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Inter-clonal competition over queen succession imposes a cost of parthenogenesis on termite colonies

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Abstract

In social insect colonies, selfish behaviour due to intracolonial conflict among members can result in colony-level costs despite close relatedness. In certain termite species, queens use asexual reproduction for within-colony queen succession but rely on sexual reproduction for worker and alate production, resulting in multiple half-clones of a single primary queen competing for personal reproduction. Our study demonstrates that competition over asexual queen succession among different clone types leads to the overproduction of parthenogenetic offspring, resulting in the production of dysfunctional parthenogenetic alates. By genotyping the queens of 23 field colonies of *Reticulitermes speratus*, we found that clone variation in the queen population reduces as colonies develop. Field sampling of alates and primary reproductives of incipient colonies showed that overproduced parthenogenetic offspring develop into alates that have significantly smaller body sizes and much lower survivorship than sexually produced alates. Our results indicate that while the production of earlier and more parthenogenetic eggs is advantageous for winning the competition for personal reproduction, it comes at a great cost to the colony. Thus, this study highlights the evolutionary interplay between individual-level and colony-level selection on parthenogenesis by queens.

Keywords: intracolonial conflict, asexual queen succession, parthenogenesis, reproductive value, social insect, termite

1. Introduction

An organismal unit consisting of lower-level units always has internal conflict, where competition at the lower level could favour selfish units that pursue their own interests at the expense of the higher unit [1]. Therefore, conflicts among the lower-level units impose costs on the higher unit. For example, a meiotic drive gene biases its own transmission during gametogenesis by subverting the usual patterns of Mendelian inheritance, gaining an advantage over the alternative non-drive gene within heterozygotes [2,3], and it also compromises the fitness of the entire individual [4–8]. To maintain its integrity, the higher unit must prevent the selfish behaviour of the lower units [9]. The conceptual framework of multilevel selection can be applied to all levels of biological hierarchy [4], including the case of altruism and selfishness for individuals interacting in social groups. The reproductive division of labour between reproductive and nonreproductive individuals is the hallmark of eusociality, which is analogous to the germ–soma divide in multicellular organisms. Thus, colonies of eusocial insects can be seen as superorganisms [10], where workers actively work for the good of the colony in a manner similar to somatic cells in an individual organism. On the other hand, insect societies are subject to internal conflicts over reproduction because they are almost always families composed of genetically different individuals, not clones [11,12]. In *Melipona* stingless bees, female larvae win the conflict with workers over caste determination, with an excess developing into queens, resulting in a diminished workforce and reduced colony reproduction [13].

In this paper, we focus on the unique reproductive system called 'asexual queen succession' (hereafter AQS) in termites as a model system to study inter-clonal conflict over queen succession within a colony and investigate its colony-level costs. The AQS system was first identified in the subterranean termite *Reticulitermes speratus*, where queens produce their neotenic replacement queens parthenogenetically but use normal sexual reproduction to produce other colony members [14] (figure 1*a*). Colonies of *R. speratus* are founded by a monogamous pair of primary reproductives (one king and one queen) derived from alates (winged adults). The primary queen produces neotenic secondary queens by parthenogenesis and is finally replaced by them. Then, secondary queens are replaced by subsequent cohorts of asexually produced secondary queens. AQS allows colonies to boost reproduction without inbreeding due to the production of a large number of secondary queens through parthenogenesis. To date, AQS has been documented in seven species including non-termitid (Rhinotermitidae) and termitid (Termitinae: *Termes* group and Syntermitinae) termites [15–17].

Queens of AQS species can produce parthenogenetic offspring in the presence of kings by closing the micropyles (sperm gates; i.e. openings for sperm entry) of their eggs [18]. The type of parthenogenesis in *R. speratus* is automictic thelytoky with terminal fusion, which results in the production of all-female broods that are diploid and almost completely homozygous [14,17 ,19]. For example, the primary queen (genotype: AB) produces secondary queens of either AA or BB. Then, secondary queens of AA and BB produce AA and BB clones, respectively. The

parthenogenetically produced daughters carrying only maternal chromosomes almost exclusively develop along the nymph pathway, i.e. the reproductive pathway, due to genomic imprinting [20,21]. Therefore, there should be a severe conflict between secondary queen clones over the production of subsequent secondary queens, a conflict we have termed 'cloneflict'. This can be seen as an intragenomic conflict (A versus B) of the primary queen (AB) over queen succession (figure 1*b*). Although this example simplifies the conflict mechanism, in reality, each allele combination across multiple loci can result in a much wider variety of putative clones produced by the primary queen (electronic supplementary material, figure S1). We can predict that earlier and greater production of parthenogenetic eggs (micropyle-less eggs) is advantageous to win the inter-clonal competition (electronic supplementary material, figure S1), which may result in the overproduction of parthenogenetic eggs and dominance by a single clone, i.e. clonal drive.

Based on the potential for clonal competition, we first sought to determine clonal drive was occurring within colonies in the field by collecting queens and performing genotyping to see whether the drive progresses with colony growth. We also investigated whether the increase in the occupancy rate of specific clones resulted from random drift or the action of certain driving factors, by examining if any alleles significantly increased in frequency following the clonal drive. Our fieldwork and genetic analysis revealed that parthenogenetic eggs, and consequently parthenogenetic female nymphs, are produced in excess, spilling over into the alate population. Based on the relatedness of sexually and parthenogenetically produced alates as viewed from each caste, we theoretically predicted the conditions under which the production of parthenogenetically produced alates becomes preferable over sexually produced alates. Following this, we assessed the relative fitness of parthenogenetically produced alates compared with their sexually produced counterparts by evaluating colony foundation success in the field. Remarkably, we found that parthenogenetically produced alates rarely succeed in founding colonies, primarily due to their smaller size and lower survival rates compared to sexually produced alates, thus resulting in a significant cost to the colony.

2. Materials and methods

(a) Clonal drive in queen population

(i) Collection of termite queens in the field

We collected 175 colonies with kings and queens of *R. speratus* in pine or Japanese cedar forests in Kyoto, Shiga, Wakayama, Nagano and Chiba, Japan from May to September 2017–2019. All termites were extracted from the nest within 10 days of collection, and the phenotypes of kings and queens (primary or secondary) and the number of each were recorded. Of the 175 total colonies, 28 were used for the genotyping analyses. Twenty secondary queens were randomly selected from each colony and stored at −80°C for genotyping. Primary queens were found in four of the colonies and were also stored for genotyping. Of the 28 colonies used for the genotyping analysis, 23 were used for taking queen weight measurements. Termites in the 23 colonies were extracted from the nest

within 2 days of collection, and fresh weights of secondary queens were measured to the nearest 0.1 mg. Prior research has established that the total weight of queens, rather than the total number of queens, is a more accurate indicator of colony size [22]. Therefore, the total weight of queens was used to analyse the correlation between the indicator of colony size and the degree of clonal drive.

(ii) Microsatellite analysis

We analysed genotypes of individuals for eight microsatellite loci: *Rf6-1*, *Rf21-1*, *Rf24-2* [23], *Rs15*, *Rs10*, *Rs78*, *Rs02* and *Rs68* [24]. Termite DNA was extracted using a modified Chelex extraction [25]. Total DNA was extracted from the heads using 50 µl Chelex® solution (10% weight per volume; TE pH 8.0) and 0.5 µl proteinase K. After incubation at 55°C for 3 h, samples were heated at 95°C for 15 min. PCR amplifications were carried out in multiplex sets (dyset1: *Rf6-1*, *Rf21-1*, *Rf24-2* and *Rs15*, dyset2: *Rs10*, dyset3: *Rs78* and dyset4: *Rs02* and *Rs68*). Primers *Rf6-1* and *Rs10* were labelled by 6-FAM fluorescent tags, *Rf21-1* and *Rs02* by VIC, fluorescent tags, *Rf24-2* and *Rs68* by NED fluorescent tags, *Rs78* and *Rs15* by PET fluorescent tags. A 15.25 µl PCR cocktail contained 2 µl of the DNA sample, 0.3 µl of 25 mM $MgCl_2$, 0.3 µl of 10 mM dNTP, 1.5 µl of 10× PCR Buffer, 0.1 µl of 5 U/µl Taq DNA polymerase (Qiagen, Valencia, CA) and 5 pmol each of the multiplex primers. The cycling conditions consisted of an initial denaturation step at 95°C for 3 min, then 35 cycles of 95°C for 30 s and 60°C for 75 s, with a final extension at 72°C for 2 min. The PCR products were mixed with Hi-Di formamide containing GS-600 (LIZ) size standard and analysed on an Applied Biosystems 3500 Genetic Analyzer. Raw data were analysed with GeneMapper 5.0 software (Applied Biosystems, Inc., Foster City, CA).

(b) Parthenogenetically-produced alates in the field

(i) Sampling of dealates walking on the ground

We collected a pre-foundation population of *R. speratus* by traps (electronic supplementary material, figure S2). Each trap consisted of a plastic board (210 × 297 mm), a guiding wall (50 mm height) and four sticky traps (16 × 80 × 90 mm). The sticky traps, which have openings on all sides, were attached at each edge of the guiding wall. The guiding wall of this trap was set to lead dealates walking on the plastic board into the sticky traps. Fifty traps (a total of 200 sticky traps) were set haphazardly on the ground of an open forest of pine trees with grassy areas in Hieidaira, Kyoto, Japan, from 5 to 7 May 2015 during the swarming season of *R. speratus*. A massive synchronized flight was observed only on 5th May in this study site. On 7 May 2015, we brought all the traps back to the laboratory and collected all the dealates from the sticky traps. The head width (the maximum distance across compound eyes) of each sample was measured under a stereoscope (Olympus, Tokyo, Japan) using a digital imaging system (FLVFS-LS; Flovel, Tokyo, Japan). Since the condition of the sticky trap samples varied among individuals, we measured head width as a stable body size indicator. The dealates were stored at −80°C for the following microsatellite analysis. We then analysed the genotypes of each individual for all eight microsatellite loci analysed.

