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RESEARCH ARTICLE

Dynamic protozoan abundance of *Coptotermes* kings and queens during the transition from biparental to alloparental care

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Abstract
An incipient termite colony initially functions as a biparental family unit. The emergence of the first workers initiates the transition from biparental to alloparental care within a colony. During this transition, the number of protozoa harbored by *Reticulitermes speratus* (Kolbe) kings and queens is dynamic. In *Coptotermes gestroi* (Wasmann), the transition to alloparental care is completed by 150 days after colony foundation. In the present study, we quantified the protozoan abundance of *C. gestroi* and *Coptotermes formosanus* Shiraki kings and queens at ten time points during the transition from biparental to alloparental care (0–400 days post colony foundation). The protozoan abundance of *C. gestroi* and *C. formosanus* kings and queens peaked on either day 35 or 60 during the biparental care period, and then progressively decreased during the remainder of the study. By day 400 the protozoan abundance of kings and queens was either similar to or less than the abundance observed within unmated dealates. Both *C. gestroi* and *C. formosanus* kings and queens still harbored protozoa at day 150 even though the transition to alloparental care was completed by this time. On days 250 and 400 for *C. formosanus*, and on day 400 for *C. gestroi*, the protozoan abundance of kings was greater than the abundance of queens. These results indicate that alloparental care can become set within a colony prior to the loss of protozoa within the royal pair, and that queens lose their protozoa earlier than kings during incipient colony development.

Keywords Subterranean termite · Biparental care · Alloparental care · Symbiosis

Introduction

Lower termite protozoa are obligate mutualists that produce digestive enzymes which enable the degradation of lignocellulose within the hindgut (Cleveland 1923; Engel and Moran 2013; Brune 2014; Peterson and Scharf 2016). These protozoa are classified within either the phylum Parabasalia or the order Oxymonadida (phylum Preaxostyla) (Ohkuma 2008). The mutualism between lower termites and their hindgut protozoa originated approximately 170 million years ago in the ancestor of termites and their sister group, the subsocial xylophagous cockroach *Cryptocercus* (Ohkuma et al. 2009; Bourguignon et al. 2015; Nalepa 2017, 2020).

Termite colonies are founded by an alate pair following a dispersal flight event (Nutting 1969). Within an incipient colony, the first larvae are cared for and fed solely by the royal pair, as workers have not developed yet, and larvae lack an established hindgut microbial community and the ability to masticate wood (Inoue et al. 2000; Shimada et al. 2013; Nalepa 2015; Du et al. 2016; Chouvenc and Su 2017). Therefore, before the emergence of the first workers, an incipient colony does not function as a eusocial group but rather as a biparental family unit similar to *Cryptocercus* (Weesner 1960; Nutting 1969; Nalepa 1988, 2015; Shellman-Reeve 1997; Chouvenc and Su 2017).

Typically, subterranean termite (Blattodea: Rhinotermitidae) larvae molt into first instar workers after two larval instars (Roisin and Lenz 1999; Lainé and Wright 2003; Shimada et al. 2013; Chouvenc and Su 2014). Unlike larvae, first instar workers harbor an established hindgut community and are able to masticate wood; therefore, workers are nutritionally independent from the king and queen and

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are capable of transferring hindgut symbionts to developing offspring and newly-molted individuals via proctodeal trophallaxis (Yamaoka et al. 1986; Kitade et al. 1997; Crosland et al. 1998; Inoue et al. 2000; Shimada et al. 2013; Du et al. 2016). The emergence of the first workers is the beginning of the transition from a biparental family unit in which the royal pair cares for their first brood, to a eusocial colony in which brood care and other essential colony functions are performed by workers that function as alloparents (Shimada et al. 2013; Nalepa 2015; Chouvenc and Su 2017). Ultimately, the transition from biparental to alloparental care releases the king and queen from their initial brood care responsibilities, and therefore allows the royal pair to invest their energy exclusively into reproduction (Nalepa 1988, 2015; Shimada et al. 2013; Chouvenc and Su 2017).

