Asexual queen succession and dynamics of intracolonial conflict in termites

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Chapter 1: General introduction

1.1 Intragroup conflicts in social insects

In social insects, the inclusive fitness theory explains the reproductive division of labor (Hamilton 1964). This theory suggests that some members of the colony give up their reproductive ability to assist in caring for the offspring of other members. Insect societies exhibit intermediate levels of relatedness, which leads to a strong motivation for altruistic behavior. That is because kin are usually in proximity and can be helped through activities such as defense or food collection. Nevertheless, this theory suggests that insect societies can also experience internal conflicts over reproduction. Even when individuals are closely related, kinship alone is not enough to eliminate selfish behavior. Therefore, social groups with lower relatedness levels are vulnerable to members exploiting group resources through free-riding (Hardin 1968; Keller 1999).

In eusocial Hymenoptera, such as bees, ants, and wasps, females have two copies of each chromosome (diploid), while males have only one (haploid) (Bourke and Franks 1995). As a result, female workers are more closely related to their full sisters (with a relatedness coefficient of 0.75) than their brothers (with a coefficient of 0.25). In contrast, the queen is equally related to male and female offspring (with a coefficient of 0.5). For optimal allocation, the queen produces an even gender ratio, while workers tend to prefer females at a ratio of three (female) to one (male) (Trivers and Hare 1976). In addition to the female-male worker conflicts resulting from kinship, male-male conflicts were attributed to mating competition; that is, the mating resource was limited because queens can store sufficient sperm in their young time for their whole life.

Cardiocondyla ants provide an example of within-nest mating conflicts. These ants have two morphological types of males. Wingless males resembling workers mate inside the nest. They engage in physical fights and use their sharp mandibles to kill each other. On the other hand, winged males mate mainly outside the nest. By chemically mimicking females, they avoid physical competition with the wingless males (Cremer et al. 2002).

According to Ratnieks 2006, kinship, coercion, and constraint can resolve those conflicts (Ratnieks et al. 2006). Therefore, identifying conflicts among nestmates and exploring reproductive strategies (such as constraint relationships between different castes within a colony) is crucial to understanding the ecological success of social insects.

1.2 Reproductive strategies in termite colonies

Termites rank among the most populous terrestrial creatures on the planet (Bar-On et al. 2018). Consistent with other eusocial insects, they have a reproductive division of labor, with specialized castes for reproduction and the rest of the colony working for them. Termites colonies are typically established by a pair of primary reproductives, also known as alate-derived reproductives, after a mating flight. The king and queen form a monogamous pair, giving birth to other colony members. In some termites, such as the lower termites, and in some higher termites, neotenic individuals, which are derived from nymphs or workers, take on the reproductive role after the death of the founding pair (Thorne et al. 1999; Korb and Hartfelder 2008). Most members are non-

reproductive workers and soldiers who forage, construct nests, care for the brood, and guard the nest (Thorne 1997; Korb 2016). The altruistic and neotenic reproductive castes are descendants of the primary parents (Figure 1.1). Royal pheromones used by kings and queens enable workers to recognize and care for these vital individuals and maintain the reproductive division of labor (Funaro et al. 2018). On the other hand, the parent-offspring commutation via trophallaxis leads to the integration of the social, nutritional, and microbial environments (Nalepa 2015).

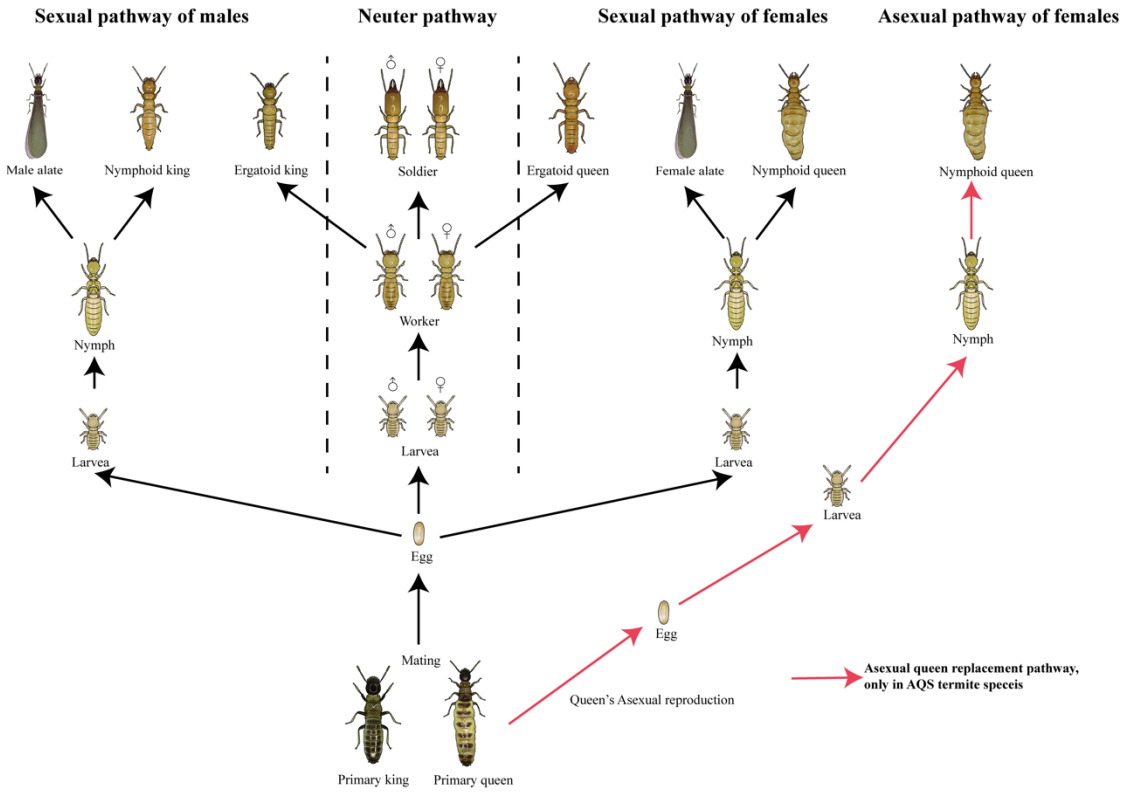

Figure 1.1 Life history of *Reticulitermes* termites. Caste differentiation pathways of *Reticulitermes* termites. Larvae differentiate into either the worker pathway (neuter pathway) or the nymph-alate pathway (sexual pathway). Nymphs develop into alates and fly out to establish new colonies. Nymphs and workers also have the potential to become nymphoids (nymph-derived neotenic reproductives) and ergatoids (worker-

derived neotenic reproductives), respectively. Scheme of asexual queen succession (AQS) is a mode of reproduction whereby workers, soldiers, and alates (dispersing reproductives) are produced sexually while neotenic queens (non-dispersing queens) arise through thelytokous parthenogenesis and eventually replace the old queen.

1.3 Caste determination and colony growth in termites

Caste determination and colony growth are fundamental aspects of the biology of social insects, known for their complex social structures and division of labor (Robinson 1992). Caste differentiation in termites is an extensively researched process by which they develop specialized roles within the colony (Snyder 1926; Roisin and Korb 2011; Pervez 2018). The phenomenon of facultative polytheism, where individuals within the same species can exhibit different phenotypes, is exemplified in termite caste differentiation. Despite having the same genetic background, termites can differentiate into various castes, each with specialized behaviors and tasks (Ye et al. 2021).

Caste differentiation is influenced by various factors, including environmental drivers (Roisin 2000; Korb and Katrantzis 2004), genetic influences (Hayashi et al. 2007; Anderson et al. 2008; Crozier and Schlüns 2008), as well as newly reported inheritable genomic imprinting components (Welch and Lister 2014; Maleszka 2016; Cardoso-Júnior et al. 2017; Matsuura et al. 2018).

Through researching field colonies and small sized incipient colonies with limited genetic diversity, a study that raised 280 laboratory termite colonies of *Reticulitermes urbis* over three years investigated how the initial composition of nymphs and workers influenced colony survival, population growth, and other dynamics, underscoring the

importance of caste composition in colony development (Ghesini and Marini 2009). Termite colony ontogeny, the developmental history of a colony, is also crucial as it encompasses the reproductive lifespan, caste ratios, and colony size, which are vital for understanding the growth and sustainability of termite colonies (Long et al. 2003). Identifying pheromones that regulate caste differentiation is another area of interest, as these chemical signals can determine the number of reproductive individuals within a colony, and therefore its reproductive capacity (Matsuura et al. 2010).

1.4 Parthenogenesis in termites

A few termite species have a unique reproductive system known asexual queen succession (AQS), where the queens produce their replacement by asexual reproduction and other nest members by mating with kings. This rare system has been found in only five out of the 2,400 described termite species *Reticulitermes speratus* (Matsuura et al. 2009), *Reticulitermes virginicus* (Vargo et al. 2012), *Reticulitermes lucifugus* (Luchetti et al. 2013), *Embiratermes neotenicus* (Fougeyrollas et al. 2015), and *Cavitermes tuberosus* (Fournier et al. 2016), and potentially in the following termite species due to their parthenogenetically reproductive capacity: *Zootermopsis angusticollis* (Light 1944), *Zootermopsis nevadensis* (Light 1944), *Bifiditermes beesoni* (Afzal and Salihah 2009), *Velocitermes* spp. (Stansly and Korman 1993), and *Reticuliterme aculabialis* (Peng et al. 2023). Termite queens can selectively switch between sexual reproduction and parthenogenetic reproduction. While workers are typically produced sexually, new queens can be produced asexually through thelytokous parthenogenesis, a strategy that

ensures the continuation of the colony even in the absence of males (Matsuura et al. 2009; Luchetti et al. 2013; Yashiro and Matsuura 2014).