(ii) Sampling of dealates after colony foundation

We collected a post-foundation population of *R. speratus* from experimentally buried brown rotten pine wood pieces that were suitable for termite nesting material [26]. The pine wood was cut into pieces of approximately $20W \times 40D \times 10H$ cm size, autoclaved and half-buried in the ground at the same site on 28 April 2015. On 14 May 2015, which was 9 days after the mating flight, all the buried pine wood was excavated and transported to the laboratory. The pine wood was meticulously dissected to extract founding units [26]. We determined the sex of each individual by examining the morphology of the terminal abdominal sterna under a microscope. The individuals were placed separately in a collection tube (1.5 ml) and stored at −25°C. Then, we analysed the genotypes of each individual for the eight microsatellite loci.

(iii) Estimation of the proportion of parthenogenetically-produced alates among the pre- and post-foundation population

The mode of parthenogenesis in *Reticulitermes* termites is automixis with terminal fusion, resulting in the production of exclusively female offspring with nearly complete homozygosity [14,19]. Therefore, females that are homozygous at all eight microsatellite loci are likely the products of parthenogenesis. Nevertheless, there remains a rare possibility that complete homozygosity at all eight loci can also arise through inbreeding. However, when such inbreeding occurs, it is expected to be equally prevalent among male alates. Therefore, the number of parthenogenetically produced alates can be calculated by subtracting the estimated number of totally homozygous females produced by inbreeding from the number of totally homozygous females. The number of totally homozygous females derived from inbreeding was calculated by multiplying the total number of females by the proportion of totally homozygous males. By employing this calculation method, we can estimate the number of parthenogenetically produced alates in the pre- and post-foundation populations, $E_{\mathrm{PA,\,pre}}$ and $E_{\mathrm{PA,\,post}}$, respectively. Then, the relative colony foundation success Φ_{PA} of the parthenogenetically produced alates to female sexually produced alates was calculated (see details in electronic supplementary materials).

(c) Survivorship of sexually- and parthenogenetically-produced alates

Three colonies (colonies A−C) containing alates were collected from pine or Japanese cedar forests in Kyoto, Japan, just before the swarming season in 2016. These colonies were maintained at 20°C to keep alates from swarming until the experiment started. Just before the experiment, each colony was transferred to a room at 28°C, and alates stimulated by high temperature emerged from their chambers in wood. Alates were then separated by sex and maintained in Petri dishes lined with a moist unwoven cloth, and used for the experiments within a day of the flight. We used 40 males and 40 females randomly chosen from each of the three colonies. After removing the wings from the alates, we measured their fresh weight and used them in the following experiment. The wings of

each individual were preserved in a test tube containing 99.5% ethanol. Then, we analysed the genotypes of each individual using the wings for four microsatellite loci: *Rf6-1*, *Rf21-1*, *Rf24-2* and *Rs15*.

To compare viability and immunity levels between sexually produced alates and parthenogenetically produced alates, we investigated the survivorship of alates both with and without exposure to the entomopathogenic fungus *Metarhizium anisopliae* (source: National Institute of Technology Evaluation Biological Resource Centre, NBRC31961) that occurs with the termites in nature [27]. Each termite was randomly assigned to one of the two treatments. See electronic supplementary material, Text S1, for the methods of pathogen treatment. In the control treatment without the pathogen, the alates were individually exposed to a conidia-free 0.025% Tween 20 solution (without pathogen). After exposure to a conidia-free solution, termites were placed individually in a well of a 24-well plate (COSTAR®3526, Corning Inc., NY) lined with filter paper moistened with distilled water. The plates were maintained at 25°C in darkness and checked every day for 30 days to investigate alate survival.

(d) Data analysis

We analysed the relationship between colony size and the number of clones in the secondary queen population using a generalized linear mixed model (GLMM) with a binomial error distribution and logit link function. Based on the alleles of eight microsatellite loci, we determined the genotype of each secondary queen, and individuals with identical genotypes were defined as belonging to the same clone type. The response variable was the number of clones per the number of secondary queens analysed, and the explanatory variable was the total weight of queens. Colony was included as a random factor. We also analysed the relationship between colony size and the proportion of the most dominant clone using a GLMM with a binomial error distribution and logit link function. The response variable was the proportion of the most dominant clone, and the explanatory variable was the total weight of queens. Colony was included as a random factor. To investigate whether the frequency of certain alleles at specific loci increases in the secondary queen population from colonies dominated by a single clone type in queens, we conducted a Fisher's exact test with Bonferroni correction. A Bonferroni-corrected significance level of 0.05/8 = 0.006 was applied. To compile the dataset for this analysis, we first defined a colony as a driven colony if over 90% of the genotyped secondary queens were composed of a single clone, which was then classified as the dominant clone type. Five colonies met this criterion (180626F, 180605I, 180721A, 180605L and 180609A). Colonies not meeting this criterion were categorized as non-driven colonies. Second, we listed the alleles found in each non-driven colony (sheet 'alleles in non-driven colonies' in data set [28]), representing the alleles present in the primary queens of these colonies. Third, to investigate the frequency of each allele present before the occurrence of clonal drive, we counted the number of each allele found in non-driven colonies for this analysis. If only one allele was detected at a locus, it means that the primary queen was homozygous for that allele, and therefore, that allele was

double counted. Fourth, we recorded the alleles of the dominant clone type in each driven colony (sheet 'alleles in dominated clones' in data set [28]) and the count of each allele was used for the analysis. Finally, we compared the observed proportion of the most frequent allele in the dominant clone type with that in the non-driven colonies using a Fisher's exact test.

To compare the observed proportion of totally homozygous females between the pre- and post-foundation populations, a Fisher's exact test was used. To compare the estimated proportion of female parthenogenetically produced alates in the pre-founding population against that of the post-foundation population, a chi-squared test was applied. To compare the number of alleles per locus between the pre- and post-foundation population, the Wilcoxon signed-rank test was performed.

We compared body size between the pre- and post-foundation populations in males and females. In the statistical analysis, the normality of the data was assessed using a Shapiro–Wilk test. If the data were found to follow a normal distribution, a *t*-test was employed for comparisons. Alternatively, in cases where the data significantly deviated from a normal distribution, the non-parametric Wilcoxon signed-rank sum test was applied. Males and females were analysed separately. To assess the cost of parthenogenesis on body size, we compared head width between the heterozygous and completely homozygous males and females in the pre-founding population using a *t*-test or Wilcoxon signed-rank sum test in a similar manner. We also compared fresh weight between the heterozygous and completely homozygous females in each colony using a *t*-test or Wilcoxon signed-rank sum test in a similar manner. Chi-squared tests were applied to compare the observed or estimated numerical sex ratio in the pre-founding population against the null hypothesis assuming that the numbers of males and females were equal.

To assess the survivorship differences among male alates, female sexually produced alates and female parthenogenetically produced alates across three colonies, we used the Kaplan–Meier survival analysis. The survival distributions were then compared using log-rank tests, considering the type of alates. For multiple comparisons, a Bonferroni-corrected significance level of 0.05/3 = 0.017 was applied. Following the Kaplan–Meier survival analysis and log-rank tests, we calculated bootstrap confidence intervals to further evaluate the survivorship estimates' reliability for each alate type. This bootstrap method involved resampling the survival times with replacement 1000 times, allowing for the construction of normal-based confidence intervals around the median survival times. These bootstrap confidence intervals provide a robust measure of uncertainty around our survival estimates, accounting for potential sample size limitations and variability inherent in the survival data.

All analyses were performed using R v. 4.2.3 [29], with package lme4. For GLMMs and GLMs, the likelihood ratio tests (LRTs) were used to determine the statistical significance of each explanatory variable. A significance value of $p < 0.05$ was considered to indicate statistical significance.