Rhinotermitid alates have consistently been shown to harbor fewer protozoa relative to other castes (Lai et al. 1983; Cook and Gold 1998; Lewis and Forschler 2004; Shimada et al. 2013). Alates inherit their protozoan community from individuals within their natal colony prior to their dispersal flight (Nutting 1969; Michaud et al. 2020). The first workers that emerge acquire their hindgut community via repeated proctodeal trophallaxis from both the king and queen, and therefore by biparental vertical transmission (Inoue et al. 2000; Nalepa et al. 2001; Ohkuma et al. 2009; Shimada et al. 2013; Nalepa 2017; Brossette et al. 2019; Michaud et al. 2020). Prior to the emergence of the first workers, the royal pair increases the number of protozoa they harbor by consuming wood, as evidenced by the behavior, expression level of the endogenous cellulase gene *RsEG*, and protozoan abundance of the king and queen during the biparental care period (Cleveland 1925; Shellman-Reeve 1990; Rosengaus and Traniello 1991; Watanabe et al. 1998; Shimada and Maekawa 2010; Shimada et al. 2013; Chouvenc and Su 2017; Brossette et al. 2019).

In the subterranean termite *Reticulitermes speratus* (Kolbe), the number of protozoa harbored by kings and queens changes during incipient colony development (Shimada et al. 2013; Inagaki et al. 2020). The protozoan abundance of *R. speratus* kings and queens increases dramatically during the first 50 days after colony foundation, coinciding with the development of the first workers (Shimada et al. 2013). After worker emergence, the number of protozoa harbored by kings and queens begins to decrease, and by day 400 protozoa are nearly absent within the royal pair (Shimada et al. 2013). Eventually, *Reticulitermes* reproductives lose their protozoa entirely, presumably because they stop consuming wood and are primarily fed through stomodeal trophallaxis from workers (Cleveland 1925; Shimada et al. 2013; Inagaki and Matsuura 2016).

The completion of the transition from biparental to alloparental care is an irreversible event that renders the royal pair nutritionally dependent upon their workers for the remainder of their lives (Cleveland 1925; Chouvenc and Su 2017). In *Coptotermes gestroi* (Wasmann), the king and queen are unable to survive if all workers are removed from the colony at 150 days after colony foundation, indicating that the transition to alloparental care is completed by this time (Chouvenc and Su 2017). The inability of the king and queen to compensate for the complete loss of their workers may stem from the absence of protozoa within the royal pair, as the king and queen may no longer harbor hindgut protozoa by 150 days after colony foundation.

We used *C. gestroi* and *Coptotermes formosanus* Shiraki to further investigate the dynamic abundance of protozoa within rhinotermitid kings and queens during incipient colony development, and to determine if protozoa are absent once the transition to alloparental care is completed (150 days post colony foundation). To do so, we quantified the protozoan abundance of *C. gestroi* and *C. formosanus* kings and queens from laboratory-reared colonies at 0 (unmated dealates), 35, 60, 75, 90, 105, 120, 150, 250, and 400 days post colony foundation. We hypothesized that the protozoan abundance of *C. gestroi* and *C. formosanus* kings and queens would increase during the biparental care period and then begin to decrease after the emergence of the first workers, and that protozoa would be nearly absent within the royal pair by the time the transition to alloparental care was completed.

Materials and methods

Alate collection and rearing unit production

Coptotermes gestroi alates were collected in Ft. Lauderdale, FL during the evening of March 19th 2018 using a light trap as described in Chouvenc et al. (2015a). *Coptotermes formosanus* alates were collected at the same location using the same method on April 25th 2018. After trapping, alates were transferred to a plastic box containing moist corrugated cardboard and kept there until they were processed the next morning. Morphology was used to identify the species and sex of collected alates (Weesner 1969; Su et al. 1997). After processing, conspecific dealate pairs were introduced into individual rearing units. Briefly, a rearing unit consisted of a transparent plastic cylindrical vial (8 cm × 2.5 cm diameter, internal volume = 37 ml, Fisher Scientific, Pittsburgh, PA) containing moistened organic soil, *Picea* sp. wooden blocks, and 3% agar solution to maintain moisture (Chouvenc and Su 2014; Chouvenc et al. 2015a). A total of 250 rearing units per species were established according to Chouvenc and Su (2014). Rearing units were capped with a lid that was punctured with a safety pin to allow airflow while still preventing termites from escaping and stored at 28 ± 1 °C and approximately 80% humidity for the duration of the study.