The study of termite chromosomes reveals a fascinating complexity. Higher termites belonging to the family Termitidae have a fixed number of chromosomes (2n = 42), a characteristic that sets them apart. In contrast, the diploid number of chromosomes in lower termites varies from 28 to 56 (Bergamaschi et al. 2007). *Reticulitermes* termites possess 2n = 42 chromosomes. In *R. speratus*, parthenogenesis results in diploid offspring (Matsuura et al. 2004). It is also worth noting that termite parthenogenesis is thelytokous, meaning only female offspring are produced due to the XY sex-determination system. In termites, males are commonly heterogametic (Roisin 2001).

As reviewed by Matsuura 2011, all cases of termite thelytoky involve automixis with terminal fusion, whereas thelytoky in social Hymenoptera involves automixis with central fusion (Matsuura 2011). Therefore, it was surprising that *E. neotenicus*, a higher termite, reproduction method was similar to automixis with central fusion (Fougeyrollas et al. 2015).

The significance of parthenogenetic reproduction in termite species has been increasingly recognized, and it is suggested that it may be more widespread than previously thought. This AQS system can protect colonies from the cost of inbreeding because the neotenic queens are not daughters of primary kings. However, given the diverse clone types among the queen population, an intracolonial conflict is potentially present; queens can use asexual reproduction to transfer all their genes to the next

generation.

Understanding the ecological and evolutionary factors that drive the variation in termite breeding systems, including parthenogenesis, represents a significant challenge in termite biology. Developmental comparisons between parthenogenetic and sexual eggs in lower termites reveal that parthenogenetic eggs have a slower rate of development and lower hatching success compared to their sexual counterparts (Vargo 2019). This suggests that while parthenogenesis is a viable reproductive strategy, it may come with inevitable trade-offs in terms of developmental efficiency (Peng et al. 2023).

1.5 Objectives and outline of this thesis

This thesis delves into refining the philosophies behind managing reproductive strategies and intragroup conflict dynamics in termite evolution by researching their native behavior and biology, mainly using *Reticulitermes speratus* and subsidiarily using *Reticulitermes amamianus* as model organisms. This was accomplished by focusing on the central questions: 1) does the potential for conflict among different types of clonal queens exist, 2) what reproductive strategies did the colonies employ to regulate offspring caste fate and population size? Chapter 2 concentrated on discovering the genotypic diversity dynamics process in the asexual neotenic queen population and its impact on colony-level adaption. Chapter 3 focused on determining asexual capacity inheritance by investigating hybrid ergatoid queens (worker-derived) asexual egg-hatching results between *R. speratus* and non-asexually reproductive species *R. amamianus*. Chapter 4 of this thesis investigated the king-specific and queen-specific

contribution to altruistic caste fate determination and colony growth by using laboratory fostering *R. speratus*, *R. amamianus,* and their hybrid colonies in different ages. Under these conditions, the soldier sex ratios, colony population size, and egg-hatching ratios were attributed mainly to the kings. Still, soldier proportions were controlled by the queens. In the end, Chapter 5 summarizes the results and findings of this thesis and discusses the large-scale implications. Through long-time empirical data and novel techniques in termite colony fostering, this study gives a deeper understanding of the biology of social insects.

1.6 References

1 Afzal, M., and Z. Salihah. 2009. Sex ratio, occurrence of parthenogenesis, ovarian development and oviposition behaviour of the primary reproductives of *Bifiditermes beesoni* (Gardner) (Isoptera, Kalotermitidae). Zeitschrift für Angewandte Entomologie 100:132–146.

2 Anderson, K. E., T. A. Linksvayer, and C. R. Smith. 2008. The causes and consequences of genetic caste determination in ants (Hymenoptera: Formicidae). Myrmecological News 11:119–132.

3 Bar-On, Y. M., R. Phillips, and R. Milo. 2018. The biomass distribution on Earth. Proceedings of the National Academy of Sciences of the United States of America 115:6506–6511.

4 Bergamaschi, S., T. Z. Dawes-Gromadzki, V. Scali, M. Marini, and B. Mantovani. 2007. Karyology, mitochondrial DNA and the phylogeny of Australian termites. Chromosome Research 15:735–753.

5 Bourke, A. F. G., and N. R. Franks. 1995. Social Evolution in Ants. Princeton University Press, Princeton (NJ).

6 Cardoso-Júnior, C. A. M., P. T. Fujimura, C. D. Santos-Júnior, N. A. Borges, C.

Ueira-Vieira, K. Hartfelder, L. R. Goulart, et al. 2017. Epigenetic modifications and their relation to caste and sex determination and adult division of labor in the stingless bee *Melipona scutellaris*. Genetics and Molecular Biology 40:61–68.

7 Cremer, S., M. F. Sledge, and J. Heinze. 2002. Chemical mimicry: male ants disguised by the queen's bouquet. Nature 419:897.

8 Crozier, R. H., and H. Schlüns. 2008. Genetic caste determination in termites: out of the shade but not from Mars. Bioessays 30:299–302.

9 Fougeyrollas, R., K. Dolejšová, D. Sillam-Dussès, V. Roy, C. Poteaux, R. Hanus, and Y. Roisin. 2015. Asexual queen succession in the higher termite *Embiratermes neotenicus*. Proceedings of the Royal Society B: Biological Sciences 282:20150260.

10 Fournier, D., S. Hellemans, R. Hanus, and Y. Roisin. 2016. Facultative asexual reproduction and genetic diversity of populations in the humivorous termite *Cavitermes tuberosus*. Proceedings of the Royal Society B: Biological Sciences 283:20160196.

11 Funaro, C. F., K. Böröczky, E. L. Vargo, and C. Schal. 2018. Identification of a queen and king recognition pheromone in the subterranean termite *Reticulitermes flavipes*. Proceedings of the National Academy of Sciences of the United States of America 115:3888–3893.

12 Ghesini, S., and M. Marini. 2009. Caste differentiation and growth of laboratory colonies of *Reticulitermes urbis* (Isoptera, Rhinotermitidae). Insectes Sociaux 56:309–318.

13 Hamilton, W. D. 1964. The genetical evolution of social behaviour. II. Journal of Theoretical Biology 7:17–52.

14 Hardin, G. 1968. The tragedy of the commons. Science 162:1243–1244.

15 Hayashi, Y., N. Lo, H. Miyata, and O. Kitade. 2007. Sex-linked genetic influence on caste determination in a termite. Science 318:985–987.

16 Keller, L. 1999. Levels of Selection in Evolution. Princeton University Press, Princeton (NJ).

17 Korb, J. 2016. Genes underlying reproductive division of labor in termites, with comparisons to social Hymenoptera. Frontiers in Ecology and Evolution 4:1–10.

18 Korb, J., and K. Hartfelder. 2008. Life history and development - a framework for understanding developmental plasticity in lower termites. Biological Reviews of the Cambridge Philosophical Society 83:295–313.

19 Korb, J., and S. Katrantzis. 2004. Influence of environmental conditions on the expression of the sexual dispersal phenotype in a lower termite: implications for the evolution of workers in termites. Evolution & Development 6:342–352.

20 Light, S. F. 1944. Parthenogenesis in termites of the genus *Zootermopsis*. University of California Publications in Zoology 43:405–412.

21 Long, C. E., B. L. Thorne, and N. L. Breisch. 2003. Termite colony ontogeny: a long-term assessment of reproductive lifespan, caste ratios and colony size in *Reticulitermes flavipes* (Isoptera: Rhinotermitidae). Bulletin of Entomological Research 93:439–445.

22 Luchetti, A., A. Velonà, M. Mueller, and B. Mantovani. 2013. Breeding systems and reproductive strategies in Italian *Reticulitermes* colonies (Isoptera: Rhinotermitidae). Insectes Sociaux 60:203–211.

23 Maleszka, R. 2016. Epigenetic code and insect behavioural plasticity. Current Opinion in Insect Science 15:45–52.

24 Matsuura, K. 2011. Sexual and asexual reproduction in termites. Pages 255–277 *in* D. E. Bignell, Y. Roisin, and N. Lo, eds. Biology of Termites: a Modern Synthesis. Springer Netherlands, Dordrecht.

25 Matsuura, K., M. Fujimoto, and K. Goka. 2004. Sexual and asexual colony foundation and the mechanism of facultative parthenogenesis in the termite *Reticulitermes speratus* (Isoptera, Rhinotermitidae). Insectes Sociaux 51:325–332.

26 Matsuura, K., C. Himuro, T. Yokoi, Y. Yamamoto, E. L. Vargo, and L. Keller. 2010. Identification of a pheromone regulating caste differentiation in termites. Proceedings of the National Academy of Sciences of the United States of America 107:12963–12968.

27 Matsuura, K., N. Mizumoto, K. Kobayashi, T. Nozaki, T. Fujita, T. Yashiro, T. Fuchikawa, et al. 2018. A genomic imprinting model of termite caste determination: not genetic but epigenetic inheritance influences offspring caste fate. The American

Naturalist 191:677–690.

28 Matsuura, K., E. L. Vargo, K. Kawatsu, P. E. Labadie, H. Nakano, T. Yashiro, and K. Tsuji. 2009. Queen succession through asexual reproduction in termites. Science 323:1687.

29 Nalepa, C. A. 2015. Origin of termite eusociality: trophallaxis integrates the social, nutritional, and microbial environments. Ecological Entomology 40:323–335.

30 Peng, X., Z. Khan, X.-M. Liu, S.-L. Deng, Y.-G. Fang, M. Zhang, X.-H. Su, et al. 2023. Embryonic development of parthenogenetic and sexual eggs in lower termites. Insects 14:640.

31 Pervez, A. 2018. Termite biology and social Behaviour. Pages 119–143 *in* K. Aslam and A. Wasim, eds. Termites and Sustainable Management. Springer International Publishing, Cham.

32 Ratnieks, F. L. W., K. R. Foster, and T. Wenseleers. 2006. Conflict resolution in insect societies. Annual Review of Entomology 51:581–608.

33 Robinson, G. E. 1992. Regulation of division of labor in insect societies. Annual Review of Entomology 37:637–665.

34 Roisin, Y. 2000. Diversity and evolution of caste patterns. Pages 95–119 *in* T. Abe, D. E. Bignell, and M. Higashi, eds. Termites: Evolution, Sociality, Symbioses, Ecology. Kluwer Academic Publishers, Dordrecht.

