Cellulolytic Protist Numbers Rise and Fall Dramatically in Termite Queens and Kings during Colony Foundation

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Among the best-known examples of mutualistic symbioses is that between lower termites and the cellulolytic flagellate protists in their hindguts. Although the symbiosis in worker termites has attracted much attention, there have been only a few studies of protists in other castes. We have performed the first examination of protist population dynamics in queens and kings during termite colony foundation. Protist numbers, as well as measurements of hindgut and reproductive tissue sizes, were undertaken at five time points over 400 days in incipient colonies of Reticulitermes speratus, as well as in other castes of mature colonies of this species. We found that protist numbers increased dramatically in both queens and kings during the first 50 days of colony foundation but began to decrease by day 100, eventually disappearing by day 400. Hindgut width followed a pattern similar to that of protist numbers, while ovary and testis widths increased significantly only at day 400. Kings were found to contain higher numbers of protists than queens in incipient colonies, which may be linked to higher levels of nutrient transfer from kings to queens than vice versa, as is known in some other termite species. Protists were found to be abundant in soldiers from mature colonies but absent in neotenics. This probably reflects feeding of soldiers by workers via proctodeal trophallaxis and of reproductive tissues via stomodeal trophallaxis. The results reveal the dynamic nature of protist numbers during colony foundation and highlight the trade-offs that exist between reproduction and parental care during this critical phase of the termite life cycle.

Long-term obligate mutualistic interactions over evolutionary time result in significant changes to the biology of each partner. Highly intricate coordination between host and symbiont may evolve and be observed from the whole-organism level down to the molecular level (1, 2). Among the most well-known examples of obligate mutualism is that between “lower” termites and the cellulolytic parabasalid and oxymonad protists present in their hindguts (3). These protists play a major role in cellulose digestion, in combination with termite-derived cellulases produced in the salivary glands of lower termites (4–7). This mutualism was established in the ancestor of termites and their sister group, the wood-feeding cockroach genus Cryptocercus (8, 9), which is thought to have existed some 130 million years ago (10). Since that time, long periods of cocladogenesis between hosts and their protist symbionts have occurred, with some occasional cases of horizontal transfer between termite species (8, 9, 11).

Although kin selection theory explains the evolution of eusociality in termites (12), cellulolytic protists are thought to have played an important role in this process (13). During colony foundation by extant lower termites, the first larval offspring are nutritionally dependent upon the incipient queen and king, since they are unable to masticate wood and lack an established protist fauna in their gut. The first offspring receive protists from their parents and typically reach the worker stage upon the molt to the third instar. Workers are nutritionally independent (i.e., able to feed on wood) and are also able to feed and care for new offspring, transferring protists to them. The trophic-shift hypothesis proposes that this transition from parental to alloparental care (including feeding and symbiont transfer) of offspring was the critical step in the evolution of eusociality of termites (13, 14). The development of the first cohort of workers and their ability to transfer protists to their siblings allows the incipient queen and king to reduce their level of parental care and instead focus on reproduction. This leads to a key division of labor within the colony.

Despite the importance of cellulolytic protists in termite colony foundation, research over the last century has focused almost solely on their role in the metabolism of wood by worker termites in mature colonies (15). No studies have examined protists during incipient colony foundation, and few studies have focused on the presence of protists in different castes since the original study of Cleveland (16) almost a century ago. Because all castes other than workers receive food either by stomodeal or proctodeal trophallaxis, it might be expected that they do not require protists. Cleveland showed that this was true in the case of neotenic reproductives; however, he found that soldiers do harbor significant numbers of protists, although fewer than workers. Cook and Gold (17) and Lewis and Forschler (18) also showed that soldiers and alates harbored protists in relatively high numbers in Reticulitermes. However, protist numbers for primary reproductives during colony foundation were not reported.

Alates must carry significant fat reserves to facilitate egg production in new colonies, as well as hindgut protists to inoculate offspring (19). Furthermore, as alates change from being food recipients in their natal colony to becoming food providers in the...
colony they initiate, they presumably need significant numbers of protists to digest wood and provide proctodeal secretions for offspring. The feeding of offspring is likely to reduce the amount of resources available for reproduction. As the colony matures and primary reproductives cease to feed offspring, their protist numbers are expected to decrease, in line with Cleveland’s observations of an absence of protists in neotenic reproductives.

Incipient termite kings and queens typically form monogamous relationships lasting several years. They display biparental care, with both parents feeding incipient offspring. It is thus expected that both kings and queens are dependent upon gut protists to digest wood and produce stomodeal and proctodeal fluids. Consequently, we might expect the number of protists harbored by each sex to be similar (per unit of wet weight) during incipient colony foundation. Females are expected to devote significantly more resources toward reproduction than males. The smaller portion of the body cavity taken up by the male sex organs compared with that taken by female sex organs could lead to a relatively large gut size in males and, therefore, increased numbers of protists.