Estimation of protozoan abundance

Both *C. gestroi* and *C. formosanus* harbor parabasalians classified within the genera *Pseudotrichonympha*, *Holomastigotoides*, and *Cononympha* (Koidzumi 1921; del Campo et al. 2017; Nishimura et al. 2020; Jasso-Selles et al. 2020). Protozoan abundance was estimated during incipient colony development by destructively sampling the king and queen from ten separate rearing units per species at 35, 60, 75, 90, 105, 120, 150, 250, and 400 days post colony foundation. During sampling, we noted the timing of the initial appearance of eggs, workers, and soldiers within rearing units (colonies). We also determined the number of workers within colonies on days 35, 60, 75, 90, 105, 120, 150, and 250. To estimate the protozoan abundance of unmated dealates (day 0), we destructively sampled ten male and female dealates per species during the morning after a dispersal flight event. Each termite was sampled separately, resulting in ten protozoan abundance estimations for each sex for each of the ten time points. Lastly, the king and queen from three 2-year-old laboratory-reared colonies per species were destructively sampled to investigate whether protozoa were present within the royal pair 2 years post colony foundation.

Dealates, kings, and queens were destructively sampled using the following protocol. The hindgut of a termite was removed from the abdomen by holding the thorax with forceps and pulling on the posterior abdominal segments with separate forceps (Lewis and Forschler 2004). After removal, the hindgut was placed in 20–250 µl of Ringer's solution (HiMedia Laboratories, West Chester, PA) depending on the number of protozoa present.

After immersion in Ringer's solution, the majority of tissues besides the hindgut were removed via forceps. The hindgut was then opened by separation with forceps to allow the hindgut contents to enter the solution. After opening, the hindgut was moved and pushed on with forceps to facilitate the expulsion of protozoa into the solution. An Olympus SZH-ILLD zoom stereo microscope and illumination base (Olympus, Tokyo, Japan) was used during hindgut dissections and homogenization. The distribution of expelled protozoa within the solution was homogenized by swirling the solution in a circular and up/down motion with forceps until it was visually apparent that homogenization had occurred and that the hindgut no longer harbored protozoa. Ten µl of the homogenized solution was then pipetted onto the counting chamber of a Reichert Bright-Line improved Neubauer hemacytometer (Hausser Scientific, Horsham, PA). The total number of protozoa within four large squares (0.4 µl) of the counting chamber was then counted using an Olympus BX51 upright compound microscope. The following formula: [(number of protozoa counted × volume of Ringer's solution used)/(volume on the hemacytometer from which cells were counted (0.4 µl))] was used to estimate the protozoan abundance within the entire homogenized solution (protozoan abundance of each sampled termite) (Lewis and Forschler 2004).