35 Roisin, Y. 2001. Caste sex ratios, sex linkage, and reproductive strategies in termites. Insectes Sociaux 48:224–230.

36 Roisin, Y., and J. Korb. 2011. Social organisation and the status of workers in termites. Pages 133–164 *in* D. E. Bignell, Y. Roisin, and N. Lo, eds. Biology of Termites: a Modern Synthesis. Springer Netherlands, Dordrecht.

37 Snyder, T. E. 1926. The biology of the termite castes. The Quarterly Review of Biology 1:522–552.

38 Stansly, P. A., and A. K. Korman. 1993. Parthenogenetic development in *Velocitermes* spp. (Isoptera, Nasutitermitidae). Sociobiology 23:13–24.

39 Thorne, B. L. 1997. Evolution of eusociality in termites. Annual Review of Ecology, Evolution, and Systematics 28:27–54.

40 Thorne, B. L., J. F. A. Traniello, E. S. Adams, and M. Bulmer. 1999. Reproductive dynamics and colony structure of subterranean termites of the genus *Reticulitermes* (Isoptera Rhinotermitidae): a review of the evidence from behavioral, ecological, and genetic studies. Ethology Ecology & Evolution 11:149–169.

41 Trivers, R. L., and H. Hare. 1976. Haploidploidy and the evolution of the social insect. Science 191:249–263.

42 Vargo, E. L. 2019. Diversity of termite breeding systems. Insects 10:52.

43 Vargo, E. L., P. E. Labadie, and K. Matsuura. 2012. Asexual queen succession in the subterranean termite *Reticulitermes virginicus*. Proceedings of the Royal Society B: Biological Sciences 279:813–819.

44 Welch, M., and R. Lister. 2014. Epigenomics and the control of fate, form and function in social insects. Current Opinion in Insect Science 1:31–38.

45 Yashiro, T., and K. Matsuura. 2014. Termite queens close the sperm gates of eggs to switch from sexual to asexual reproduction. Proceedings of the National Academy of Sciences of the United States of America 111:17212–17217.

46 Ye, C., Z. Song, T. Wu, W. Zhang, N. U. Saba, L. Xing, and X. Su. 2021. Endocuticle is involved in caste differentiation of the lower termite. Current Zoology 67:489–499.

Chapter 2: Termite colonies incur a cost of parthenogenesis due to inter-clonal competition over queen succession

2.1 Introduction

Organisms composed of lower-level units experience internal conflict, where competition within the lower level can favor self-serving units that prioritize their interests, often at the expense of the higher-level units (Burt and Trivers 2006). When the units are at a lower-level conflict, it costs the higher unit. A gene that influences meiotic drive can distort the propagation of gametes, which undermines typical Mendelian inheritance patterns. Our results show a competitive advantage over the alternate allele that does not promote drive in heterozygous combinations (Crow 1979; Dawe et al. 2018), and it also compromises the fitness of the entire individual (Wilson 1997; Ardlie 1998; Dyer et al. 2007; Higgins et al. 2018; Larner et al. 2019). To maintain its integrity, the higher unit must prevent the selfish behavior of the lower units (Szathmáry and Smith 1995). The theoretical concept of hierarchical selection can be applied to various levels of biological organization (Wilson 1997). We can examine self-interest and selflessness among cooperative society members by adjusting our perspective to a higher level. The reproductive division of labor between reproductive and sterile individuals is the hallmark of eusociality, akin to the germ-soma divide in multicellular organisms. The colonies of eusocial insects can be considered superorganisms (Hölldobler and Wilson 2009), where workers actively work for the colony's good in a manner similar to somatic cells in an individual organism. Besides, insect societies are subject to internal conflicts over reproduction because insect

societies are almost always families, not clones (Trivers and Hare 1976; Ratnieks et al. 2006). In stingless bees *Melipona*, the female larvae win the conflict with the workers over their caste fate determination, with an excess developing into neotenic queens, leading to the diminishment of the workforce and reduction of colony reproduction (Wenseleers and Ratnieks 2004).

In this study, we focus on the asexual queen succession of termites as a system to invest in inter-clonal conflict over half-clonal queen succession. We investigate its colony-level costs. The AQS mechanism was first reported in *Reticulitermes speratus* termites (Matsuura et al. 2009), where queens produce their neotenic replacement queens asexually and use sexual reproduction to produce other colony members (Figures 1.1 and 2.1a). *R. speratus* colonies are founded by a monogamous pair of primary reproductives (one king and one queen) derived from dealates. The primary queens (PQ) produce secondary queens (SQs) via parthenogenesis and are eventually replaced by them. Afterward, the initial SQs are substituted by successive groups of half-clonal SQs. AQS enables colonies to enhance reproduction without inbreeding by producing multiple SQs through thelytoky. Until, AQS has been documented in seven species from lower (Rhinotermitidae) and higher (Termitinae: *Termes* group and Syntermitinae) termites (Hellemans et al. 2019; Hellemans and Roisin 2020; Matsuura 2020).

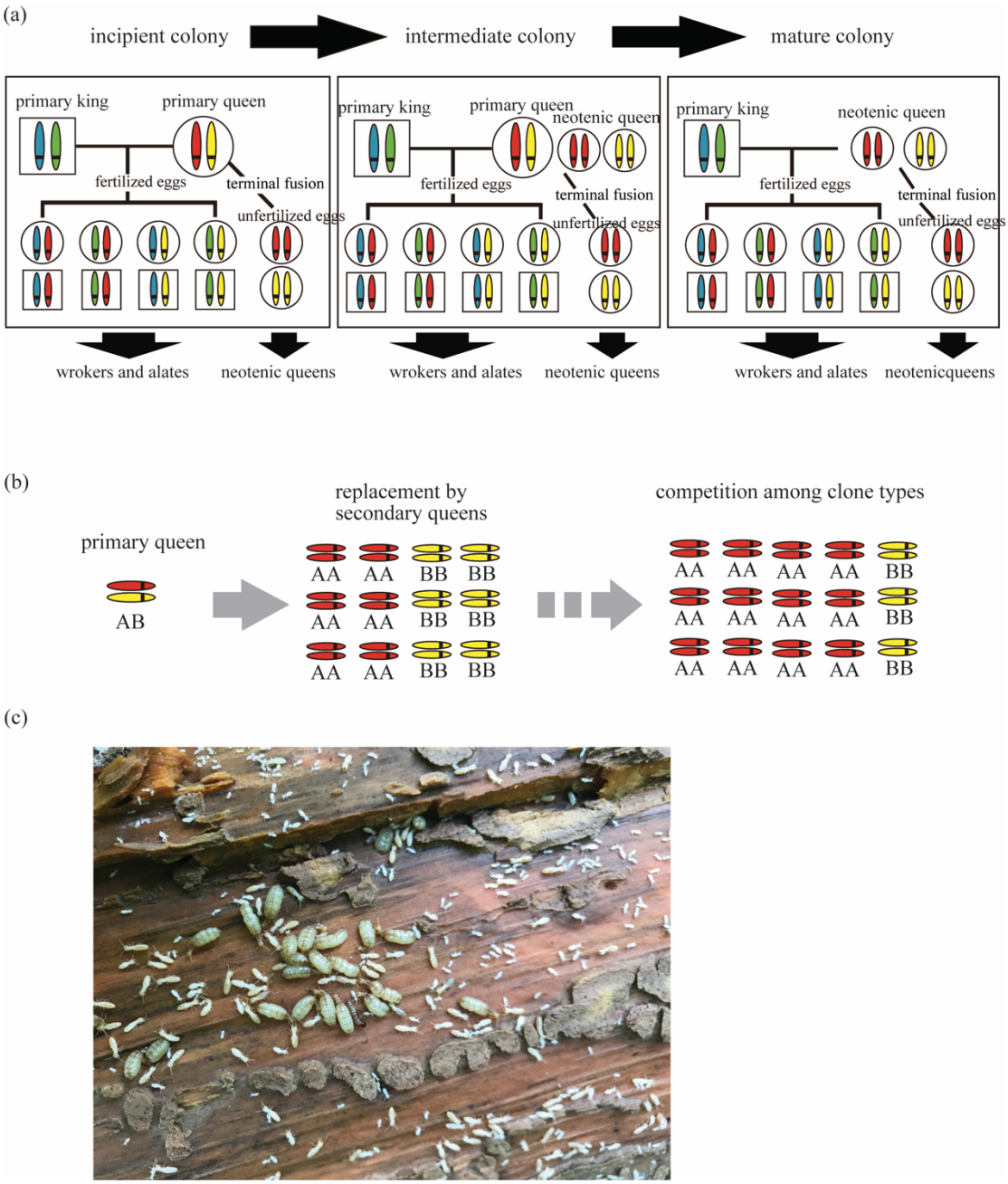

Figure 2.1 Asexual queen succession and intracolonial conflict over queen position. (a) Scheme of asexual queen succession (AQS) in *Reticulitermes* termites. Secondary queens (SQs) are produced asexually (automixis with terminal fusion) by the primary queen (PQ), which differentiates within the colony and supplements egg production, eventually replacing the primary queen. PK indicates primary king. Circles and squares indicate females and males, respectively. (b) Process of queen succession and hypothetical clonal drive. 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. Severe conflicts between secondary queen clones over the next queen position may occur, resulting in a monopoly by a single clone. (c) A photo of a mature *R. speratus* termite colony.

