and divided by the number of individuals analyzed per caste, resulting in the unit: ng/ml/individual. These concentrations (ng/ml/individual) were subjected to a one-way ANOVA with caste/instar as the explanatory variable. Means were separated using Tukey's HSD test (α = 0.05). All analyses were done using JMP (JMP 15.0.0, SAS Institute, Cary, NC).

Results and Discussion

We confirmed through direct observation that workers consumed IgG-marked exuviae and that the IgG marker was detected in both workers and dependent individuals, which confirms that exuviae are recycled and redistributed within the colony.

Workers consumed the IgG-marked exuviae within 4 h after they were introduced to the Petri dishes (no visible exuviae when checked). The IgG marker, originating from the marked exuviae, was detected in every caste/instar except the L_1. The proportions of individuals per caste/instar positive for the marker are presented in Fig. 1 and the concentrations of marker per caste/instar are presented in Fig. 2. In the proportion positive analysis (Fig. 1), the threshold for a positive sample is higher than the one used for the concentration analysis, since it follows Hagler and Machtley (2016), which is why it appears that there was no marked exuviae in the L_1 in Fig. 1 and that there was marked exuviae in Fig. 2. The proportion of individuals positive for the marker varied by caste/instar (F = 5.5; df = 7, 31; P = 0.0008) (Fig. 1), with the queens always being positive for the marker. The queens had a significantly higher proportion of being positive for the marker than the eggs, L_1, L_2, and S; however, the proportion of W_1, W_{2+}, and kings positive for the marker did not significantly differ from the queens (Fig. 1). The marker was always detected in the workers and reproductives. The concentration of IgG per individual also varied by caste/instar (F = 4.9; df = 7, 31; P = 0.0015) (Fig. 2). The queens and W_{2+} had significantly higher concentrations of the marker than the eggs and L_1 (Fig. 2).

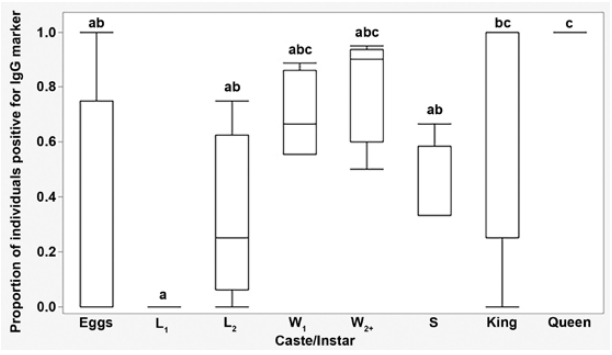

Fig. 1. Proportions of individuals positive* for rabbit immunoglobulin (IgG) marker by caste of subsets of *C. gestroi* colonies (n = 4) 2 d after being fed 20 IgG-marked exuviae. L_1: first-instar larvae, L_2: second-instar larvae, W_1: first-instar workers, W_{2+}: second- or higher-instar workers, S: soldiers. Means with the same letter are not statistically different (one-way ANOVA followed by Tukey's HSD test, α = 0.05).**A sample was considered positive if its optical density (OD) reading was greater than or equal to the mean OD of the negative controls plus six times the standard deviation OD of all the negative controls* (Hagler and Machtley 2016).

The concentration of marker in the L_2 caste ranged from 0 to 34.7 ng/ml/individual, and the mean was 6.8 ng/ml/individual. The individuals with high concentrations may have fed directly on the marked exuviae, since L_2 have been directly observed feeding on exuviae in this and other experiments (pers. obs.). In *C. formosanus*, L_2 can move on their own and engage in cannibalism (Du et al. 2016), which may suggest some capability of digesting exuviae. Although Du et al. (2016) did not observe L_2 helping nestmates molt or consuming exuviae, they opportunistically fed on marked exuviae during this experiment.