Statistical analysis

A two-way ANOVA with primary reproductive sex and time (number of days since colony foundation) as factors, and estimated protozoan abundance as the dependent variable was performed for both *C. gestroi* and *C. formosanus*. We decided to not statistically test for any differences in estimated protozoan abundance between *C. gestroi* and *C. formosanus* because the detection of any differences between species was not the focus of this study. In addition, observations from 2-year-old kings and queens were not included in either two-way ANOVA because of the limited number of replicates (three per species). Estimated protozoan abundance data were first $(x+1)$ transformed because protozoa were absent in some individuals on days 250 and 400, and the data needed to be square root transformed to meet the assumptions of a two-way ANOVA. Estimated protozoan abundance data present within figures are untransformed for clarity. We chose to not report or further analyze the main effect of either factor because of a significant interaction between factors for both *C. gestroi* and *C. formosanus*. Instead, we analyzed the simple main effects of time for *C. gestroi* and *C. formosanus* kings and queens, and subsequently performed pairwise comparisons within each significant simple main effect. Pairwise comparisons between the estimated protozoan abundance of kings and queens at each of the ten time points were also performed for *C. gestroi* and *C. formosanus* separately. Simple main effects and pairwise comparisons were determined to be significant or non-significant based upon Bonferroni-adjusted *P* values ($\alpha = 0.05$). We used R (R Core Team 2019) with the packages 'car' (Fox and Weisberg 2019), 'dplyr' (Wickham et al. 2019), 'ggplot2' (Wickham 2016), 'ggpubr' (Kassambara 2019), 'phia' (Rosario-Martinez 2015), and 'tiff' (Urbanek 2013) for statistical analyses, data management, and figure production.

Results

Initial appearance of eggs, workers, and soldiers

Eggs, workers, and soldiers were first observed on days 35, 60, and 75, respectively (Table 1). Eggs and one or more workers were observed in all *C. gestroi* and *C. formosanus* colonies sampled on days 35 and 60, respectively (Table 1). After the initial emergence of workers on day 60, the number of workers within both *C. gestroi* and *C. formosanus* colonies progressively increased during the remainder of

Table 1 The timing of the initial appearance of eggs, workers, and soldiers within colonies

Species	Eggs on day 35	Workers on day 60	Soldiers on day 75
Coptotermes gestroi	10/10	10/10	8/10
Coptotermes formosanus	10/10	10/10	8/10

Values indicate the number of colonies in which eggs, workers, and soldiers were observed on days 35, 60, and 75, respectively ($n = 10$ colonies per time point per species)

the study (Table 2). On day 75, one or more soldiers were observed within most *C. gestroi* and *C. formosanus* colonies (Table 1).

Coptotermes gestroi protozoan abundance

The results of a two-way ANOVA indicated that the interaction between time (number of days since colony foundation) and primary reproductive sex was significant for *C. gestroi* kings and queens ($F = 1.951$; $df = 9$, 180; $P = 0.0475$). An analysis of simple main effects revealed that time significantly affected the protozoan abundance of both *C. gestroi* kings ($F = 18.962$; $df = 9$, 180; $P < 0.0001$) and queens ($F = 21.081$; $df = 9$, 180; $P < 0.0001$).

The protozoan abundance of *C. gestroi* kings significantly increased from day 0 to day 60, and then significantly decreased by day 120 relative to the peak abundance observed on day 60 (Fig. 1A). By day 250 the protozoan abundance of *C. gestroi* kings was statistically equivalent to the abundance observed on day 0 (Fig. 1A). A similar trend was observed for *C. gestroi* queens with a significant peak in protozoan abundance observed on day 60, and a significant decrease by day 105 relative to the abundance observed on day 60 (Fig. 1B). By day 400 the protozoan abundance of *C. gestroi* queens was significantly less than the abundance observed on day 0 (Fig. 1B). Two-year-old *C. gestroi* kings and queens harbored no protozoa in their hindgut ($n = 3$ kings and queens).

Coptotermes formosanus protozoan abundance

Similar to *C. gestroi*, the results of a two-way ANOVA indicated that there was a significant interaction between time and primary reproductive sex for *C. formosanus* kings and queens ($F = 3.546$; $df = 9$, 180; $P < 0.001$). Simple main effects analysis revealed that time significantly affected the protozoan abundance of both *C. formosanus* kings ($F = 22.137$; $df = 9$, 180; $P < 0.0001$) and queens ($F = 32.933$; $df = 9$, 180; $P < 0.0001$).