Queens of AQS species can produce asexual offspring with the presence of kings by closing micropyles (sperm gates) on their eggs (Yashiro and Matsuura 2014). The parthenogenesis in *R. speratus* is the automictic thelytoky type with terminal fusion. That leads to the production of all-female broods, which are diploid and completely homozygous (Matsuura et al. 2004, 2009; Matsuura 2017). For example, the primary queen (genotype AB) produces neotenic queens of either AA or BB. Then, SQs of AA / BB produce AA / BB clones, respectively. Most daughters produced by parthenogenesis develop into a reproductive pathway, relying on genomic imprinting due to carrying only maternal genomes (Matsuura et al. 2018; Tamaki et al. 2021). SQ clones will likely experience intense conflict when determining who will become the next queen. There is an intra-genomic conflict between two different alleles of PQ over queen succession (Figure 2.1a). Based on our predictions, the production of asexual eggs (micropyle-less eggs) occurs earlier and in larger quantities before a single clone dominates the competition (as shown in Figure 2.S1). This overproduction of asexual eggs could result in a single clone possessing a monopoly, leading to complete clonal drive.

Based on the possibility of a conflict, we investigated whether clonal drive occurs in the SQ population of field colonies. Our aim was to determine whether SQs produce

excessive parthenogenetic eggs and, if so, what happens to these eggs. Through our field sampling and genetic analysis, we discovered that overproduced asexual eggs result in an overflow of parthenogenetic female nymphs into alates. We conducted a study to compare the fitness of asexual alates to sexual alates by examining their survivorship and colony foundation success ratios. Surprisingly, the asexual alates produced by parthenogenesis were not successful in establishing colonies because their numbers were smaller due to lower survivorship compared to the sexually-produced alates. As a result, they imposed a high cost on the colony.

2.2 Materials and methods

2.2.1 Clonal drive in the queen populations

2.2.1.1 Collection of termite queens in the field

We collected 175 colonies of *R. speratus* with queens and kings in pine or Japanese cedar forests in Kyoto, Shiga, Wakayama, Nagano, and Chiba, Japan, from May to September 2017 through 2019. All the termites were removed from the nest within ten days after collection, and the characteristics of the kings and queens (primary or secondary) and the number of each were noted. Out of the 175 colonies, 28 were selected for the genotyping analysis. Twenty secondary queens were randomly chosen 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 two days of collection, and fresh

weights of secondary queens were measured to the nearest 0.1 mg.

2.2.1.2 Microsatellite analysis

We analyzed the genotypes of individuals for eight microsatellite loci: *Rf6-1*, *Rf21-1*, *Rf24-2* (Vargo 2000), *Rs15*, *Rs10*, *Rs78*, *Rs02*, and *Rs68* (Dronnet et al. 2004). The termite DNA extraction process involved using a modified Chelex extraction method (Walsh et al. 1991). DNA was extracted from the heads using 50 µL Chelex® solution (10% weight per volume with TE pH 8.0) and 0.5 µL proteinase K. After incubation at 55 °C for three hours, samples were thereby heated at 95 °C for 15 minutes. PCR amplifications were carried out in multiplex (dyset1: *Rf6-1*, *Rf21-1*, *Rf24-2*, and *Rs15*, dyset2: *Rs10*, dyset3 *Rs78*, dyset4 *Rs02* and *Rs68*). Primers *Rf6-1* and *Rs10* were labeled with fluorescent tags: 6-FAM (FAM), *Rf21-1* and *Rs02* (VIC), *Rf24-2* and *Rs68* (NED), *Rs78* and *Rs15* (PET). To prepare a 15.25 µL PCR cocktail, you need to add 2 µL of the DNA sample, 0.3 µL of 25 mM MgCl2, 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, USA), and five pmol each of the multi-plex primers. The cycling process involved an initial denaturation step at 95 °C for 3 minutes, followed by 35 cycles of 95 °C for 30 seconds and 60 °C for 75 seconds, with a final extension at 72 °C for 2 minutes. The PCR products were mixed with Hi-Di formamide that contained GS-600 (LIZ) size standard and were analyzed on an Applied Biosystems 3500 Genetic Analyzer. The sample was analyzed using the GeneMapper 5.0 software (Applied Biosystems, Inc., located in Foster City, CA, USA).

2.2.2 Parthenogenetically-produced alates in the field

2.2.2.1 Sampling of dealates walking on the ground

We obtained a pre-foundation population of *R. speratus* using traps (Figure 2.S2). Each trap was composed of a plastic board measuring 210 × 297 mm, a guiding wall that was 50 mm in height, and four sticky traps that measured 16 × 80 × 90 mm. The sticky traps, which had openings on all sides, were attached to each edge of the guiding wall. The guiding wall was designed to guide dealates walking on the plastic board into the sticky traps. We randomly placed 50 traps (200 sticky traps) on the ground of an open forest of pine trees with grassy areas in Hieidaira, Kyoto, Japan, from May 5th to May 7th, 2015, during the swarming season of *R. speratus*. We only observed a massive and synchronized flight on May 5th at this study site. On May 7th, 2015, we collected all dealates from the sticky traps and returned all traps to the laboratory. We used a digital imaging system (FLVFS-LS; Flovel, Tokyo, Japan) to measure the maximum distance across the compound eyes of each sample under a stereoscope (Olympus, Tokyo, Japan). To ensure consistency, we measured the head width of each sample as a stable indicator of body size since the condition of the sticky trap samples varied among individuals. The dealates were stored at -80°C and genotyped using eight microsatellite loci.

2.2.2.2 Sampling of dealates after the colony foundation

We collected a population of *R. speratus* from brown rotten pine woods suitable for termite nesting material after the foundation (Kusaka and Matsuura 2017). The pine

woods were cut into approximately 20W × 40D × 10H cm pieces size. These pieces were then autoclaved and placed on the same site from April 28th to May 14th, 2015. On May 14th, 2015, all the pine boards were brought back to the laboratory and carefully examined to find nests of newly flown dealates, which are founding units. We determined the sex of each individual by examining the morphology of the terminal abdominal sterna under a microscope. The individuals were then placed individually in a collection tube of 1.5 mL and stored at -25ºC. Finally, we analyzed the genotypes of each individual for eight microsatellite loci.

2.2.2.3 Estimation of the proportion of parthenogenetically-produced alates among the pre- and post-foundation population

Homozygous individuals are produced by inbreeding or parthenogenesis. In *R. speratus*, only females can be produced through parthenogenesis. Thus, totally homozygous males must be produced through inbreeding. We estimate the minimum number of colonies producing parthenogenetically-produced offspring, known as alates, by that assuming inbred colonies produce both male and female alates. To estimate the alleles of parthenogenetically-produced alates, we exclude the alleles of totally homozygous male alates from those of totally homozygous female alates, as homozygous males and females from the identical colony share alleles. The number of colonies that produce parthenogenetic alates is determined by dividing the number of alleles in the alates by two, which is the maximum number of alleles in the mother at the most polymorphic locus. Assuming inbred colonies produce alates at the same sex ratio as other colonies,

we can estimate the number of parthenogenetically-produced alates in the pre- and post-foundation population $E_{PA,\ pre}$, and $E_{PA,\ post}$, respectively. Subtract the estimated number of totally homozygous females produced by inbreeding from the number of totally homozygous females to determine these values. The total number of homozygous females resulting from inbreeding was determined by multiplying the number of females by the percentage of homozygous males. Then, the relative fitness w_{PA} of the parthenogenetically-produced alates to female sexually-produced alates was calculated (see details in supplementary materials).

2.2.3 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 of 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 the woods. 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 analyzed the genotypes of each individual using the wings for four microsatellite loci: *Rf6-1*, *Rf21-1*, *Rf24-2*, and *Rs15*.

We compared the survival rates and immunity of sexually-produced alates and parthenogenetically-produced alates. We observed their survivorship under two conditions: with and without exposure to the fungus *Metarhizium anisopliae*, which is found in the natural habitat of termites. This study was conducted by the National Institute of Technology Evaluation Biological Resource Centre (document number 1) (Zoberi 1995). The alates were exposed to one of two substances - a 0.025% Tween 20 solution (without any pathogen) or a 1.0 × 105 (conidia / mL) suspension of *M. anisopliae* (which contained the pathogen). We used a suspension of 1.0 × 105 (conidia / mL) as it proved sufficient to measure the level of immunity in our preliminary experiment. Each termite was randomly assigned to one of the two treatments. After exposure, each termite was 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 kept in darkness at 25ºC and checked daily for 30 days to observe the survival rate of the alates.

2.2.4 Data analysis

We analyzed 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

analyzed, and the explanatory variable was the total weight of queens. Colony was included as a random factor. We also analyzed 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 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, 180609A), with three out of the five being composed of a single clone type (180721A, 180605L, 180609A). Conversely, colonies not meeting this criterion were categorized as non-driven colonies. Secondly, we listed the alleles found in each non-driven colony (sheet "alleles in non-driven colonies" in Dataset 2.S1), representing the alleles present in the primary queens (PQ) of these colonies. Thirdly, we counted the number of alleles found in the non-driven colonies for this analysis. This step aims to investigate the frequency of each allele present before the occurrence of clonal drive. Thus, if only one allele was detected at a locus, it means that the PQ was homozygous for that allele, and therefore, that allele was double counted. Fourthly, we recorded the alleles of the dominated clone type in each driven

colony (sheet “alleles in dominated clones” in Dataset 2.S1), and the count of each allele was used for the analysis. Finally, we compared the observed proportion of the most frequent allele in the dominated clone type with that in the non-driven colonies.

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

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 the 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 analyzed 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 utilized the Kaplan-Meier survival analysis. The survival distributions were then compared using log-rank tests, considering the type of alates and their colony of origin. A Bonferroni-corrected significance level of 0.05/3 = 0.017 was applied for multiple comparisons.

All analyses were performed using R version 4.2.3 (R Core Team 2020) with the package lme4. For GLMMs and GLMs, the likelihood ratio tests (LRTs) were used to determine the statistical significance of each explanatory variable. The statistical significance was determined using a significance value of $p < 0.05$.