3. Results

(a) Clonal drive in queen population

(i) Progression of clonal drive in field colonies

We collected 14 colonies with a primary queen (intermediate colonies) and 161 colonies without a primary queen (mature colonies, see definition for figure 1*a*). The colonies without a primary queen had more secondary queens than those with a primary queen (GLM, LRT: $\chi^2 = 106.570$, d.f. = 1, $p < 0.001$, figure 2*a*). Twenty-eight colonies were randomly selected and 20 secondary queens were genotyped from each. In total, 557 secondary queens were successfully genotyped, as three samples failed to produce reliable data. Microsatellite genotyping showed that there is notable variation in the number of clone types among the colonies. Of those colonies, 23 colonies in which queens were weighed within 2 days of collection were analysed to evaluate the association between the number of clone types and the total weight of queens, an indicator of colony size. The number of clone types decreased as the total weight of queens in the colony increased (GLMM, LRT: $\chi^2 = 6.555$, d.f. = 1, $p = 0.010$, figure 2*b*). The proportion of the most dominant clone type increased as the total weight of queens in the colony increased (GLMM, LRT: $\chi^2 = 6.066$, d.f. = 1, $p = 0.014$, figure 2*c*). Subsequently, to investigate whether specific alleles increase in frequency as the number of clone types decreases, data from all 28 genotyped colonies were used to compare the frequency of the allele in the dominant clone type in driven colonies with its frequency in non-driven colonies. The results revealed that a certain allele (274 bp allele) at the microsatellite locus *Rs15* tended to be over-represented among the prevailing clones (Fisher's exact test, 95% CI = 0.001–0.507, odds ratio = 0.042, $p = 0.004$, Bonferroni-adjusted significance level $p < 0.006$, figure 2*d*). In loci other than *Rs15*, no over-representation of specific alleles was detected (Fisher's exact test, *Rf24-2*: 95% CI = 0.026–3.898, odds ratio = 0.279, $p = 0.209$; *Rf6-1*: 95% CI = 0.003–1.857, odds ratio = 0.166, $p = 0.152$; *Rf21-1*: 95% CI = 0.006–0.961, odds ratio = 0.087, $p = 0.023$; *Rs10*: 95% CI = 0.017–2.819, odds ratio = 0.193, $p = 0.133$; *Rs78*: 95% CI = 0.005–2.851, odds ratio = 0.256, $p = 0.354$; *Rs68*: 95% CI = 0.011–1.509, odds ratio = 0.148, $p = 0.061$, Bonferroni-adjusted significance level $p < 0.006$, figure 2*e*).

(ii) Field study

Pre-foundation population

We collected 154 walking dealates in sticky traps, consisting of 111 females and 43 males (figure 3*a*). Forty-six out of the 111 females (41.4%) were completely homozygous at the eight loci examined, whereas only 3 out of 43 males (7.0%) were completely homozygous (figure 3*a*). Thus, the number of totally homozygous females derived from inbreeding can be estimated to be 7.7 (=7.0% of 111 females). In addition, our microsatellite analysis of parthenogens produced by female–female pairs in the laboratory showed that recombination and mutation rate are so low (recombination rate = 0, and mutation rate = 0.00236) that parthenogenesis results in complete loss of heterozygosity. Therefore, the number of parthenogenetically produced alates was estimated to

be

$$E_{PA,\,pre} = 46 - 7.7 = 38.3,$$

and that of female sexually-produced alates to be

$$E_{SA,\,pre} = 111 - 38.3 = 72.7,$$

where parthenogenetically-produced alates comprised 34.5% of all trapped female dealates.

The sex ratio of dealates of the pre-foundation population was significantly female-biased (43 males and 111 females: chi-squared test, $\chi^2 = 30.026$, d.f. = 1, $p < 0.001$, electronic supplementary material, figure S3*a*). This trend was consistent even after removing parthenogenetic individuals (43 males and 72.7 females; chi-squared test, $\chi^2 = 7.624$, d.f. = 1, $p = 0.006$).

Post-foundation population

From the artificially buried brown rotten pine wood pieces, we obtained 60 founding units. Most of these units (51 units) were male–female pairs and the other units were single male, single female, male–male pair, single male with two females, single female with two males, single male with three females and two males with two females (electronic supplementary material, figure S3*b*). In the male–female units, 5 out of the 51 females (9.8%) were completely homozygous, and 1 out of 51 males (2.0%) was completely homozygous (figure 3*b*). The proportion of parthenogenetically produced alates in the post-foundation population is significantly smaller than that in the pre-founding population (Fisher's exact test, 95% CI = 2.318–22.393, $p < 0.001$). Thus, the number of totally homozygous females derived from inbreeding can be estimated to be 1.0 (= 2.0% of 51 females). The number of parthenogenetically produced alates was estimated to be

$$E_{PA,\,post} = 5 - 1.0 = 4.0,$$

which comprised 7.8% of all female founders. This proportion was significantly lower than that in the pre-foundation population (34.5%; chi-squared test: $\chi^2 = 11.50$, d.f. = 1, $p < 0.001$).

The pre- and post-foundation populations shared 85.9 (± 4.4 s.e.) % of alleles. There was no significant difference in the number of alleles per locus between the pre- and post-foundation populations (Wilcoxon signed-rank test: $Z = -1.73$, $n = 8$ loci, $p = 0.250$). Thus, we concluded that these individuals belonged to the same population.

Thus, the estimated relative fitness Φ_{PA} of parthenogenetically produced alates to female sexually produced alates (see electronic supplementary material, Text S1) was

$$\Phi_{PA} = \frac{E_{PA,post}}{E_{PA,pre}} \Big/ \frac{E_{SA,post}}{E_{SA,pre}} = \frac{4.0}{38.3} \Big/ \frac{47.0}{72.7} = 0.162\,.$$

Body size comparison

Head width of individuals in the post-foundation population was significantly larger than that in the pre-foundation population for both sexes (for males: *t*-test, $t = 5.6634$, d.f. = 90.903, $p < 0.001$; for females: Wilcoxon signed-rank sum test, $W = 4431$, $p < 0.001$, figure 4*a*). In the pre-foundation population, the head width of totally homozygous female dealates was significantly smaller than that of the heterozygous female dealates (Wilcoxon signed-rank sum test, $W = 989.5$, $p = 0.002$, figure 4*b*). However, this trend was not found in the males of the pre-foundation population (*t*-test, $t = 0.71752$, d.f. = 2.2815, $p = 0.5394$). Comparison of fresh weight between female sexually and parthenogenetically produced alates revealed that parthenogenetically produced alates were significantly smaller than female sexually produced alates (*t*-test for colony A: $t = -6.7527$, d.f. = 17.658, $p < 0.001$; for colony B: $t = -4.9666$, d.f. = 14.161, $p < 0.001$; for colony C: $t = -5.0064$, d.f. = 6.5623, $p = 0.002$, figure 4*c*).

(iii) Comparison of survivorship between sexually- and parthenogenetically-produced alates in the laboratory

A total of 238 alates were successfully genotyped excluding two samples. As a result of microsatellite analysis, the control and pathogen treatments used 60 and 60 males, 47 and 51 female sexually-produced alates, and 11 and 9 parthenogenetically-produced alates, respectively. Then, we analysed the survivorship of males, female sexually and parthenogenetically produced alates in control treatment (see electronic supplementary material, Text S1, for the results of pathogen treatment). Owing to the inherently smaller sample size of parthenogenetically produced alates ($n = 20$) compared with sexually produced females (98) and males (120), we employed bootstrap confidence intervals to provide a more robust assessment of survivorship variability among these groups, taking into account the sample size limitations. Parthenogenetically produced alates had a significantly shorter survival than males and female sexually produced alates (log-rank test: $\chi^2 = 6.8$ and 8.4, d.f. = 1 and 1, $p = 0.027$ and 0.008, respectively, figure 4*d*). Bootstrap confidence intervals for median survival times, calculated using a normal approximation, further elucidate these findings. Female sexually produced alates have a normal confidence interval of 22.4–43.7 days, parthenogenetically produced alates at 2.3–15.5 days and male alates at 9.5–28.5 days, respectively. These intervals underscore the variability in survivorship among the different alate types and highlight the significantly shorter survival of parthenogenetically produced alates compared to both sexually produced and male alates. There was no significant difference in survivorship between male and female sexually produced alates (log-rank test: $\chi^2 = 0.7$, d.f. = 1, $p = 0.80$, figure 4*d*).

4. Discussion

In the present study, we found that clone diversity in the secondary queen population reduces as the

colony develops, eventually leading to dominance by a single clone. The clonal drive signifies intense competition among clone types for the queen position, a phenomenon we have termed 'cloneflict'. Therefore, the earlier and greater production of parthenogenetic eggs than other secondary queens is advantageous to win the inter-clonal competition. This situation meets the conditions for the tragedy of the commons [13,30], which predicts the overproduction of parthenogenetic eggs over what is necessary for queen succession. From a mechanistic point of view, the production of parthenogenetic eggs is determined by a decrease in the number of micropyles as the queen ages [18]. More proximately, the genes or epigenetic agents controlling the age-dependent reduction of the number of micropyle-forming oocytes [18] should be involved in this clonal drive. Our prior research has dismissed the emergence of parthenogenetic eggs owing to sperm depletion, as it has been shown that all queens are fertilized and every egg with even a single micropyle undergoes sexual reproduction, even during the season when the number of supplementary queens peaks [18]. We have identified an allele (274 bp allele at the microsatellite locus *Rs15*) that exhibits a significant increase in proportion as clonal drive progresses (figure 2). This result indicates that the rise in dominance of specific clones cannot be explained by random drift alone and suggests the presence of genes in the vicinity of this locus that are involved in the regulation of micropyle formation or that confer advantages in inter-clonal competition.