The protozoan abundance of *C. formosanus* kings significantly increased from day 0 to a peak at day 35, and then significantly decreased by day 90 relative to the peak abundance observed on day 35 (Fig. 1C). By day 400 the protozoan abundance of *C. formosanus* kings was statistically equivalent to the abundance observed on day 0 (Fig. 1C). A similar pattern was observed for *C. formosanus* queens with a significant peak in protozoan abundance observed on day 60, and a significant decrease by day 90 relative to the abundance observed on day 60 (Fig. 1D). By day 150 the protozoan abundance of *C. formosanus* queens was statistically equivalent to the abundance observed on day 0 (Fig. 1D). Further decreases in protozoan abundance were observed on days 250 and 400 for *C. formosanus* queens (Fig. 1D). Similar to *C. gestroi*, 2-year-old *C. formosanus* kings and queens did not harbor any protozoa in their hindgut ($n = 3$ kings and queens).

Differences in protozoan abundance between kings and queens

For most time points, pairwise comparisons within each species indicated that the number of protozoa harbored by kings was not significantly different from that of queens. However, for *C. gestroi*, a significant difference was found between the protozoan abundance of kings and queens on day 400 ($F = 10.889$; $df = 1$, 180; $P = 0.0116$). Significant differences were also found between the protozoan abundance of *C. formosanus* kings and queens on days 250 ($F = 13.912$; $df = 1$, 180; $P = 0.0025$) and 400 ($F = 19.241$; $df = 1$, 180; $P < 0.001$). In all three instances, the protozoan abundance of kings was significantly greater than that of queens.

Table 2 The number (mean ± SD) of workers (first instar or older) within colonies at eight time points during incipient colony development ($n = 10$ colonies per time point per species)

Species	Day 35	Day 60	Day 75	Day 90	Day 105	Day 120	Day 150	Day 250
Coptotermes gestroi	0	2.3 ± 3.5	6.6 ± 5.4	13.1 ± 8.3	17.8 ± 6.8	24.1 ± 5.7	29.4 ± 5.3	78.4 ± 14.9
Coptotermes formosanus	0	2.1 ± 1.3	6.3 ± 5.1	11.4 ± 6.7	16.7 ± 6.6	22.1 ± 9.1	28.1 ± 7.5	79.4 ± 17.8