2.3 Results

2.3.1 Clonal drive in the queen population

2.3.1.1 Progression of clonal drive in the field colonies

We collected 14 colonies with a primary queen (intermediate colonies) and 161 without a primary queen (mature colonies; see definition for Figure 2.1a). The colonies without a primary queen had more secondary queens than the ones with a primary queen (GLM, LRT: $\chi 2 = 106.570$, $df = 1$, $p < 0.001$, Figure 2.2a). We randomly selected 28 colonies and genotyped 20 secondary queens from each. We successfully genotyped 557 secondary queens, as three samples failed to produce reliable data. Our microsatellite genotyping showed that there was considerable variation in the number of clone types among the colonies. We analyzed 23 colonies in which queens were weighed within

two days of the collection for the association between the number of clone types and queen weight. We found that the number of clone types decreased as the total weight of queens in the colony increased (GLMM, LRT: $\chi 2 = 6.555$, $df = 1$, $p = 0.010$, Figure 2.2b). Additionally, the proportion of the most dominant clone type increased as the total weight of queens in the colony increased (GLMM, LRT: $\chi 2 = 6.066$, $df = 1$, $p = 0.014$, Figure 2.2c). We further investigated whether specific alleles increase in frequency as the number of clone types decreases. To do this, we used data from all 28 genotyped colonies to compare the frequency of the allele in the dominated clone type in driven colonies with its frequency in non-driven colonies. Our results revealed that a certain allele (274bp 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 2.2d). We did not find any notable variation in loci other than this one. 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 2.2e).

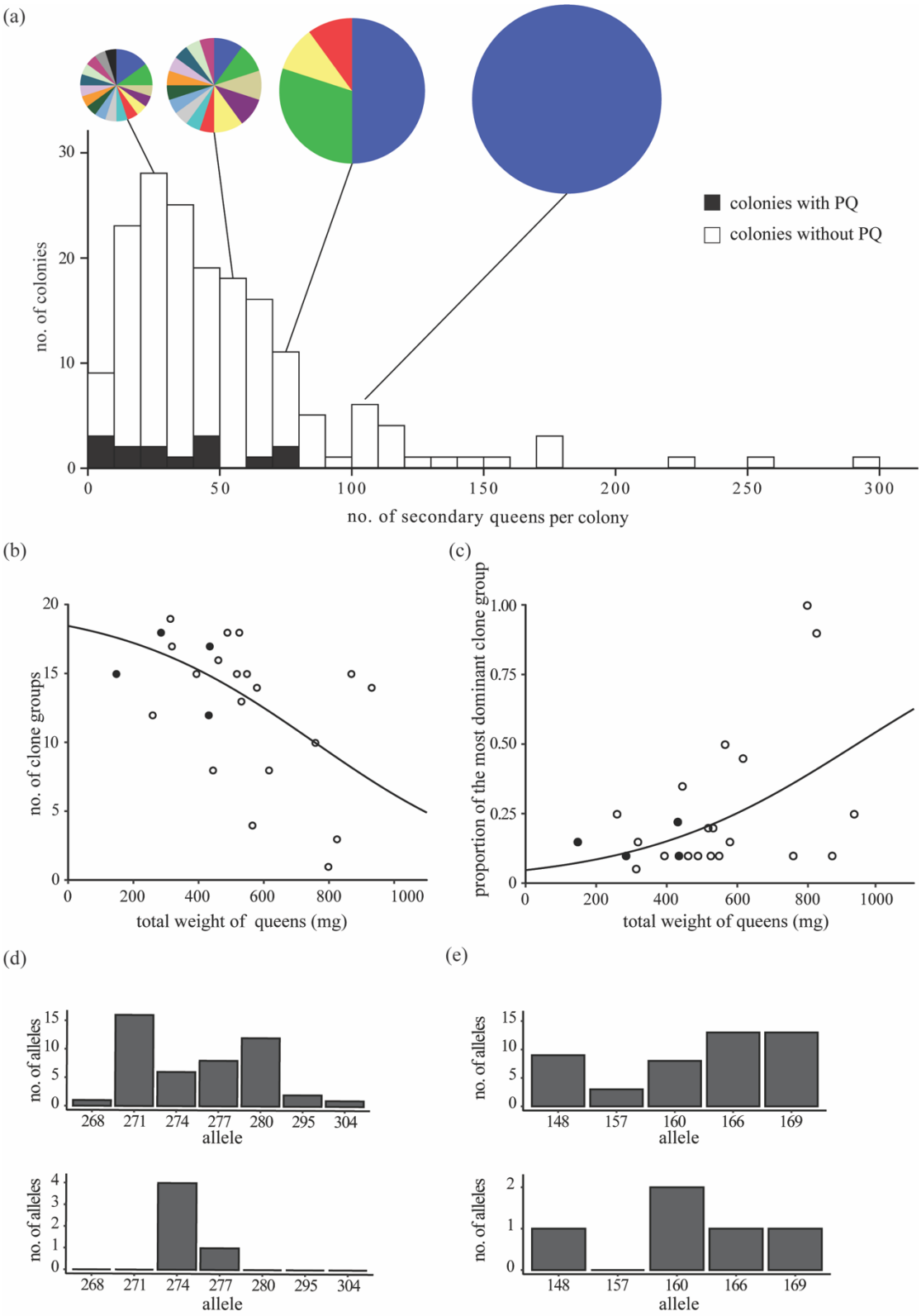

Figure 2.2 As the colony develops, there is a correlation between the progression of clonal drive. (a) Distribution of the number of secondary queens per colony. The charts

display the clone composition of specific representative colonies, where each color represents a different clone. The size of the pie chart is in proportion to the number of secondary queens. (b) Relationship between the total weight of queens in a colony and the number of clone types present. (c) Relationship between total queen weight and dominant clone proportion in a colony. Closed circles represent colonies with primary queens (n = 4), and open circles represent those without (n = 19). (d and e) Number of alleles in colonies where the clonal drive has not yet progressed (above) and in colonies dominated by a single clone type in queens (below). (d) The proportion of the 274bp allele at the microsatellite locus *Rs15* increases significantly during clonal progression. (e) No additional alleles were observed at the other seven microsatellite loci. *Rs68* is representative (Fisher's exact test followed by Bonferroni correction, $p < 0.05$).

2.3.2 Field study

2.3.2.1 Pre-foundation population

We captured 154 walking dealates using sticky traps, consisting of 111 females and 43 males (as shown in Figure 2.3a). Among the females, 46 out of 111 (41.4%) were found to be entirely homozygous across the eight gene loci examined, while only 3 out of 43 males (7.0%) were homozygous (Figure 2.3a). Based on these results, we estimate that the number of females who are homozygous due to inbreeding is 7.7 (= 7.0% of 111 females). Furthermore, our analysis of parthenogens produced by female-female pairs in the laboratory showed that the recombination and mutation rates are very low (recombination rate = 0, and mutation rate = 0.00236). Therefore, the number of parthenogenetically-produced alates (below is PA) was estimated to be

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

and that of female sexually-produced alate (below is SA) to be

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

where parthenogenetically-produced alates comprised 34.5% of all trapped female dealates. The coefficient of relatedness between completely homozygous females and the other females was 0.0194 (± 0.0056 SE) and 0.0029 (± 0.0038 SE), respectively. We estimated that 14.8 % of the colonies contributed to the population of totally homozygous female dealates.

The ratio of male and female dealates in the pre-foundation population was significantly imbalanced towards females. There were 43 males and 111 females, as confirmed by a Chi-squared test ($\chi 2 = 30.026$, $df = 1$, $p < 0.001$, Figure 2.S3a). Even after removing parthenogenetic individuals, this trend remained consistent with 43 males and 72.7 females (Chi-squared test, $\chi 2 = 7.624$, $df = 1$, $p = 0.006$).

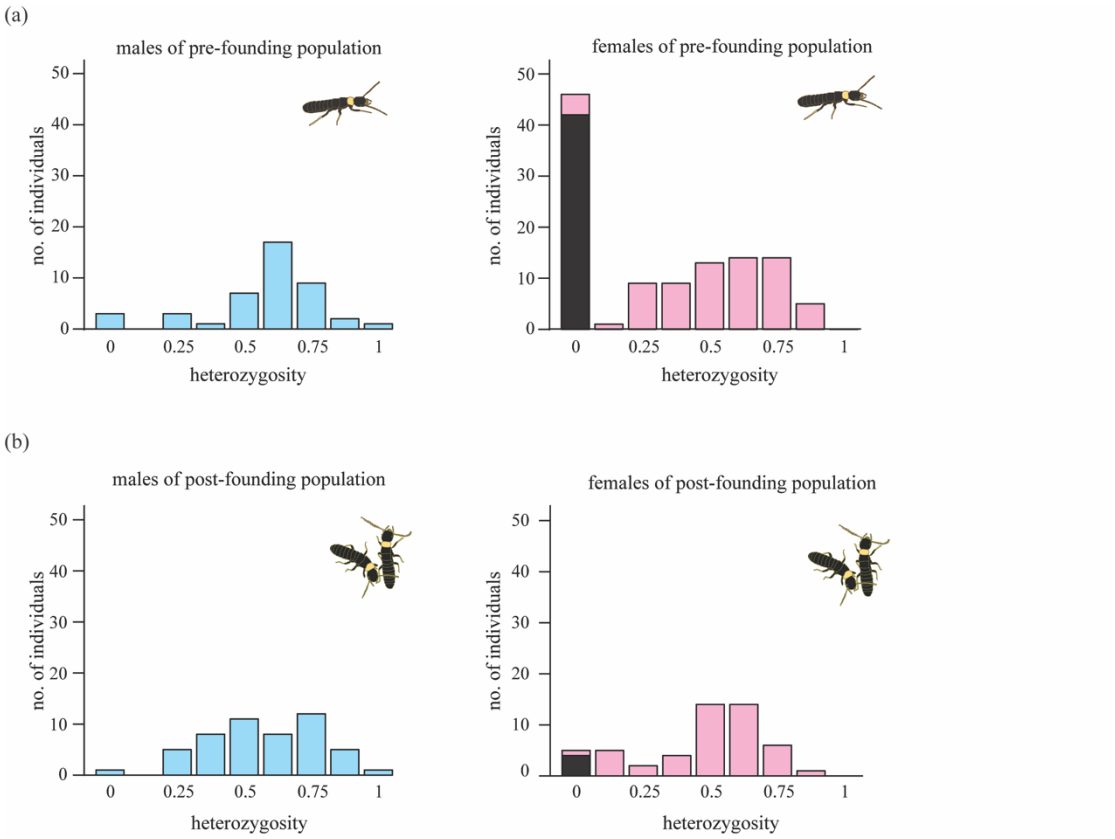

Figure 2.3 Comparison of the frequency of asexual females between pre- and post-foundation populations. (a) The pre-founding population's males (left) and females (right) were collected by sticky traps. (b) Males (left) and females (right) of founding pairs extracted from rotten wood. Black bars indicate 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, $p < 0.001$).