Because parthenogenetic offspring are all females and have an epigenetic predisposition to develop into the nymph pathway [20,21], their caste fate is either to become neotenic secondary queens or to become female alates. Our earlier studies of genotyping queens in field mature colonies identified all successful primary queens as being derived from sexually produced alates [14,18 ,20], which has concealed the existence of parthenogenetic alates. Our field sampling of dealates walking on the ground (pre-founding population) showed that 34.5% of female dealates were parthenogenetically produced. After genotyping 40 female alates each from three different field colonies, the proportions of asexually produced female alates within the female alates of the three colonies were found to be 10.5%, 27.5% and 12.5%, respectively. This indicates that many parthenogenetically produced offspring that have been left out of the musical chairs game for reproductive privileges overflow into alates (electronic supplementary material, figure S4). However, the parthenogenetically produced alates rarely succeed in pairing and colony foundation, likely due to their smaller body size and lower survivorship than sexually produced alates (see also electronic supplementary material, Text S1, for survivorship under exposure to pathogens). Because parthenogenetically produced alates have a twofold higher degree of relatedness than sexually produced alates (electronic supplementary material, figure S5), it is advantageous for secondary queens to produce alates parthenogenetically if the relative fitness Φ_{PA} of parthenogenetically produced alate to sexually produced alate is greater than 0.5 (electronic supplementary material, Text S1 and figure S5). In fact, the relative fitness Φ_{PA} was 0.162, a value so low that the production of parthenogenetic alates is a loss for colony members, even for the mother queen (electronic supplementary material, figure S5). However, parthenogenetically produced alates that almost never

achieve colony foundation should not necessarily be considered a waste for the colony. This is because they may reduce the predation pressure on sexually produced alates from the same colony through a dilution effect.

Within a colony, secondary queens producing excess parthenogenetic eggs would have higher fitness than those that produce only the small number of parthenogenetic eggs sufficient for queen succession. But colonies having excess parthenogenetic offspring and thus raising many dysfunctional parthenogenetically produced alates should have a lower colony fitness, that is, contribute to fewer successful founders in the larger population, than colonies producing only sexually produced alates. The overall outcome depends on the balance between the individual- and colony-level selective forces [31,32]. This clonal drive in AQS termites is analogous to the female meiotic drive in maize [33], although the selection level is different. The abnormal chromosome 10 (Ab10), which is a well-known selfish genetic element in maize, encodes a meiotic drive system that exhibits strong preferential segregation [34]. Despite this transmission advantage, Ab10 imposes fitness costs at the individual level such as decreased pollen viability, decreased seed set and decreased seed weight [7], which may explain why Ab10 is present at low frequencies in natural populations. Similarly, the timing and amount of parthenogenetic egg production depend on both the intensity of competition for queen succession and the cost of overproducing parthenogenetic eggs. A future study using a mathematical model is needed to predict equilibrium conditions.

The AQS system is employed to increase the number of queens through parthenogenesis of primary queens, thereby enhancing reproduction without inbreeding [14,17]. In this system, sexual reproduction produces workers and alates, whereas parthenogenesis is solely used for the production of secondary queens. These queens remain in the nest and are sheltered in a royal chamber, where they are fed by workers and solely engaged in egg production. Consequently, they experience a lower workload than female alates, which establish new colonies on their own. Our study found that when parthenogenetic daughters differentiate into alates, their fitness is considerably lower than that of sexual daughters. It is well-known across a broad range of organisms, including insects, that loss of heterozygosity in offspring produced by automictic parthenogenesis leads to a reduction in body size [35]. The genotype of offspring produced by parthenogenesis, and the associated costs, vary significantly depending on the mode of parthenogenesis [17,36 ,37]. In *R. speratus*, parthenogenesis leads to a rapid loss of heterozygosity because diploidy is restored through terminal fusion automixis [14,17 ,19]. Moreover, the low mating success of parthenogenetically produced alates may also result from mate choice, as male *R. speratus* tend to select larger females as mates [38], or due to the inability for pairs to coordinate tandem movements [39]. In contrast, the asexual lineage of the termite *Glyptotermes nakajimai* reproduces solely through parthenogenesis, with heterozygosity maintained by clonal (apomictic) reproduction [40,41]. The origin of parthenogenesis in *G. nakajimai* is thought to be hybrid, arising from two lineages with distinct karyotypes [41]. Parthenogenesis with hybrid origins, which maintains high heterozygosity, has low fitness costs [42].

It is noteworthy that the relative fitness of parthenogenetically produced alates was extremely low, yet it was not zero. This is because the result implies that a selection pressure, albeit weak, consistently operates for parthenogenetically produced alates to function as founding queens. Throughout our many years of field research and genotyping, we have not found a single instance where a colony founded by parthenogenetically produced alates grew large enough to produce alates. However, if selection pressure operates over a long evolutionary timescale, there is a substantial possibility that parthenogenetically produced alates could evolve to function as founding queens. Interestingly, parthenogenetically produced primary queens have been reported in *Cavitermes tuberosus*, *Inquilinitermes inquilinus* and *Spinitermes trispinosus*, even though offspring produced through parthenogenesis via gamete duplication become completely homozygous [15]. Therefore, to understand the role that parthenogenesis plays in each termite species, it will be necessary to consider a complex set of factors, including not just the cytogenetic mechanisms, but also the evolutionary time since its origin and the unique ecological background of each species.

Ethics

This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility

The dataset supporting this article are available via Dryad (doi: 10.5061/dryad.cjsxksndz).

Supplementary material is available online at [43].

Declaration of AI use

We have not used AI-assisted technologies in creating this article.

Authors' contributions

Y.W.: data curation, formal analysis, investigation, methodology, resources, visualization, writing—original draft; T.F.: data curation, investigation, methodology, resources, visualization, writing—original draft; Y.N.: data curation, investigation, methodology, resources, visualization, writing—original draft; K.K.: conceptualization, data curation, investigation, methodology, resources, writing—original draft; M.T.: formal analysis, funding acquisition, resources, validation, visualization, writing—original draft, writing—review and editing; E.L.V.: conceptualization, supervision, writing—original draft, writing—review and editing; K.M.: conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, visualization, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration

We declare we have no competing interests.

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Footnotes

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Figures

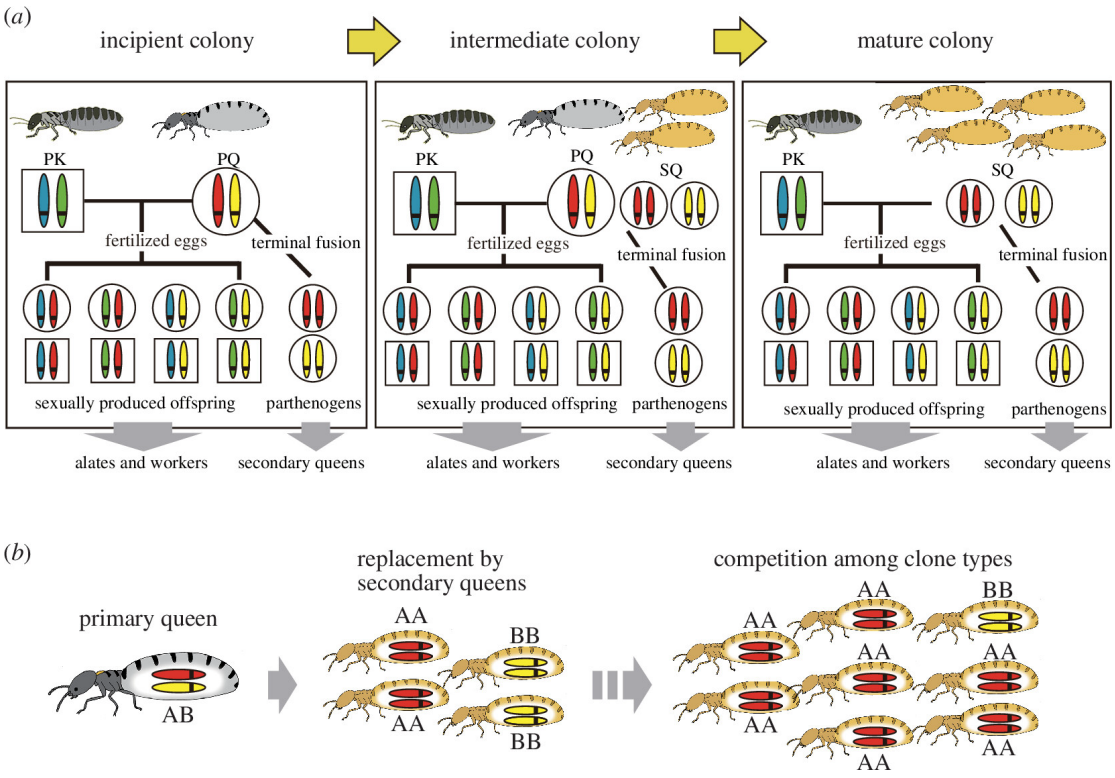

Figure 1. AQS and intraclonal conflict over queen position. (*a*) Scheme of AQS in *Reticulitermes* termites. Secondary queens (SQs) produced asexually (automixis with terminal fusion) by the primary queen (PQ) differentiate within the colony and supplement egg production, eventually replacing the PQ. An 'incipient colony' refers to an early-stage colony consisting solely of a pair of primary king (PK) and PQ. An 'intermediate colony' describes a state where both the PQ and SQs are present. A 'mature colony' is defined as a state where the PQ has been completely replaced by multiple SQs. Circles and squares indicate females and males, respectively. (*b*) Process of queen succession and hypothetical clonal drive. The PQ (genotype: AB) produces SQs of either AA or BB. Then, SQs of AA and BB produce AA and BB clones, respectively. There should be a severe conflict between SQ clones over the next queen position, which may result in the position being dominated by a single clone.