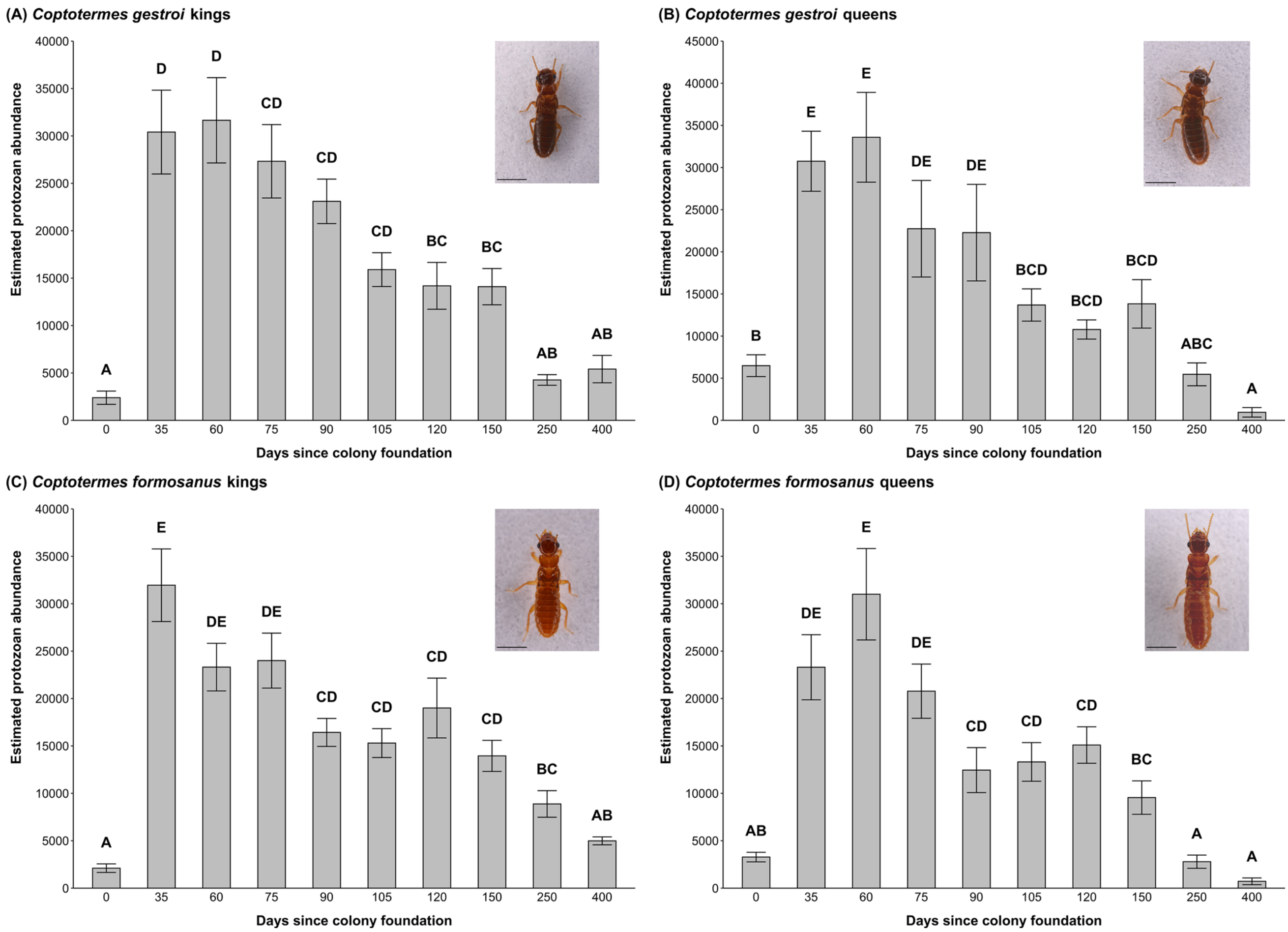

Fig. 1 The protozoan abundance (mean ± SE) of *C. gestroi* kings (**A**) and queens (**B**), and *C. formosanus* kings (**C**) and queens (**D**) at ten time points during incipient colony development. Ten kings (**A**, **C**) or ten queens (**B**, **D**) were destructively sampled for each time point. Time points with the same letter were not significantly different based on pairwise comparisons that were performed within each significant simple main effect. Pairwise comparison results within each panel (**A**–**D**) are only applicable to that particular panel and are not comparable between panels. Pairwise comparisons were determined to be significant or non-significant using Bonferroni-adjusted *P* values ($\alpha = 0.05$). All scale bars denote 2 mm

Discussion

Our results demonstrate that the protozoan abundance of *C. gestroi* and *C. formosanus* kings and queens is dynamic during incipient colony development (Fig. 1). Similar to *Reticulitermes*, the number of protozoa harbored by *C. gestroi* and *C. formosanus* dealates was relatively low (Cook and Gold 1998; Lewis and Forschler 2004; Shimada et al. 2013). By day 35 the protozoan abundance of *C. gestroi* and *C. formosanus* kings and queens had significantly increased relative to day 0, peaking on either day 35 or 60. Within laboratory-reared *C. gestroi* colonies the first eggs are laid 20–25 days after colony foundation, the first larvae emerge by 36–42 days, and by day 60–65 the first workers are present (Chouvenc et al. 2015b). During this study, eggs were present in all *C. gestroi* and *C. formosanus* colonies sampled on day 35, and one or more workers were observed in all colonies of both species on day 60 (Tables 1, 2). Therefore, similar to *R. speratus*, the peak protozoan abundance of *C. gestroi* and *C. formosanus* kings and queens coincides with the emergence of the first workers during the biparental care period (Shimada et al. 2013).