As the L_2, W_1, and W_{2+} fed directly on the marked exuviae, they can be considered donors, and the remaining castes/instars may be considered recipients. If the concentrations of IgG are analyzed separately for donors and recipients (L_2, W_1, and W_{2+} vs. eggs, L_1, S, kings, and queens), there are no significant differences among donors (F = 1.0; df = 2, 9; P = 0.4078), but there are significant differences among recipients (F = 12.8; df = 4, 15; P< 0.0001), and the queens received more IgG-marked exuviae than the eggs, L_1, S, and kings. Using *R. flavipes*, Suárez and Thorne (2000) reported ~1 to 2% of each donor's original gut load was transferred to one or more recipients. Recipients may also become secondary donors in a 'trophallactic cascade' (Suárez and Thorne 2000). In the same species, the amount of IgG-marked paper towel passed from donors to recipients ranged from ~1.4 to 2.7% per individual (Buczkowski et al. 2007). Donors had significantly higher levels of marker than recipients (Buczkowski et al. 2007); thus, individuals with high levels of marker likely fed directly on the marked food source.

In *C. formosanus*, only the W_{2+} proctodeally fed the queen and king (Du et al. 2016). In this study, the average IgG concentration for W_{2+} was ~11.2 ng/ml/individual. If *C. gestroi* W_{2+} transfers gut load similarly to *R. flavipes* (i.e., transferring 1.5% of their gut load), ~56 proctodeal trophallaxis events from W_{2+} to the queen, and ~25 events from W_{2+} to the king occurred per colony within 2 d (where the number of trophallaxis events equals the average recipient concentration divided by 1.5% of the average donor concentration). Soldiers are recipients of proctodeal trophallaxis from the L_2, S, W_1, and W_{2+} (Du et al. 2016). Applying the average IgG concentrations of L_2, W_1, and W_{2+}, ~39 events occurred per S. However, fewer events may have occurred between individuals with large amounts of marker, or if gut loads are passed differently for nitrogen-rich foods. The recipient of proctodeal trophallaxis usually initiates the exchange by grooming the abdomen of the donor (Du et al. 2016,

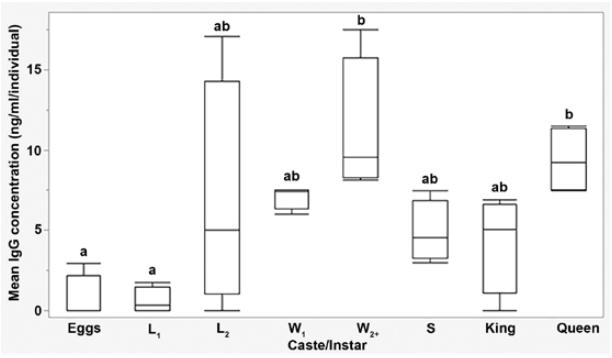

Fig. 2. Concentrations (ng/ml/individual) of rabbit immunoglobulin (IgG) marker by caste of subsets of *C. gestroi* colonies (n = 4) two days after being fed 20 IgG-marked exuviae. L_1: first-instar larvae, L_2: second-instar larvae, W_1: first-instar workers, W_{2+}: second- or higher-instar workers, S: soldiers. Means with the same letter are not statistically different (one-way ANOVA followed by Tukey's HSD test, α = 0.05).

Machida et al. 2001), with nitrogen-deficient termites soliciting more frequently (Machida et al. 2001). However, between workers and soldiers, the workers initiate and determine if trophallaxis occurs (Su and La Fage 1987). It is unknown if the queens and kings solicited proctodeal trophallaxis or if the feedings were initiated by the W_{2+}.

Regardless of the initiator, the transfers of nitrogen-rich exuviae to the reproductives were recorded in this study. The transfer of IgG marker originating from marked exuviae, which contain ~11.2% N by dry weight (Tong et al. 2021), to the kings and queens may be an example of proctodeal trophallaxis allowing for a prioritization of nitrogen allocation within the colony (Leach and Granovsky 1938, Nalepa 1994, Nalepa 2015), such that the queen may meet the high demands of egg production (Mattson 1980, Mullins and Su 2018, Shellman-Reeve 1990). Because the queens were always fed marked exuviae, and the amount of marked exuviae found in the queens was significantly higher than in other castes/instars, it is possible that the queen is a primary recipient for exuviae within the colony.