2.3.2.2 Post-foundation population

We were able to get 60 founding units from the artificially buried, brown, rotten pine woods. The majority of units (51 units) were male-female pairs, and the other units were single male or 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 (Figure

2.S3b). In the male-female units, 5 out of the 51 females (9.8 %) were utterly homozygous, and 1 out of 51 males (2.0 %) was completely homozygous (Figure 2.3b). The proportion of parthenogenetically-produced alates in the post-foundation population is significantly smaller than 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: $\chi^2 = 11.50$, $df = 1$, $p < 0.001$).

The pre-foundation and post-foundation populations shared 85.9 (± 4.4 SE) % of alleles. There was no significant difference in the number of alleles per locus between the pre-foundation and post-foundation population (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 of parthenogenetically-produced alates to female sexually-produced alates (see supplementary information) was

2.3.2.3 Body size comparison

The 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, $t = 5.6634$, $df = 90.903$, $p < 0.001$; for females: Wilcoxon rank sum test, $W = 4431$, $p < 0.001$, Figure 2.4a). In the pre-foundation population, the head width of totally homozygous female dealates was significantly smaller than that of the heterozygous female dealates (Wilcoxon rank sum test, $W = 989.5$, $p = 0.002$, Figure 2.4b). However, this trend was not found in the males of the pre-foundation population (t-test, $t = 0.71752$, $df = 2.2815$, $p = 0.5394$). Comparison of fresh weight between female sexually-produced alates and parthenogenetically-produced alates revealed that parthenogenetically-produced alates were significantly smaller than female sexually-produced alates (t-test for colony A: $t = -6.7527$, $df = 17.658$, $p < 0.001$; for colony B: $t = -4.9666$, $df = 14.161$, $p < 0.001$; for colony C: $t = -5.0064$, $df = 6.5623$, $p = 0.002$, Figure 2.4c).

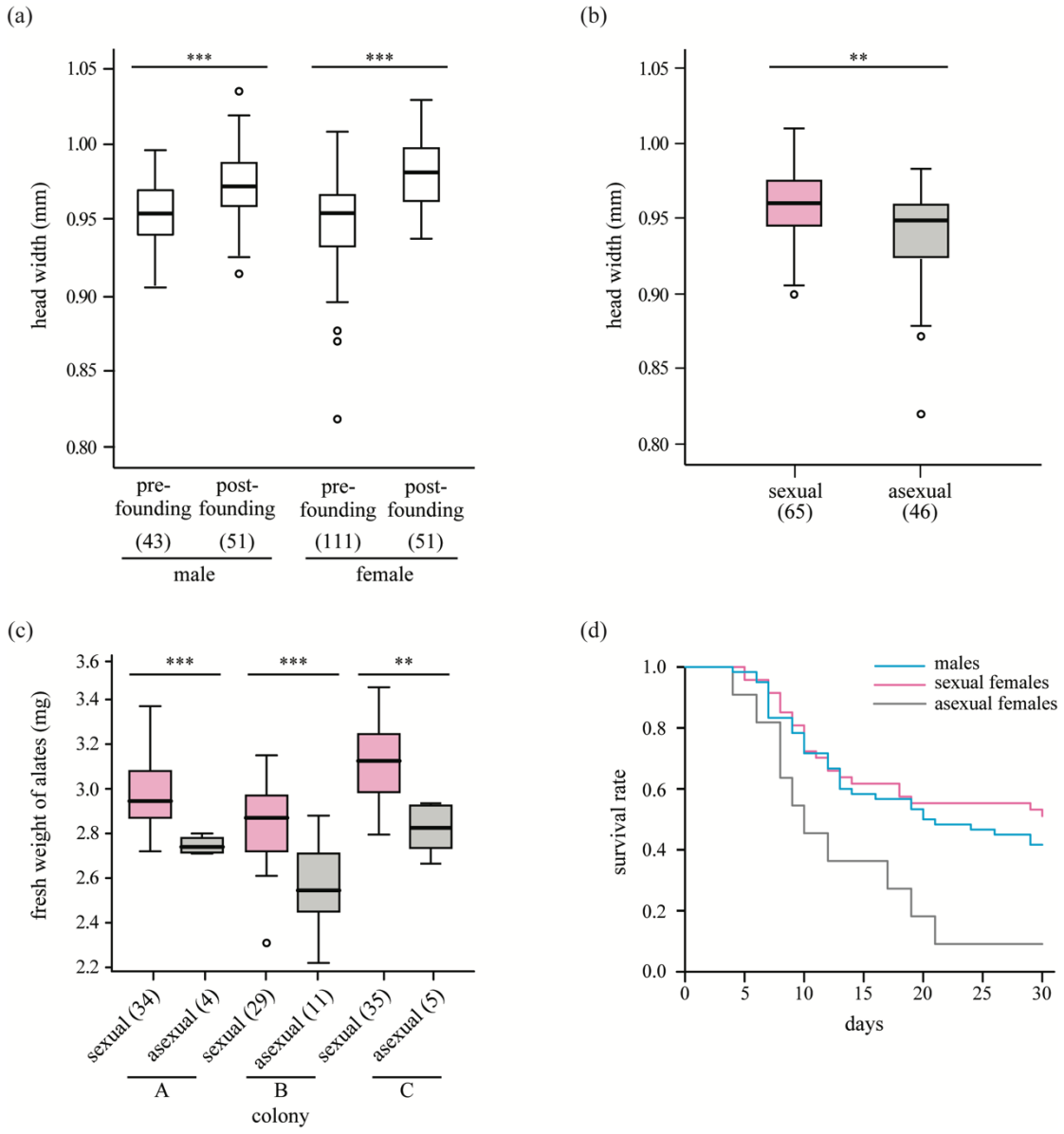

Figure 2.4 Comparison of fitness components between sexual and asexual (parthenogenetically-produced) alates. (a) Comparisons of head widths of pre- and post-foundation individuals for both sexes. Successfully paired founders had significantly larger head sizes than pre-founding dealates in males and females. (b) Comparisons of head widths between sexual and asexual females in the pre-founding population. (c) Comparison of body weights between sexual and asexual alates collected from three natal colonies. The number of samples is indicated in parentheses (t-test or Wilcoxon rank sum test, $**p < 0.01$, $***p < 0.001$). (d) Kaplan-Meier's analysis of the survival times of males (blue), sexual females (red), and asexual females

(gray) that were kept individually on moist filter paper. Asexual females had a shorter survival time (pairwise comparisons by log-rank test followed by Bonferroni correction, $p < 0.05$).

2.3.3 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, 60 and 60 males, 47 and 51 female sexually-produced alates, and 11 and 9 parthenogenetically-produced alates were used without pathogen treatment and with pathogen treatment, respectively. After analyzing the survivorship of male, female, and parthenogenetically-produced alates in each treatment, we found that parthenogenetically-produced alates had a shorter survival rate compared to male and female sexually-produced alates in the without pathogen treatment (log-rank test: $\chi^2 = 6.8$ and 8.4, $df = 1$ and 1, $p = 0.027$ and 0.008, respectively, Figure 2.2d). There was no significant difference between the survivorship of males and female sexually-produced alates (log-rank test: $\chi^2 = 0.7$, $df = 1$, $p = 0.80$, respectively, Figure 2.2d). There was no significant difference in survivorship between female sexually-produced and parthenogenetically-produced alates under pathogen exposure, likely due to a high mortality rate in the early stages of the experiment (log-rank test: $\chi^2 = 0.2$, $df = 1$, $p = 1$). There was no significant difference in survivorship between male alates and female alates, whether they were sexually or parthenogenetically produced. (log-rank test: $\chi^2 = 1.6$ and 0.8, $df = 1$ and 1, $p = 0.4$ and 0.8, respectively).

2.4 Discussion

This study found that clonal diversity in the SQ population reduces as the colony grows, eventually resulting in a monopoly of a single clone. This clonal drive suggested severe competition among clone types over the queen position. Thus, producing more asexual eggs earlier than other SQs is beneficial towards inter-clonal competition. This cycle meets the conditions for the tragedy of the commons (Hardin 1968; Wenseleers and Ratnieks 2004), which predicts the overproduction of asexual eggs more than necessary for queen succession. Given those asexual offspring are all females and have an epigenetic predisposition to develop into nymph-pathway (Matsuura et al. 2018; Tamaki et al. 2021), their caste fate is either to become neotenic SQs or to become female alates. Our previous studies of genotyping queens in natural mature colonies identified all successful PQs as being derived from sexually-produced alates (Matsuura et al. 2009, 2018; Yashiro and Matsuura 2014), which has concealed the existence of asexual alates. Field sampling of dealates walking on the ground (pre-founding population) indicated that 34.5% of female dealates were asexually produced. This shows that the high number of parthenogenetically-produced offspring that fail in queen replacement overflow as alates (Figure 2.S4).

Nevertheless, the parthenogenetically produced alates cannot succeed in pairing colony foundations due to their smaller body size and lower survivorship. Given that parthenogenetically produced alates have a two-fold higher degree of relatedness than sexually-produced alates (Figure 2.S5), it is preferred by the SQs to produce parthenogenetically-produced alates if the relative fitness of parthenogenetically-

produced alate to sexually-produced alate is more than 0.5. De facto, the relative fitness of asexually produced alate was 0.162, a value relatively low that the production of asexual alates is a cost for colony members, even for the mother queen (Figure 2.S5).