The protozoan abundance of *C. gestroi* and *C. formosanus* kings and queens began to decrease shortly after the emergence of the first workers. By day 120 the number of protozoa harbored by kings and queens had significantly decreased relative to peak protozoan abundance. Remarkably, *C. gestroi* and *C. formosanus* kings and queens still harbored a relatively large number of protozoa on day 150 despite the transition to alloparental care having been completed by this time (Chouvenc and Su 2017). These results imply that the irreversible transition from biparental to alloparental care is completed prior to the loss of protozoa within the royal pair. Therefore, while the progressively decreasing protozoan abundance of kings and queens is indicative of the transition to alloparental care and a useful indicator of the progression of incipient colony development, the number of protozoa alone does not reveal when alloparental care is set within a colony. Rather than the loss of protozoa, the irreversibility of the transition from biparental to alloparental care may stem from the exhaustion of the finite initial metabolic reserves of the king and queen. Metabolic reserves such as lipids, nitrogenous compounds, and proteins are depleted during colony foundation and while caring for the first brood (Van der Westhuizen et al. 1987; Nalepa 1988; Shellman-Reeve 1990; Costa-Leonardo et al. 2013; Mullins and Su 2018; Chouvenc 2019; Inagaki et al. 2020). The exhaustion of these reserves during incipient colony development combined with the degradation of jaw muscles, may be why the royal pair is nutritionally dependent upon the alloparents they invested in despite still harboring protozoa (Cleveland 1923, 1925; Chouvenc and Su 2017).

Protozoa may persist within the king and queen after the transition to alloparental care is completed via the small amount of proctodeal trophallaxis they receive relative to stomodeal trophallaxis from workers (Du et al. 2016, 2017). By day 400 the protozoan abundance of *C. gestroi* and *C. formosanus* kings and queens was either statistically equivalent to or significantly less than the abundance observed on day 0. Furthermore, similar to observations on *Reticulitermes*, we observed no protozoa within *C. gestroi* and *C. formosanus* kings and queens from 2-year-old laboratory-reared colonies (Cleveland 1925; Shimada et al. 2013; Inagaki and Matsuura 2016). These results indicate that eventually protozoa are absent from the hindgut of rhinotermitid reproductives, and that the loss of protozoa may occur well after the transition from biparental to alloparental care is completed.

Pairwise comparisons between the protozoan abundance of kings and queens revealed three instances in which the number of protozoa harbored by kings was significantly greater than the protozoan abundance of queens. On day 250, the protozoan abundance of *C. formosanus* kings was significantly greater than that of *C. formosanus* queens, and on day 400, both *C. gestroi* and *C. formosanus* kings harbored significantly more protozoa than their respective queens. Similar to our results, *R. speratus* kings harbored substantially more protozoa than *R. speratus* queens at 400 days after colony foundation (Shimada et al. 2013). These results suggest that after transitioning to alloparental care, queens lose their protozoa earlier than kings. *Reticulitermes flavipes* (Kollar) and *Reticulitermes grassei* Clément queens were significantly more likely to donate trophallaxis and spent significantly more time doing so compared to kings during the first 6 months of colony development (Brossette et al. 2019). Conceivably, queens may lose their protozoa earlier than kings because they donate more proctodeal fluid during incipient colony development. The earlier loss of protozoa by queens relative to kings may facilitate ovarian development, as the loss of protozoa likely reduces the size of the hindgut, which consequently may provide more space for the development of the reproductive organs (Shimada et al. 2013).

In conclusion, the number of protozoa within the king and queen dramatically increases as the royal pair cares for their first brood during the biparental care period. The first workers that emerge are inoculated with the hindgut community of the royal pair via repeated proctodeal trophallaxis, producing the first functioning alloparents within the colony. After transmitting their hindgut community to their first workers, the protozoan abundance of the king and queen begins to decline, as the royal pair is transitioning from feeding themselves to being fed by their workers. As the transition from biparental to alloparental care proceeds to completion, the number of protozoa harbored by the king and queen continues to decrease, and eventually, the protozoan community of the royal pair is lost entirely.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00040-021-00808-6.

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Data availability Protozoan abundance data are present within Tables S1–S4.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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