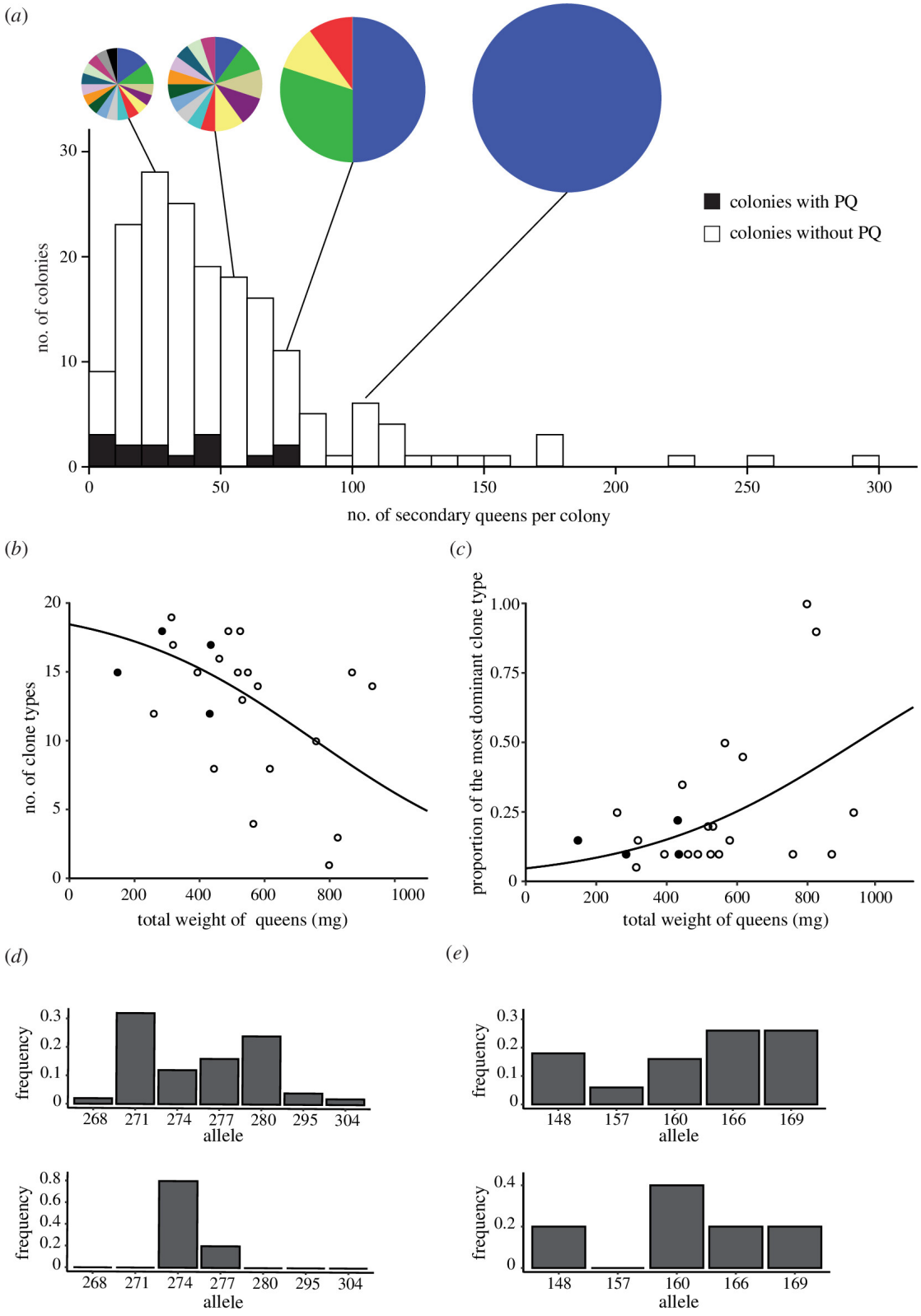

Figure 2. Progression of clonal drive correlated with colony development. (*a*) Distribution of the number of secondary queens per colony. Pie charts show the clone composition of selected representative colonies (different colours indicate different clones). The size of each pie chart is in proportion to its number of secondary queens. (*b*) Relationship between total weight of queens in a colony and the number of clone types. (*c*) Relationship between total weight of queens in a colony

and proportion of the most dominant clone type. The curves in (*b,c*) represent logistic fit to the data. Closed circles indicate the data of colonies with primary queens ($n = 4$) and open circles indicate those without primary queens ($n = 19$). Given that the total weight of queens is an indicator of colony size [22], these graphs illustrate that clonal drive progresses as the colony grows. (*d,e*) Numbers of alleles in colonies where clonal drive has not yet progressed (above) and in colonies dominated by a single clone type in queens (below). (*d*) Allele frequencies at the microsatellite locus *Rs15* before (above) and after (below) clonal drive. The 274 bp allele exhibits a significant increase in proportion as clonal drive progresses. (*e*) No such allele is observed at the other seven microsatellite loci. Allele frequency at locus *Rs68* is shown as representative. (Fisher's exact test followed by Bonferroni correction, $p < 0.05$).

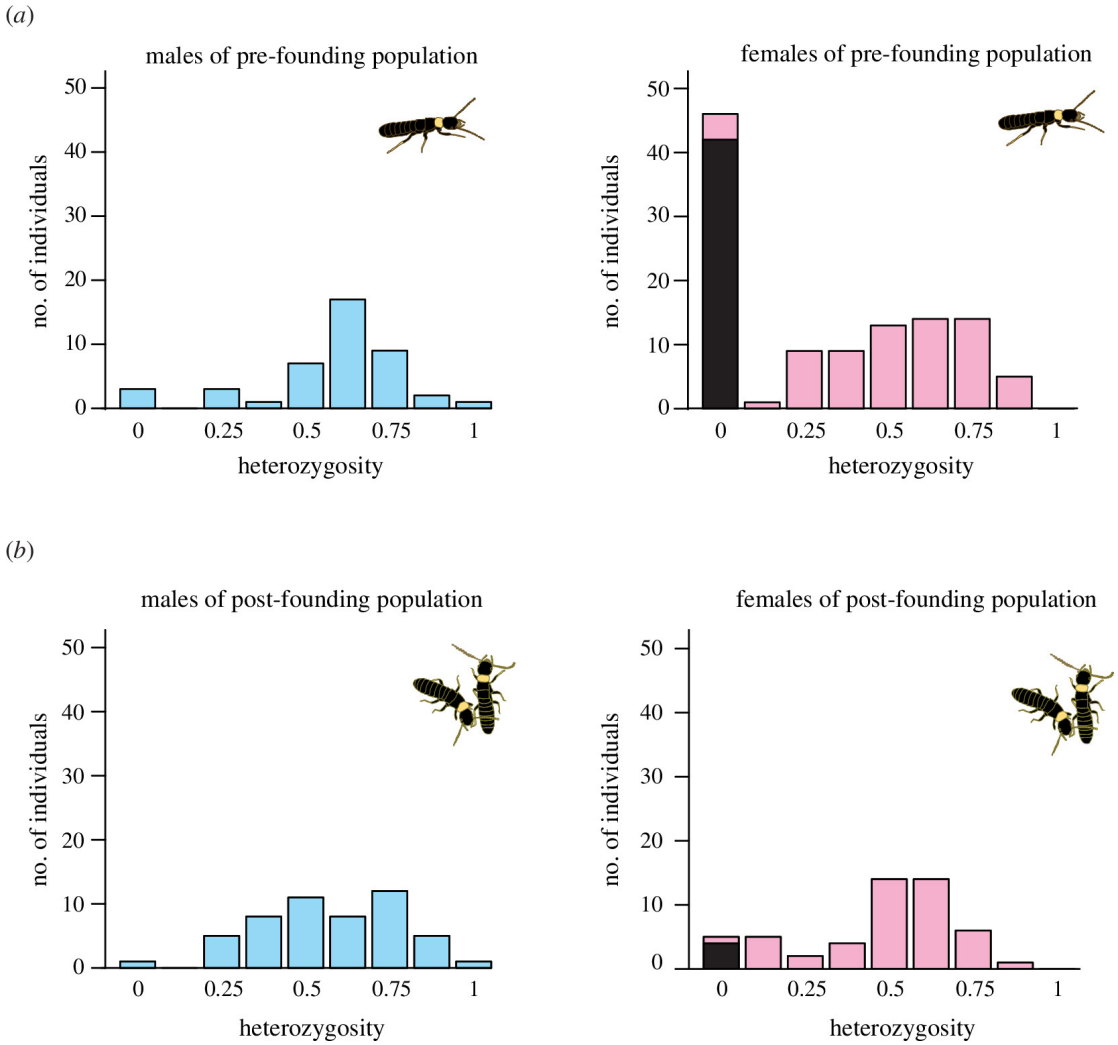

Figure 3. Comparison of the frequency of asexual (parthenogenetically produced) females between pre- and post-foundation population. (*a*) Males (left) and females (right) of pre-founding population were collected by sticky traps. (*b*) Males (left) and females (right) of founding pairs extracted from rotten wood. Black bars indicate the estimated number of asexual females. The proportion of asexual females in the post-foundation population is significantly smaller than that in the pre-founding population (Fisher's exact test, $p < 0.001$).