In a colony, SQs producing over-parthenogenetic eggs would have higher fitness than those who make only a few parthenogenetic eggs sufficient for queen succession. However, colonies that have excess thelytokous offspring and hence raise many dysfunctional asexually produced alates should have a lower colony fitness, which results in fewer founders to the global population than colonies that produce only sexually produced alates. The outcome relies on balancing individual- and colony-level selective forces (Wilson 1975; Okasha 2006). Considering the underlying mechanics, the production of asexual eggs is determined by decreased micropyles as the queen ages (Yashiro and Matsuura 2014). At present, the genes or epigenetic agents regulating the age-dependent reduction of the number of micropyle-forming cells (Yashiro and Matsuura 2014) should be involved in this clonal drive.

This clonal drive in AQS termites is akin to the female meiotic drive in maize (Hall and Dawe 2018), even though the selection level differs. The abnormal chromosome 10 (Ab10), a well-known selfish genetic element in maize, encodes a meiotic drive system that exhibits strong preferential segregation (Rhoades 1942). Although seen as a transmission advantage, Ab10 imposes fitness costs at individual levels, such as decreased pollen viability, decreased seed set, and weight (Higgins et al. 2018), which may explain why Ab10 is present at low frequencies among natural populations. Similarly, the timing and amount of parthenogenetic egg production would rely on the

intensity of competition for queen succession and the cost of overproducing asexual eggs. A future study using a mathematical model is necessary to predict equilibrium conditions.

The AQS system increases the queen population through the parthenogenesis of primary queens, thereby enhancing reproduction without inbreeding (Matsuura et al. 2009; Matsuura 2017). In this system, sexual reproduction produces alates and workers, whereas parthenogenesis is solely utilized to produce neotenic queens. These queens remain in the colony and are sheltered in a royal chamber, are fed by workers, and are exclusively engaged in egg production. Therefore, they underwent a lower workload than female alates, who founded new colonies independently. This study revealed that when asexual daughters differentiate into alates, their fitness is relatively lower than that of sexual ones. Moreover, the genotype of offspring produced by parthenogenesis and the associated costs vary significantly depending on the mode of parthenogenesis (Templeton 1982; Engelstädter 2008; Matsuura 2017). In *R. speratus*, parthenogenesis leads to a rapid loss in heterozygosity, as diploidy is restored via terminal fusion automixis (Matsuura et al. 2004, 2009; Matsuura 2017). This loss of heterozygosity is likely responsible for the small body size and low survival rate of asexually produced alates.

Furthermore, the low mating success rate of asexually produced alates may also result from mate choice, as males tend to select larger females as mates (Matsuura and Nishida 2001). In contrast, the asexual lineage of the termite *Glyptotermes nakajimai* reproduces only through parthenogenesis, with heterozygosity maintained by clonal

(apomictic) reproduction (Yashiro et al. 2018, 2021). The origin of parthenogenesis in *G. nakajimai* is thought to be hybrid, arising from two lineages with distinct karyotypes (Yashiro et al. 2021). Parthenogenesis with hybrid origins, which keeps high heterozygosity, has low fitness costs (Kearney et al. 2022). Notably, the application of parthenogenesis in life history varies depending on the type of parthenogenesis and the genetic characteristics of the resulting offspring. AQS in termites exemplifies the pros and cons of asexual and sexual reproduction, as well as the associated conflicts.

2.5 Supplementary materials

2.5.1 Supplementary materials and methods

2.5.1.1 Intra-colonial conflict over parthenogenetic alate production

As parthenogenetically produced alates have only maternal alleles, relatedness from colony members to parthenogens differs. Since *R. speratus* colony members can only transmit genes through alates, this difference in relatedness would incur intra-colony conflict (Figure 2.1b). Sexually produced alates are equally related to the primary king (PK), the primary queen (PQ), and workers by 0.5. On the other hand, parthenogenetically produced alates are related to PK, PQ, and workers by 0, 1.0, and 0.5, respectively. We can predict the conflicts among castes from the reproductive values of sexual and parthenogenetic alates for each caste. This is calculated by multiplying relatedness to sexual and parthenogenetic alates by relative fitness. This leads to three predictions. First, suppose the relative fitness of parthenogenetic alates compared to sexual alates w_p is more significant than 1.0. In that case, PQ and workers

benefit from the production of parthenogenetic alates, whereas PK does not. So, the conflict between PK and the other members of the colony occurs over the production of sexual versus asexual alates. Second, if the relative fitness w_p is from 0.5 to 1.0, only the PQ benefits from producing parthenogenetic alates, whereas the PK and workers do not. In this case, there is a conflict between the PQ and other colony members. Third, if the relative fitness w_p is smaller than 0.5, no colony members benefit from the production of parthenogenetic alates. Thus, if $w_p < 0.5$, parthenogenetic alate production should be maladaptive for colony members and, therefore, can be the inevitable cost of the AQS system.

2.5.1.2 Genetic analysis of pre- and post-foundation population

Individual degrees of inbreeding for pre- and post-foundation populations were estimated using individual heterozygosity, measured as the proportion of heterozygous loci in an individual (Coltman et al. 1998). The heterozygosity was zero if an individual was homozygous at all the examined loci. The proportion of colonies producing homozygous female dealates can be estimated from relatedness measurements by modification of the method used to calculate the proportion of colonies contributing to each swarm aggregation (DeHeer and Vargo 2006; Husseneder et al. 2006). We estimated relatedness within totally homozygous and the other female dealates of the pre-founding population using KINGROUP v.2.0.8 (Konovalov et al. 2004), and then we calculated the proportion by dividing the relatedness of individuals with heterozygous loci by the that of totally homozygous individuals. To investigate whether

the pre- and post-foundation populations represented the same population, we measured the genetic similarity between the pre- and post-foundation population by calculating the percentage of alleles shared by these two populations, that is, the number of alleles shared by the two populations divided by the sum of the alleles possessed by both populations (Tsutsui et al. 2000). Furthermore, to investigate the difference in allelic diversity between the two populations, we compared the number of alleles per locus between the pre- and post-foundation populations using Wilcoxon's signed rank Wilcoxon's.

2.5.1.3 Estimation of the relative fitness of parthenogenetically- and sexually-produced alate

The relative fitness w_{PA} of the parthenogenetically-produced alates to female sexually-produced alates is given by W_{PA} / W_{SA}, where W_{PA} and W_{SA} are colony foundation success rates of parthenogenetic and sexual females, respectively. (PA hereafter is parthenogenetically-produced alate, SA hereafter is sexually-produced alate)

Where the subscripts indicate either parthenogenetic (*PA*) or sexual (*SA*) female alates and *pre*- or *post*-foundation populations, N is the number 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 fitness w_{PA} can be calculated from the estimated

numbers of female alates produced by a specific reproductive mode in the *pre*- and *post*-foundation populations, $E_{PA,\ post}$, $E_{PA,\ pre}$, $E_{SA,\ post,}$ and $E_{SA,\ pre}$.

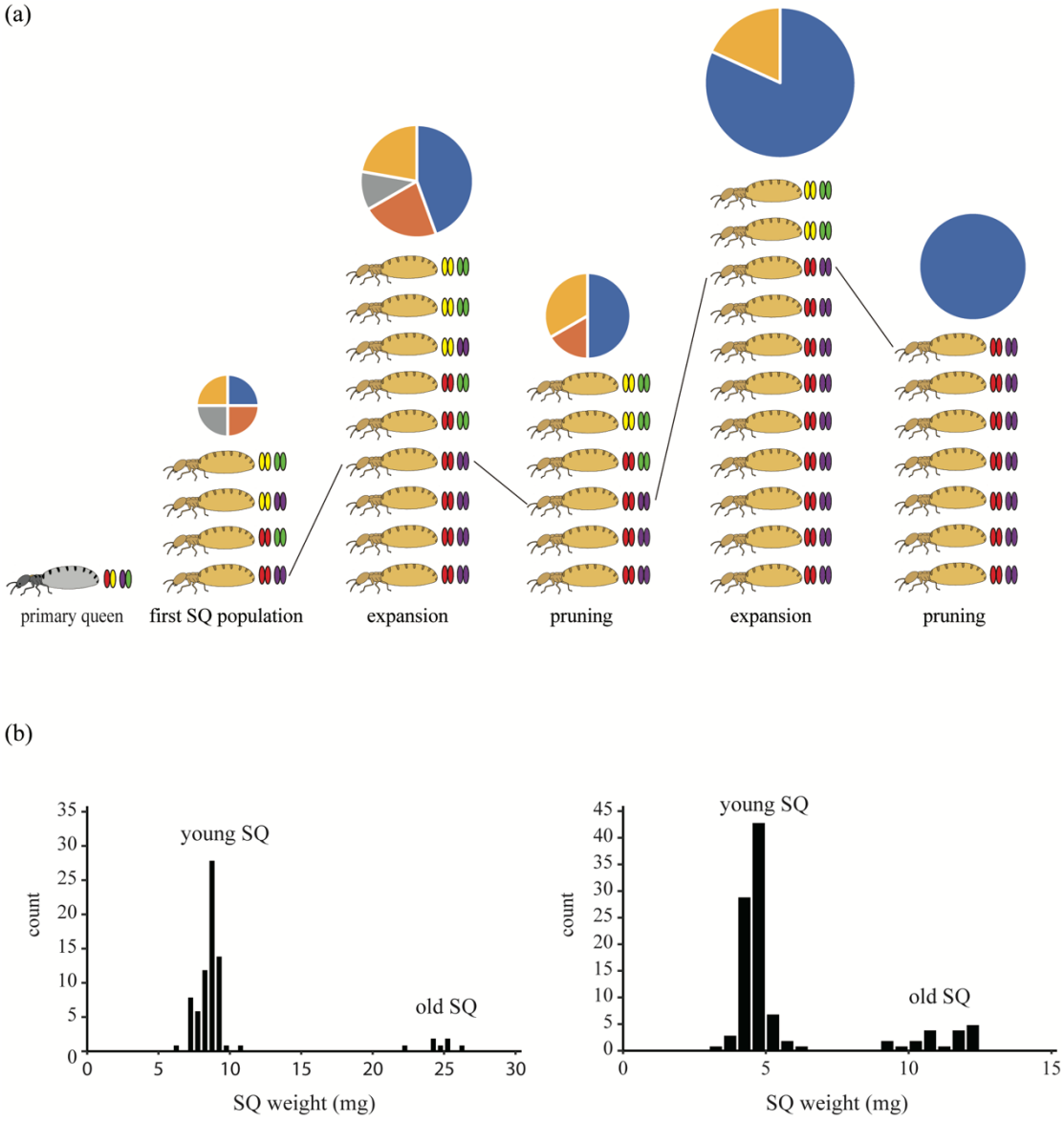

Figure 2.S1 Process of queen succession and hypothetical clonal drive. (a) Scheme of asexual queen succession (AQS) in *Reticulitermes* termites. Secondary queens are produced asexually (automixis with terminal fusion) by the primary queen. Excessive numbers of neotenic queens emerge synchronously with 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.