To test hypotheses concerning protist dynamics during incipient colony foundation, we used the termite Reticulitermes speratus as a model. Our data come from experiments conducted in the laboratory and kept in plastic cases in constant darkness. Field primary colonies from rotten wood in laurel forests in Toyama and Ishikawa Prefectures, Japan, were collected in the spring. The feeding of offspring is likely to reduce the amount of resources available for reproduction. As the colony matures and primary reproductives cease to feed offspring, their protist numbers are expected to decrease, in line with Cleveland’s observations of an absence of protists in neotenic reproductives.

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To test hypotheses concerning protist dynamics during incipient colony foundation, we used the termite Reticulitermes speratus as a model. Progeny numbers were determined at the following five time points: days 0 (unmated alates), 30, 50, 100, and 400 after colony foundation. We examined gut size and reproductive tissue development at these five time points. For comparison, we examined protist numbers in various castes in mature colonies (workers, soldiers, and neotenic reproductives).

### MATERIALS AND METHODS

**Insects.** Several mature colonies of Reticulitermes speratus were collected from rotten wood in laurel forests in Toyama and Ishikawa Prefectures, Japan, in 2008 to 2012. Pieces of nest wood were brought back to the laboratory and kept in plastic cases in constant darkness. Field primary colonies contained nymphs (neotenic reproductives differentiated from nymphs) with functional reproductive organs, workers (6th or 7th instars [20]), and soldiers were collected from mature colonies. Ergatoids (neotenic reproductives differentiated from workers) with functional reproductive organs were obtained from other colonies maintained under laboratory conditions.

**Colony foundation and sample collection.** Incipient colonies were set up as described by Maekawa et al. [21], and primary queens and kings were sampled at 30, 50, 100, and 400 days after colony foundation. The details of colony members of each period are shown in Table 1. For anatomical observations of hindguts and reproductive organs (see below), reproducitives were dissected carefully, fixed in formaldehyde-ethanol-acetic acid (6:16:1, vol/vol/vol) for at least 24 h, and stored in 70% ethanol.

### TABLE 1 Mean numbers of eggs, larvae, workers, soldiers, and colony sizes and percentages of workers at different periods after colony foundation in the termite Reticulitermes speratus

| Colony parameter | Mean value ± SD on day: |
|------------------|------------------------|
|                  | 30         | 50         | 100        | 400        |
| Colonies examined| 26         | 21         | 20         | 22         |
| Eggs             | 8.6 ± 3.1  | 8.0 ± 2.3  | 1.2 ± 2.0  | 23.0 ± 47.7|
| Larva            | 0          | 2.9 ± 1.2  | 1.7 ± 2.1  | 4.9 ± 8.1  |
| Workers          | 0          | 2.2 ± 1.6  | 13.9 ± 3.6 | 105.3 ± 27.4|
| Soldiers         | 0          | 0          | 0.9 ± 0.4  | 4.2 ± 1.2  |
| Total termites in colony | 10.6 ± 3.1 | 15.0 ± 3.8 | 19.6 ± 4.6 | 139.3 ± 54.4|
| % of workers     | 0          | 16.0 ± 9.9 | 80.5 ± 14.8| 81.5 ± 18.7|

**Counts of gut protist numbers.** The enlarged part of the hindgut of R. speratus known as the paunch harbors more than 10 species of symbiotic protist (22–24). In this study, we estimated the numbers of the major symbiotic protist species Trichonympha agilis, Pyronymphpha spp., and Dienesymphtha exilis, previously shown to be the primary wood decomposers (25). Because it was difficult to discriminate three described Pyronymphpha species (P. grandis, P. modesta, and P. affinis) by observation of living materials (24), the numbers of them were estimated as a whole (hereinafter called Pyronymphpha spp.). We also estimated the numbers of all protists (including the above-named species) without distinguishing species (hereinafter referred to as “all protists”). To estimate protist numbers, termite hindguts were pulled out carefully with forceps and dissected in a drop of 0.45% NaCl solution. After careful removal of most intestinal tissue, the volume of suspended intestinal fluid was adjusted to 20 μl (40 μl in case of reproductives at day 30 to 100) by pipetting additional NaCl solution. The protists present in the resuspended intestinal fluid were then counted. The focal species were then counted in 1/200 (for T. agilis, 1/4) of the solution (20 μl) using a Thoma hemacytometer (Sunlead Glass, Saitama, Japan) and BX40 bright-field microscope (Olympus, Tokyo, Japan). We then estimated the total numbers of each protist. In the case of counting all protists, the total numbers were estimated by subsampling 1/3,200 of the solution, including all gut contents. Pictures of gut protists were taken with a BX51 microscope equipped with differential interference contrast (Olympus).