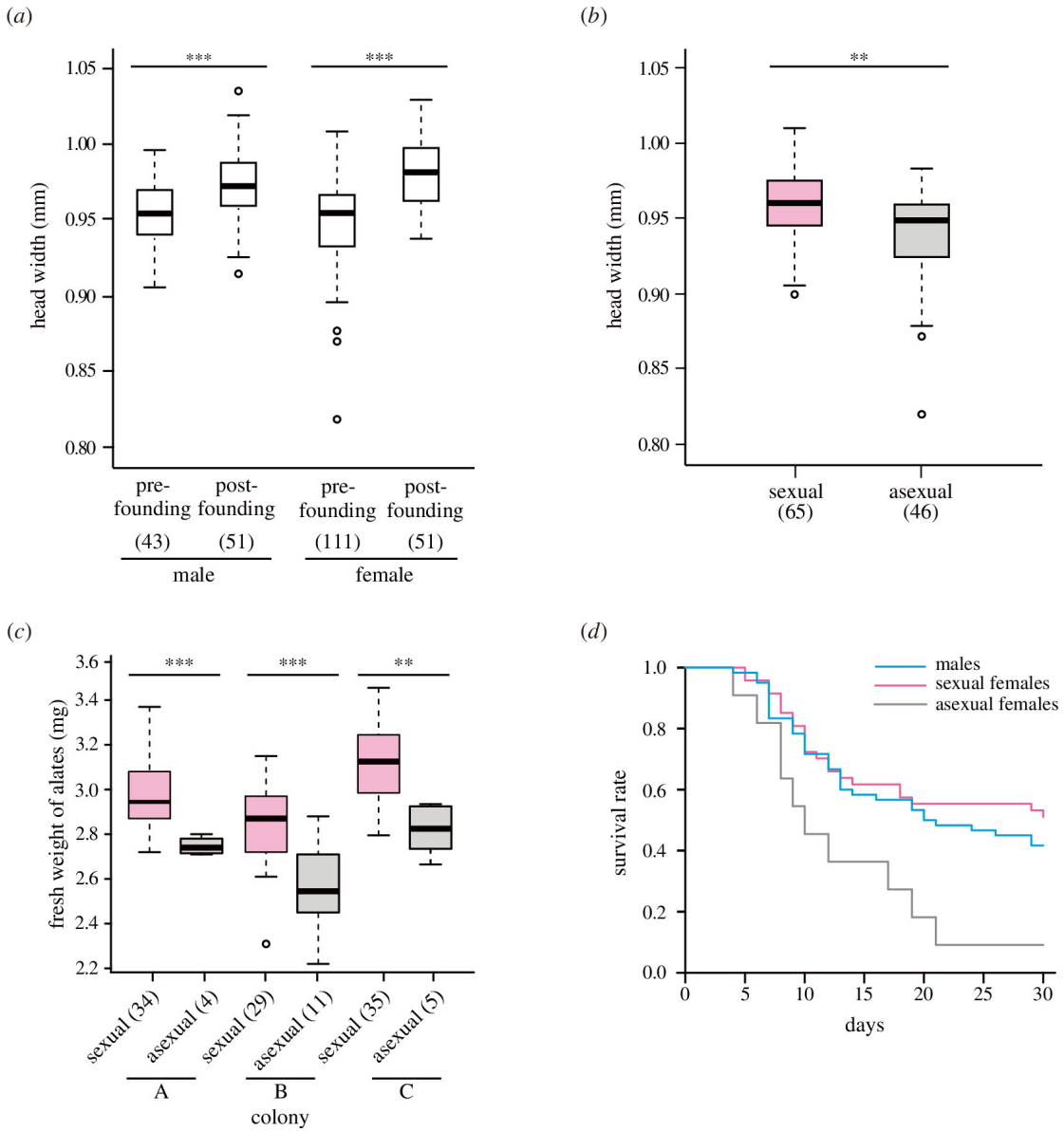

Figure 4. Comparison of fitness components between sexual and asexual (parthenogenetically produced) alates. (*a*) Comparisons of head widths of pre- and post-foundation individuals for both sexes. Successfully paired founders had significantly larger head size than pre-founding dealates both in males and females. (*b*) Comparisons of head widths between sexual and asexual females in pre-founding population. (*c*) Comparison of body weights between sexual and asexual alates collected from three natal colonies. The numbers of samples are indicated in the parentheses. $^{**}p < 0.01$, $^{***}p < 0.001$ (*t*-test or Wilcoxon signed-rank sum test). (*d*) Kaplan–Meier analysis of survival of males (blue), sexual females (red) and asexual females (grey) kept individually on moist filter papers. Asexual females had a shorter survival time (pairwise comparisons by log-rank test followed by Bonferroni correction, $p < 0.05$).

Supplementary Materials for

Inter-clonal competition over queen succession imposes a cost of parthenogenesis on termite colonies

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This file includes:

Text S1: Supplementary Methods and Results

Intra-colonial conflict over parthenogenetic alate production

As parthenogenetically produced alates have only maternal alleles, relatedness from colony members to parthenogens differ. Since *Reticulitermes speratus* colony members can only transmit genes through alates, this difference in relatedness would incur intra-colony conflict (figure 1b). Whether the production of sexually or parthenogenetically produced alates is preferable for the primary king (PK), the primary queen (PQ), secondary queens (SQ), and workers depends on their relatedness to sexually and parthenogenetically produced alates from the perspective of each caste, as well as the relative fitness Φ_{PA} of parthenogenetically produced alates compared to sexually produced alates (figure S5). From the perspective of PK, the relatedness to sexually produced alates ($g_{PK,SA}$) is 0.5, whereas the kinship to parthenogenetically produced alates ($g_{PK,PA}$) is 0. Therefore, regardless of the value of Φ_{PA}, the production of sexually produced alates is always preferable. On the other hand, from the perspective of SQ, the relatedness to sexually produced alates ($g_{SQ,SA}$) is 0.25, whereas the kinship to parthenogenetically produced alates ($g_{SQ,PA}$) is 0.5. Therefore, the production of parthenogenetically produced alates becomes preferable when $\Phi_{PA} > 0.5$ (figure S5). From the perspective of workers, the kinship to both sexually produced alates ($g_{worker,SA}$) and parthenogenetically produced alates ($g_{worker,PA}$) is equal at 0.5. Therefore, the production of parthenogenetically produced alates becomes preferable when $\Phi_{PA} > 1.0$. Thus, if $\Phi_{PA} < 0.5$, the production of parthenogenetically produced alates is not preferable from the perspective of any caste within the colony.

Estimation of the relative fitness of parthenogenetically- and sexually-produced alates

Empirically measuring the lifetime fitness of parthenogenetically- and sexually-produced alates to determine their relative fitness Φ_{PA} is extremely challenging. Instead, we estimated their relative fitness Φ_{PA} by comparing survival rates during the most critical period for survival, from swarming to colony foundation. The relative colony foundation success Φ_{PA} of the parthenogenetically-produced alate (PA) to sexually-produced female alate (SA) is given by W_{PA} / W_{SA}, where W_{PA} and W_{SA} are colony foundation success rates of parthenogenetic and sexual females, respectively.

$$\Phi_{PA} = \frac{W_{PA}}{W_{SA}} = \frac{N_{PA,post}}{N_{PA,pre}} \Big/ \frac{N_{SA,post}}{N_{SA,pre}} = \left(\frac{E_{PA,post}}{E_{PA,pre}} \cdot \frac{S_{pre}}{S_{post}}\right) \Big/ \left(\frac{E_{SA,post}}{E_{SA,pre}} \cdot \frac{S_{pre}}{S_{post}}\right)$$

$$= \frac{E_{PA,post}}{E_{PA,pre}} \Big/ \frac{E_{SA,post}}{E_{SA,pre}}$$

where the subscripts indicate either parthenogenetic (PA) or sexual (SA) female alates and either *pre*- or *post*-foundation populations. N is the numbers of individuals produced by each reproductive mode in the pre- and post-foundation populations. S_{pre} and S_{post} are the sampling efficiency of our methods for the *pre*- and *post*-foundation populations, respectively. Thus, the relative pairing success Φ_{PA} can be calculated from the estimated numbers of female alates produced by a certain reproductive mode in the *pre*- and *post*-foundation populations, $E_{PA,\,post}$, $E_{PA,\,pre}$, $E_{SA,\,post}$, and $E_{SA,\,pre}$.

721

722 **Survivorship of sexually- and parthenogenetically-produced alates under pathogen**
723 **treatment**

724 We investigated the survivorship of alates both with exposure to the entomopathogenic
725 fungus *Metarhizium anisopliae* (source: National Institute of Technology Evaluation
726 Biological Resource Centre, NBRC31961). The alates were individually exposed to a 1.0 ×
727 105 (conidia / mL) suspension of *M. anisopliae* (under exposure to pathogen). The suspension
728 of 1.0 × 105 (conidia / mL) is sufficient to measure the level of immunity based on data from
729 our preliminary experiment. After exposure to a conidia suspension, termites were placed
730 individually in a well of a 24-well plate (COSTAR®3526, Corning Inc. NY) lined with filter
731 paper moistened with distilled water. The plates were maintained at 25ºC in darkness and
732 checked every day for 30 days to investigate alate survival. To assess the survivorship
733 differences among male alates, female sexually-produced alates, and female
734 parthenogenetically-produced alates across three colonies, we utilized the Kaplan-Meier
735 survival analysis and calculated bootstrap confidence intervals, employing the same approach
736 as used for the control treatment in the main text.