Larvae and workers may benefit from directly feeding on or receiving exuviae to recycle proteins needed to synthesize new cuticle during their upcoming molting event, and, like other organisms that have limited dietary nitrogen, may exhibit enhanced growth when supplemented with nitrogen (Mattson 1980, Dadd 1985). Although soldiers have reached a final molt, they may benefit from increased nitrogen for possible use in their large frontal gland secretions, which, in *C. formosanus*, contain small amounts of glycosaminoglycans, lysozyme, and ceramides (Ohta et al. 2007).

The eggs were positive for IgG in one colony subset. It is unknown if the marker can be passed from the queen to the eggs; however, IgG marker has been shown to persist through different stages (larval to pupal) in the pink bollworm (Hagler and Miller 2002). It is not conclusive if the presence of the marker in the eggs is a result of IgG-marked exuviae being fed to the queen and utilized in egg production or if the marker was transferred to the eggs via egg-grooming behavior (Du et al. 2016, Chouvenc and Su 2017). In *R. flavipes*, Buczkowski et al. (2007) suggested that IgG marker is not transferred during allogrooming. Further, this study examined the marker presence after 2 d; if the study had lasted longer, more eggs may have tested positive for marker, since it takes time for the queen to allocate ingested proteins to produce eggs. This may be explored in a future study with a different marker, as rabbit IgG concentrations in the termites significantly decreased 5 d after initial exposure in preliminary trials.

The nonrandom redistribution of exuviae, especially in termites confined to the central nest, was observed. This result also implies a centralization of the nitrogen recycling of exuviae in subterranean termites, and the hypothesis that workers molt in the central nest to provision the queen or larvae with nitrogen recycled from exuviae is supported, but further studies are needed to determine if nitrogen from exuviae goes to the eggs. In addition, although the behavior of molting at the central nest may contribute to the centralization of the nitrogen conservation of exuviae, a more important factor may be the patterns of provisioning nitrogen within the colony. For example, cockroaches may feed on their own exuviae and also competitively feed on the exuviae of their nestmates (Bell et al. 2007), and *Reticulitermes* Holmgren (Blattodea: Rhinotermitidae) termites may also feed on the exuviae of their nestmates or their own exuviae after molting (Grassé 1949). In *Coptotermes* colonies, exuviae are only consumed by nestmates (Raina et al. 2008, Xing et al. 2013). The patterns of proctodeal trophallaxis of the nitrogen from exuviae may also differ between these groups, especially since the former two are more likely to eventually molt into reproductives than *Coptotermes* workers. A conservation strategy in which more nitrogen is allocated to the primary reproductives may lead to increased reproduction, larger colony sizes, and may represent a relevant factor in the transition from lower to higher termites (Higashi et al. 2000, Chouvenc et al. 2021, Konishi et al. 2023).

This study identified which individuals fed on and received IgG-marked exuviae within *C. gestroi* colony subsets and experimentally demonstrated the recycling of exuviae into the colony. Exuviae were also fed to dependent castes through trophallaxis. This study mapped the trophic path of a nitrogen food source within a subterranean colony as opposed to a cellulosic food source (e.g., Suárez and Thorne 2000, Buczkowski et al. 2007). Studies in older colonies may be able to determine if nymphs solicit more proctodeal food than other caste members. Future studies may also be performed to determine the trophic path of exuviae within one-piece colonies versus separate-piece colonies, especially in regard to proctodeal feeding relationships comparing colonies containing totipotent versus effectively sterile individuals.

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Author Contributions

RLT: Conceptualization; Formal analysis; Investigation; Methodology; Visualization; Writing - original draft. E-KC: Investigation; Methodology; Supervision. KU: Investigation; Methodology; Supervision; Writing - review & editing. TC: Conceptualization; Resources; Supervision; Writing - review & editing. N-YS: Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Supervision; Writing - review & editing.

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