**Measurements of the sizes of hindguts and reproductive organs in queens and kings.** The outer morphology and color of hindguts of queens and kings were observed using an SZX7 or SZX10 stereomicroscope (Olympus). Hindgut sizes were evaluated by measuring the maximum width of the paunch (i.e., the most enlarged part of the hindgut). The degree of ovarian and testicular development was evaluated in accordance with the methods used in previous studies (26, 27). The degree of ovarian development was evaluated by measuring each ovary's width and the number of vitellogenic ovarioles (containing oocytes more than 0.28 mm in length) (28). For the evaluation of testicular development, the diameter of each testis at its widest point was measured (29). All measurements were carried out using a stereomicroscope and 3-chip charge-coupled-device (3CCD) digital camera XD250-2D (Olympus).

**Statistical tests.** Two-way analysis of variance (ANOVA) and Tukey’s multiple comparison test were used for statistical analyses using Ekuseru-Toukei 2010 (Social Survey Research Information Co., Ltd., Tokyo, Japan). P values less than 0.05 were considered significant.

### RESULTS

**Colony development at each time point.** At day 0, males and females were paired for colony initiation. By day 30, only the primary pair and eggs were present in all colonies (Table 1). By day 50, a total of 15.0 ± 3.8 (mean ± standard deviation [SD]) individuals were present (including the 2 primary reproductives), including 8.0 ± 2.0 eggs. There were 2.2 ± 1.6 workers (3rd or 4th instar) at day 50, but no soldiers were observed in any colony. At day 100, colonies contained 19.6 ± 4.6 individuals, ~80% of which were workers. The egg number dropped to 1.2 ± 2.0 eggs, while the number of soldiers was 0.9 ± 0.4. At day 400, colonies contained about 139.3 ± 54.4 individuals, including 3 to 5 soldiers, but the worker ratio did not change (about 80%). The egg number had increased to 23.0 ± 47.7 by this time point.

**Quantities of gut protists in reproductives and other castes.** Although the patterns of protist numbers in queens and kings were similar over the course of incipient colony development (Fig. 1), two-way ANOVA showed a statistical difference between queens and kings for protist numbers (Pyronymphpha spp., df = 1, F = 4.6496, P = 0.0361; D. exilis, df = 1, F = 10.3930, P = 0.0023;
all protists, df = 1, F = 12.8782, P = 0.0008), except for T. agilis (df = 1, F = 3.1004, P = 0.0846). Significant differences were also found among periods following colony foundation (T. agilis, df = 4, F = 28.5415, P < 0.0001; Pyrsonympha spp., df = 4, F = 16.7912, P < 0.0001; D. exilis, df = 4, F = 19.4830, P < 0.0001; all protists, df = 4, F = 81.9686, P < 0.0001). No interaction was detected between sexes and periods in all cases (T. agilis, df = 4, F = 1.0494, P = 0.3918; Pyrsonympha spp., df = 4, F = 1.7054, P = 0.1643; D. exilis, df = 4, F = 1.5369, P = 0.2065; all protists, df = 4, F = 2.3306, P = 0.0693).

In incipient queens and kings, protist numbers increased significantly from day 0 to day 30 in all cases and continued to increase at day 50 (Fig. 1; see also Tables S1 and S2 in the supplemental material). At day 100, protist numbers dropped significantly in all cases compared to the numbers at day 50. By day 400, protist numbers in queens were very low, and protists had disappeared completely in the cases of T. agilis and Pyrsonympha spp. Unlike the case for queens, protists were found in the guts of kings at day 400, albeit in small numbers. However, in three primary kings collected from field colonies (the ages of which were unknown), no gut protists at all were found (see Table S2 in the supplemental material).

Among other castes in mature colonies (the ages of which were unknown), the numbers of gut protists in workers were significantly higher than the numbers in soldiers, with the exception of Pyrsonympha spp. (Fig. 2; see also Table S3 in the supplemental material). Ergatoids with functional reproductive organs had no protists, except for one individual which had low numbers (200 D. exilis, 400 Pyrsonympha spp., and 3,200 of all protists). Nymphoids with functional reproductive organs had no gut protists at all.

Development of hindguts and reproductive organs during colony development. For hindgut width, two-way ANOVA showed no statistical difference between queens and kings (df = 1, F = 0.0819, P = 0.7765). On the other hand, significant differences were found among periods following colony foundation (df = 4, F = 19.7234, P < 0.0001). No interaction was detected between sexes and periods (df = 4, F = 1.9674, P = 0.1211).