737 As a result of microsatellite analysis, 60 males, 51 female sexually-produced alates, and
738 9 parthenogenetically-produced alates were used for the pathogen treatment. Under exposure
739 to pathogen, there was no significant difference between the survivorship of female sexually-
740 and parthenogenetically-produced alates, likely due to the early demise of most individuals
741 involved in the experiment (log-rank test: $\chi^2 = 0.2$, $df = 1$, $p = 1$). Similarly, no significant
742 differences were observed in the survival rates between males and female alates, regardless of
743 their mode of reproduction (log-rank test: $\chi^2 = 1.6$ and 0.8, $df = 1$ and 1, $p = 0.4$ and 0.8,
744 respectively). Bootstrap confidence intervals for median survival times, calculated using a
745 normal approximation under pathogen exposure, reveal narrower ranges: 7.1–11.0 days for
746 female sexually-produced alates, 2.8–9.4 days for parthenogenetically-produced alates, and
747 5.0–14.9 days for male alates. These reduced intervals under pathogen challenge are notably
748 shorter when compared to those in the control environment.

749

750 **Text S2: Supplementary Discussion**

751 **Contribution of parthenogenetically produced alates through predation dilution effect**

752 AQS species have the advantage of maintaining large numbers of secondary queens and
753 avoiding inbreeding compared to non-AQS species, allowing for greater colony expansion.
754 Despite the costs associated with producing asexually produced alates, AQS colonies can
755 produce more alates over their lifetime than non-AQS colonies. Mature colonies under clone
756 drive, although incurring costs from producing asexual female alates, are larger and thus can
757 produce more sexually reproduced alates than younger, smaller colonies.

758 Furthermore, the presence of asexually produced alates may contribute to the
759 reproductive success of their sexually produced siblings. The most critical period for the
760 survival of alates is the time from swarming to colony foundation, when they are outside the
761 nest and exposed to predators. This study revealed that the colony foundation success by

parthenogenetically produced alates is extremely low, with most dying during this critical period. This outcome may seem, at first glance, like a complete waste, as if the colony is investing resources in producing non-functional alates. However, colonies that produce such parthenogenetically produced alates are also larger and more mature, and they produce a large number of sexually reproduced alates as well. Our previous research demonstrated that males prefer to pair with larger females [1] and that solitary individuals have a higher predation rate when encountered by predators compared to those in tandem walking [2], suggesting that asexually produced female alates are likely to be preyed upon at a higher rate than sexually produced female alates. Therefore, by parthenogenetically produced alates facing relatively higher predation pressure, it's possible they enhance the survival rates of their sexually produced siblings through a dilution effect. This indirect contribution of parthenogenetically produced alates to the colony's overall fitness may reduce the cost of producing these alates and diminish the potential for counter-adaptations that would completely suppress their production.

Challenging issues to be addressed in future field studies

It is challenging to obtain data on the total number of alates produced by mature field colonies. The complexity of accurately capturing the entirety of a colony, especially for subterranean species like *Reticulitermes* termites that nests across multiple sites, makes it extremely difficult. We have recently developed a method to accurately estimate the colony size of *R. speratus* [3]. Technically, understanding the total number of alates in field colonies is an ambitious goal. However, how clone drive, alate production numbers, and the number of asexually produced alates evolve with colony growth presents an intriguing future research direction.

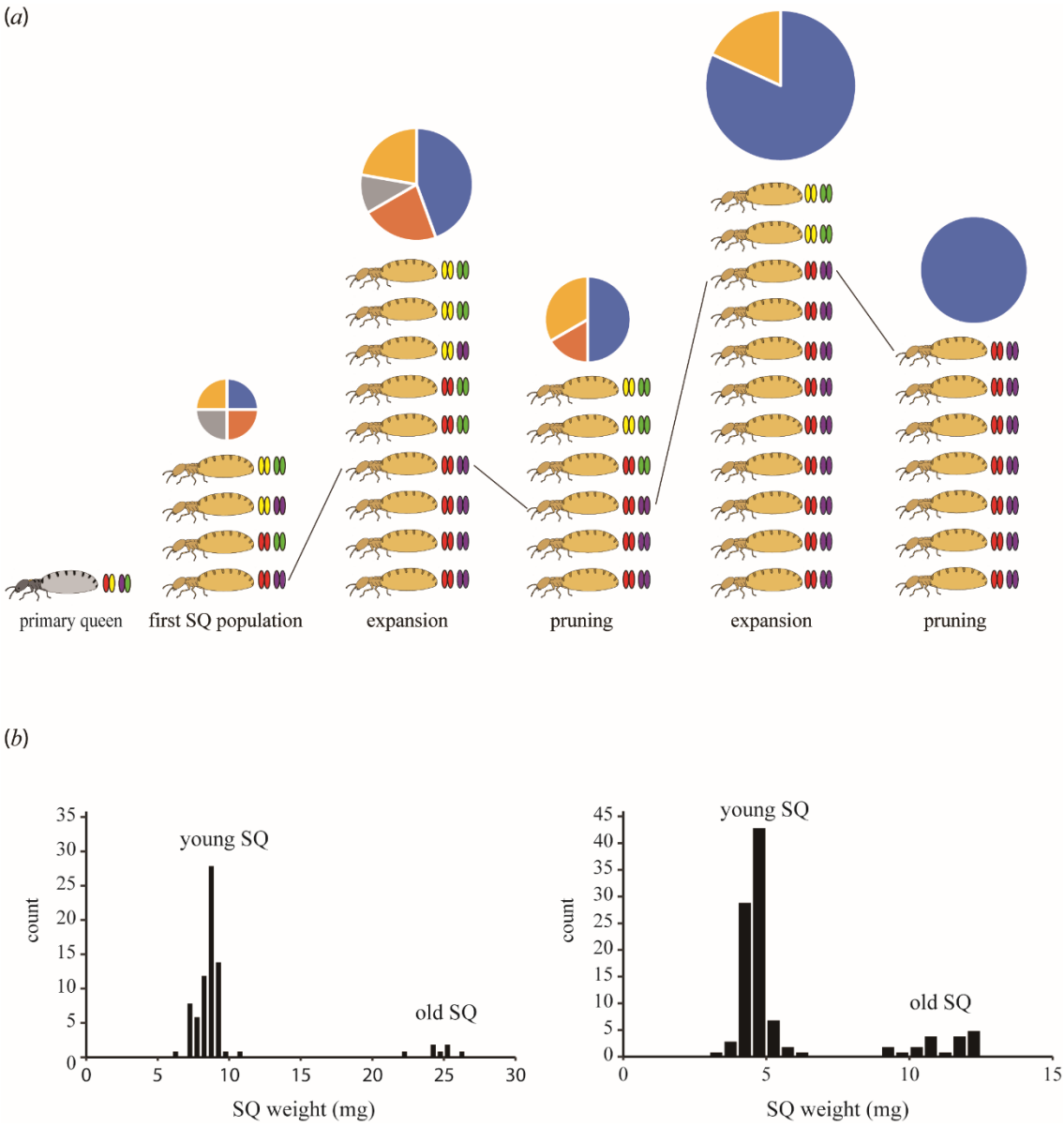


Figure S1. Process of queen succession and hypothetical clonal drive. (*a*) Scheme of asexual queen succession (AQS) in *Reticulitermes* termites. Secondary queens produced asexually (automixis with terminal fusion) by the primary queen. Excessive numbers of neotenic queens emerge synchronously with the differentiation of alates, while only a limited number of them inherit the queen position due to pruning before mid-summer. (*b*) The number of newly differentiated secondary queens and old physogastric secondary queens in representative colonies.

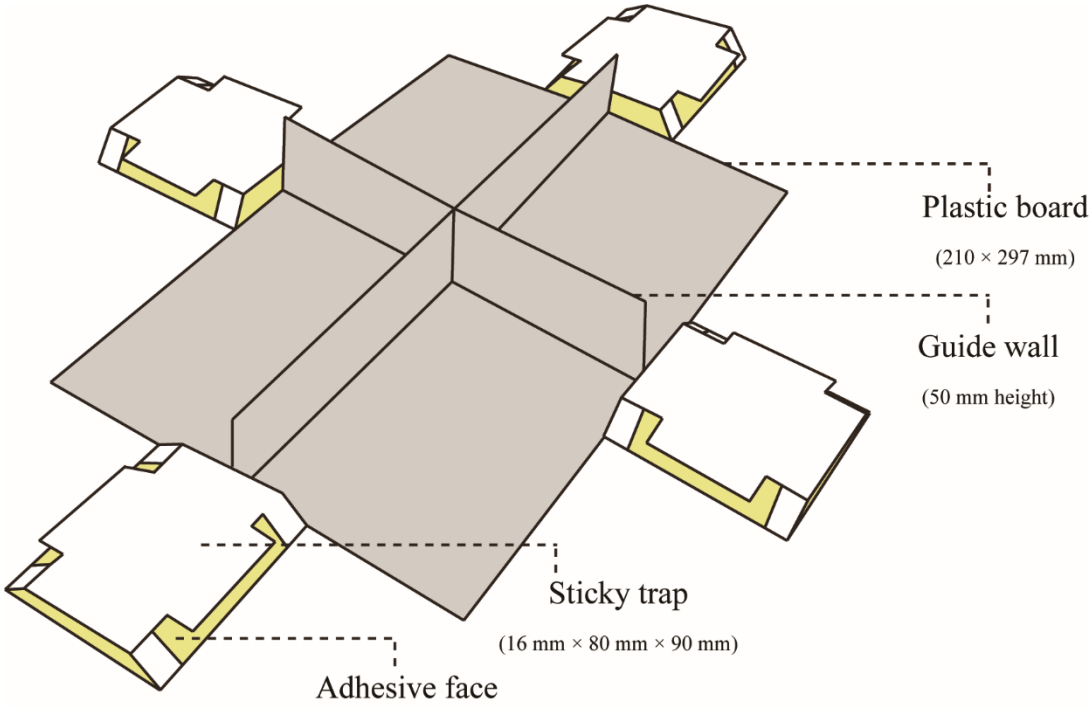


Figure S2. Schematic diagram of the traps used to collect walking dealates following mating flights. Each trap consists of guiding walls and four sticky traps arranged so as to introduce walking termites into sticky traps.

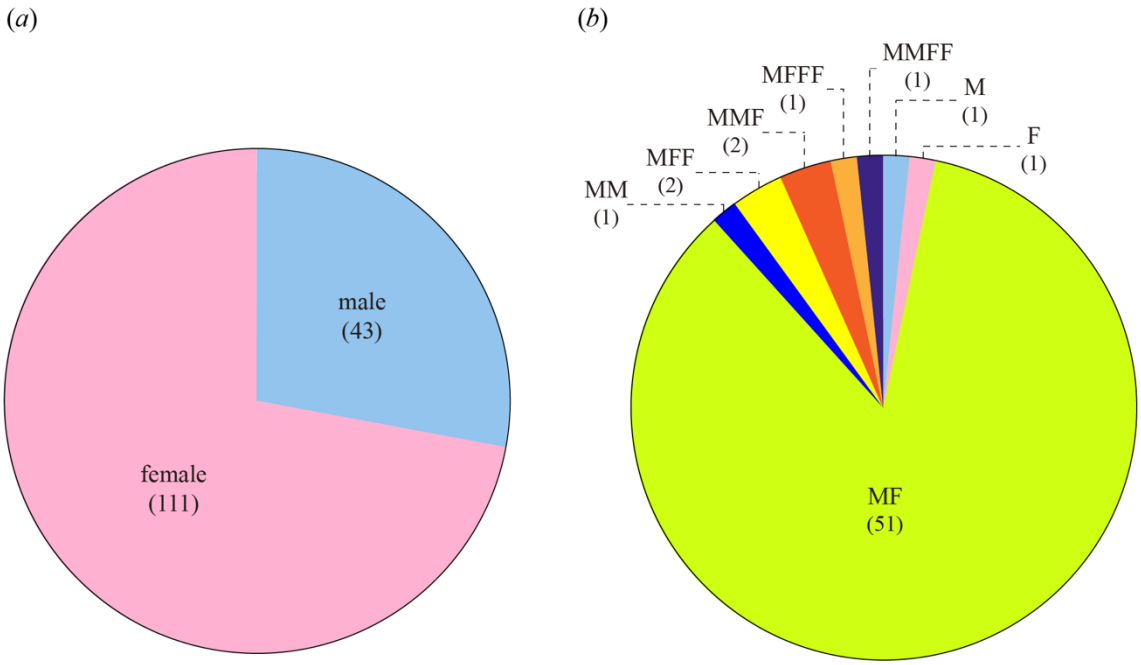


Figure S3. Composition of field-collected dealate and founding individuals. (*a*) Proportion of male and female dealates in pre-foundation population. (*b*) Composition of founding units. M: single male, F: single female, MF: male-female pair, MM: male-male pair, MFF: single male with two females, MMF: single female with two males, MFFF: single male with three females, and MMFF: two males and two females. The number of samples are indicated in the parentheses.

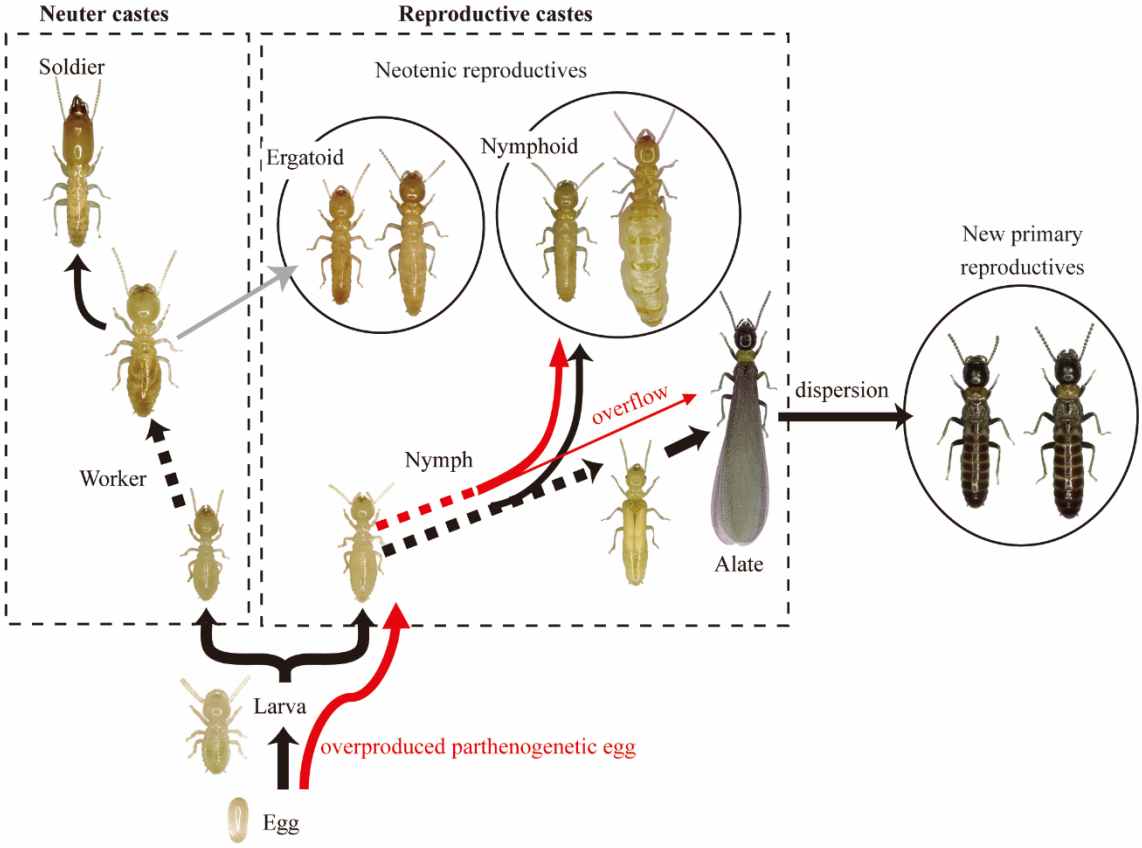
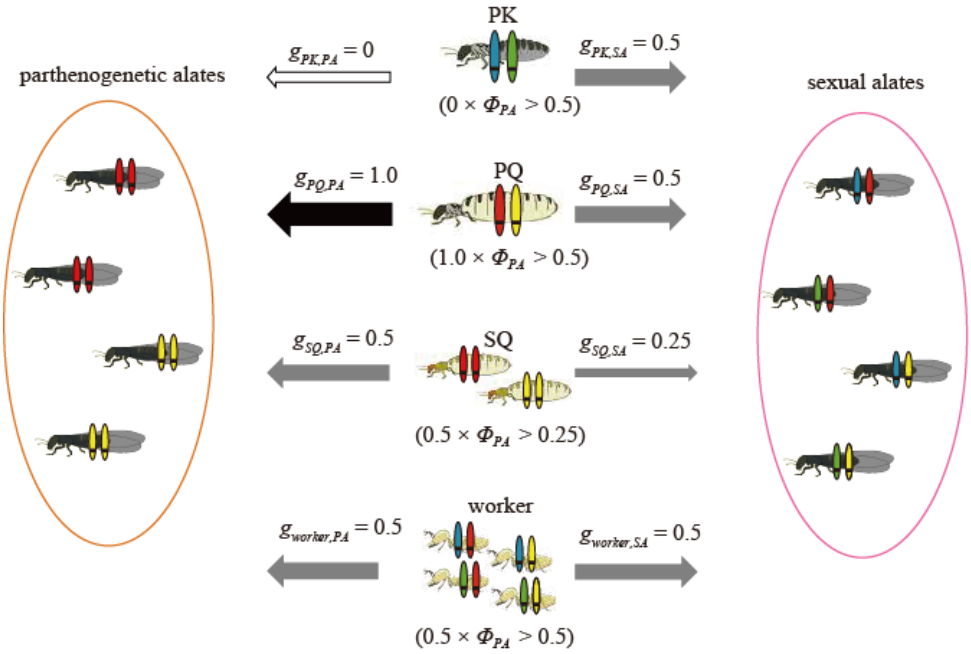


Figure S4. The scheme of caste differentiation of parthenogenetically-produced offspring. Parthenogenetically-produced offspring exclusively develop into nymphs and then into nymphoid secondary reproductives. However, overproduced parthenogenetic females overflow into alates.



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815 **Figure S5. Relative fitness of sexually and parthenogenetically produced alates as viewed**
816 **by each caste, and conditions under which the production of parthenogenetically**
817 **produced alates is preferable**. PK: primary king, PQ: primary queen, SQ: secondary queen.
818 $g_{caste,SA}$ and $g_{caste,PA}$ indicate the relatedness to sexually (SA) and parthenogenetically (PA)
819 produced alates from each caste (PK, PQ, SQ, and worker), respectively. Φ_{PA}: the relative
820 fitness of parthenogenetically produced female alates compared to sexually produced female
821 alates. Conditions favoring the production of parthenogenetic alates over sexual alates are
822 indicated in the parentheses.
823

824 **References**

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