At day 0, female and male alates had relatively small hindguts, with maximum widths of 0.42 ± 0.04 and 0.50 ± 0.05 mm, respectively (Fig. 3A). By day 30, these had increased significantly in width, to 0.63 ± 0.12 mm (queens) and 0.63 ± 0.15 mm (kings). These queens and kings had enlarged brownish hindguts filled with a large amount of wood particles (Fig. 3B). The hindgut widths did not change significantly between days 30 and 100 (Fig. 3A), but at
day 400, queens and kings had significantly reduced hindguts (0.32 ± 0.05 and 0.36 ± 0.10 mm, respectively) (Fig. 3A). Their hindguts were pale and transparent, containing no woody materials (Fig. 3C).

The ovary widths of female alates were 0.26 ± 0.04 mm (Fig. 4A). The ovaries of queens developed slightly by days 30 (0.42 ± 0.07 mm) and 50 (0.42 ± 0.09 mm), although the development was not significant compared with the ovary widths at day 0. At day 100, ovary width decreased significantly (0.27 ± 0.05 mm), but then it increased significantly and ovaries became well-developed by day 400 (0.67 ± 0.08 mm) (Fig. 4A). Many vitellogenic ovarioles were observed in ovaries of the queens at this stage (26).

Similarly, male alates and kings from day 30 to 100 had only slightly developed testes, but kings at day 400 had well-developed testes (Fig. 4B), as shown in a previous study (26). Three primary kings collected from field colonies (age unknown) had better-developed testes than any other kings (maximum testis width, 1.17 ± 0.23 mm; n = 3).

**DISCUSSION**

**Protist numbers and hindgut sizes in queens and kings change significantly during colony foundation.** Following colony foundation, rhinotermitid and termitid incipient queens typically lay 10 to 20 eggs within the first month (19, 21). We found that the first eggs in *R. speratus* incipient colonies hatch 30 days after colony foundation. This was matched by an approximately 7-fold increase in protist numbers (all protists) in queens and kings by day 30 compared with the protist numbers at day 0 (Fig. 1), equivalent to ~2 to 3 times the numbers present in workers of mature colonies (Fig. 2). These increases in protist numbers were accompanied by significant increases in hindgut width. In a previous study, we found that endogenous cellulase gene expression increased from day 0 in incipient queens and kings, peaking from day 30 to 100 (26). This suggests increased levels of wood consumption over this period. Together, increases in protist numbers and endogenous cellulase activity by day 30 are likely to facilitate feeding of the first offspring that hatch at this time and the production of eggs and sperm by queens and kings, respectively. We did not measure changes in fat content of incipient queens and kings, but we assume fat reserves, as well as recycling of flight muscle protein, are both used early in colony development, particularly to facilitate egg production in the case of the queen.

By day 50, an average of 2.2 ± 1.6 workers were present in the

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**FIG 2** Numbers (mean ± SD) of *Trichonympha agilis* (A), *Pyronympha* spp. (B), *Dinenympha exilis* (C), and all protists (D) in hindguts of workers (W), soldiers (S), ergatoids (ER), and nymphoids (NP). The numbers of individual termites examined are indicated in parentheses. Different letters over the bars denote significant differences among castes (Tukey’s test, P < 0.05).

**FIG 3** (A) Changes in size (mean ± SD) of hindguts of queens (black columns) and kings (white columns) during colony development. Different letters denote significant differences (two-way ANOVA followed by Tukey’s test, P < 0.05). The numbers of individual termites examined are indicated within the columns. (B and C) Midguts (MG) and hindguts (HG) of primary queens at 100 days (B) and 400 days (C) after colony foundation. Each size bar indicates 0.5 mm.
Protist Dynamics in Termite Reproductives

![Graph](image)

**FIG 4** Changes in size (mean ± 5D) of reproductive organs of queens (A) and kings (B) during colony development. Different letters denote significant differences (Tukey’s test, *P* < 0.05). The numbers of individual termites examined are indicated in parentheses. Testis size data were obtained from a previous report (26).

Conclusions. We have performed the first examination of pro-
tist dynamics in termite queens and kings during colony foundation. The results indicate that protists play a key role in this process, increasing in numbers over the first 50 days before starting to decline by day 100. Protists are likely to contribute significantly to the energy resources of incipient queens and kings for wood digestion and brood care. Kings have larger numbers of protists than queens, suggesting that kings probably act as donors of nutrients to the queen and contribute more to offspring feeding than the queen. By day 400, both kings and queens lose their protists entirely and become completely dependent upon their offspring for feeding. Therefore, our study shows that protist numbers during colony foundation are highly plastic and reveals the trade-offs between reproduction and parental care during this important phase of the termite life cycle.

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