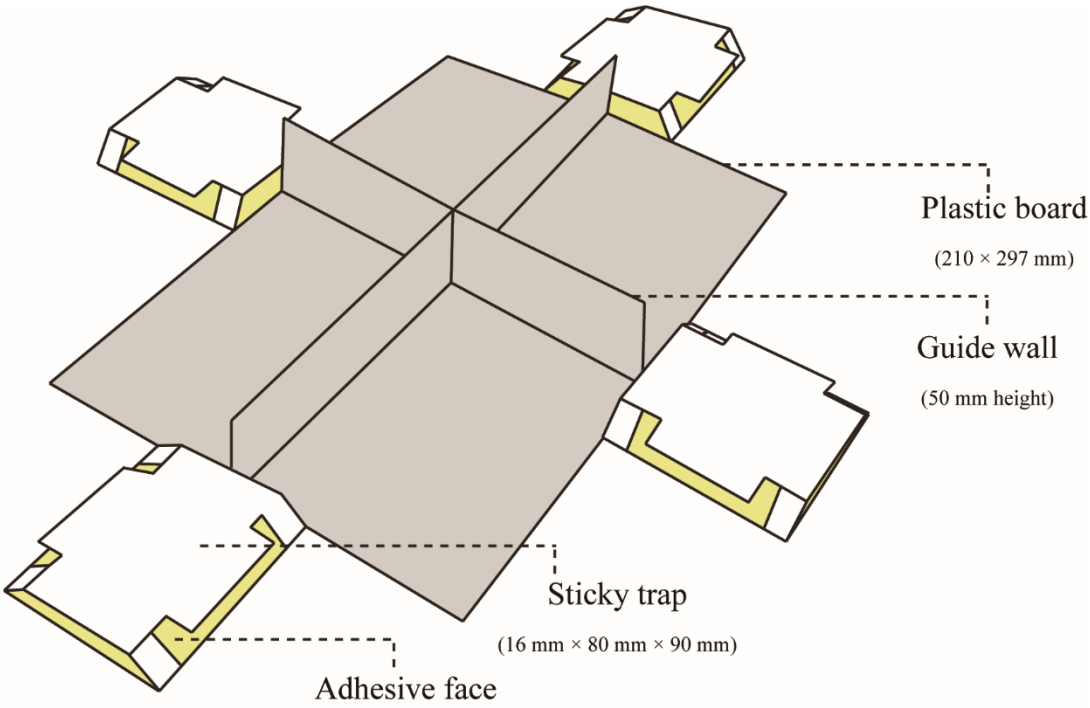

Figure 2.S2 Schematic diagram of the traps that collect walking dealates following mating flights. All traps consist of guiding walls and four sticky traps arranged to introduce walking termites into sticky traps.

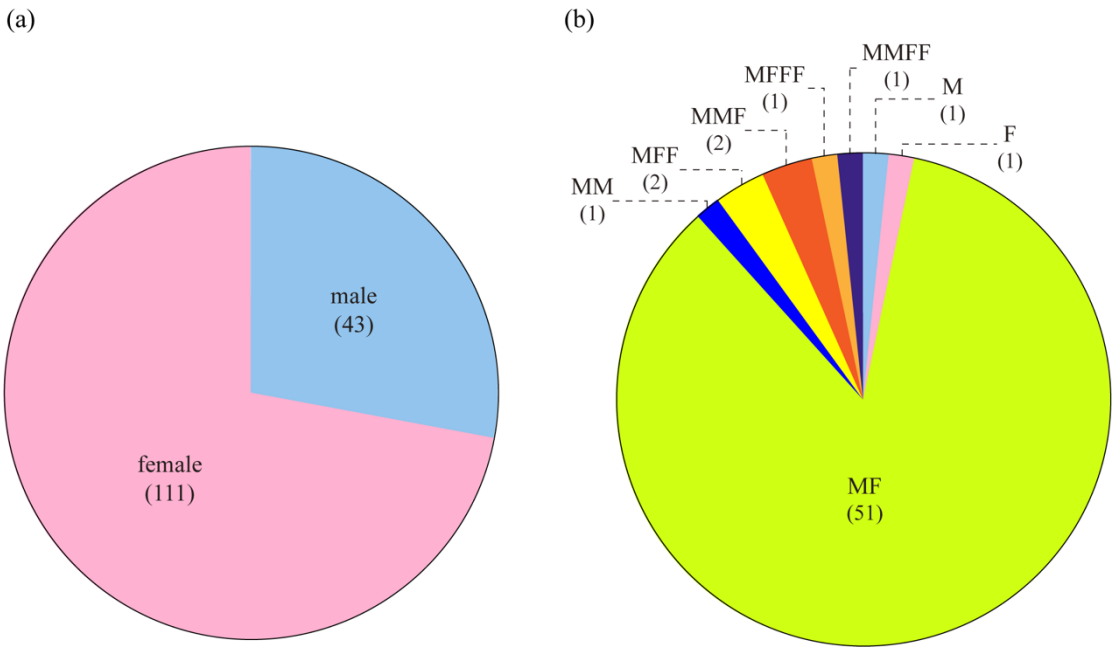

Figure 2.S3 Composition of field-collected dealates and founding individuals. (a) The proportion of male and female dealates in the pre-foundation population. (b) Composition of founding units. M: single male, F: single female, MF: male-female pair, MM: male-male pair, MFF: single male with two females, MMF: single female with two males, MFFF: single male with three females, and MMFF: two males and two females. The number of samples is indicated in parentheses.

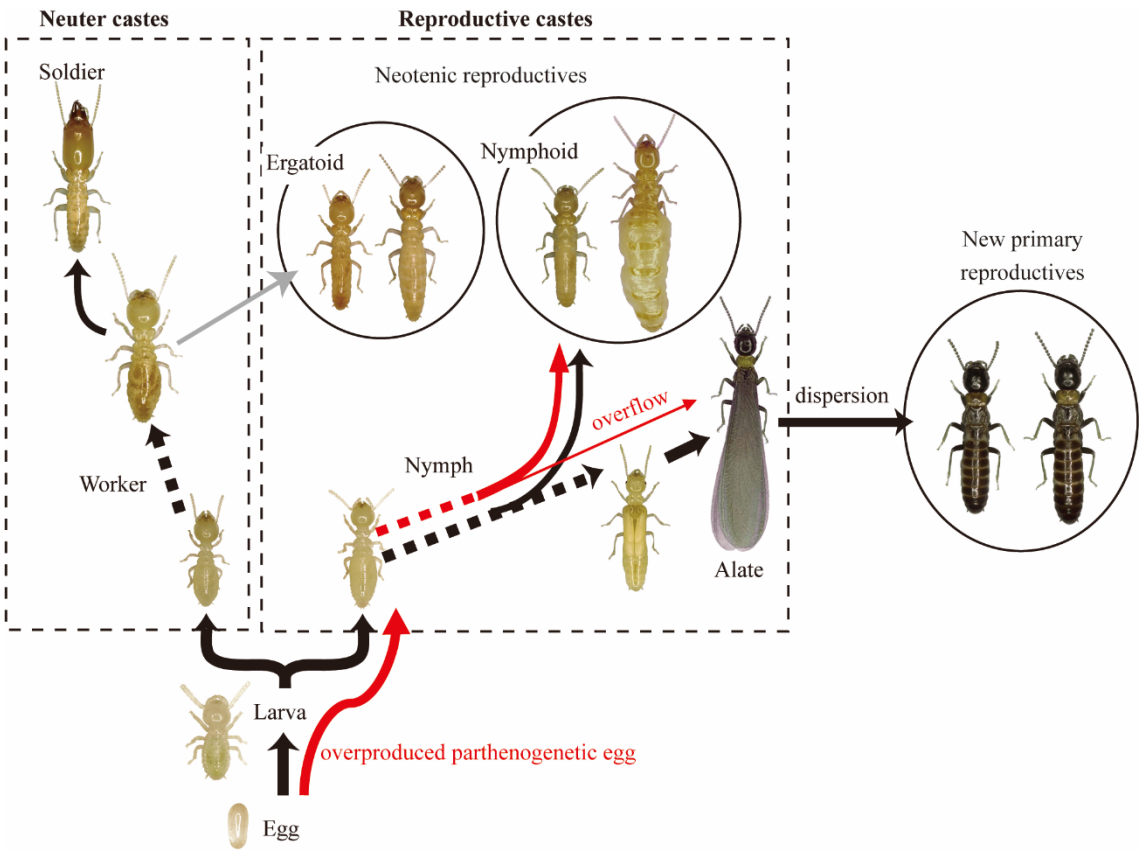

Figure 2.S4 The scheme of caste differentiation of parthenogenetically-produced daughters. Parthenogenetically-produced daughters exclusively develop into nymphs and then into nymphoid secondary reproductives. However, the overproduced parthenogenetic females overflow into alates.

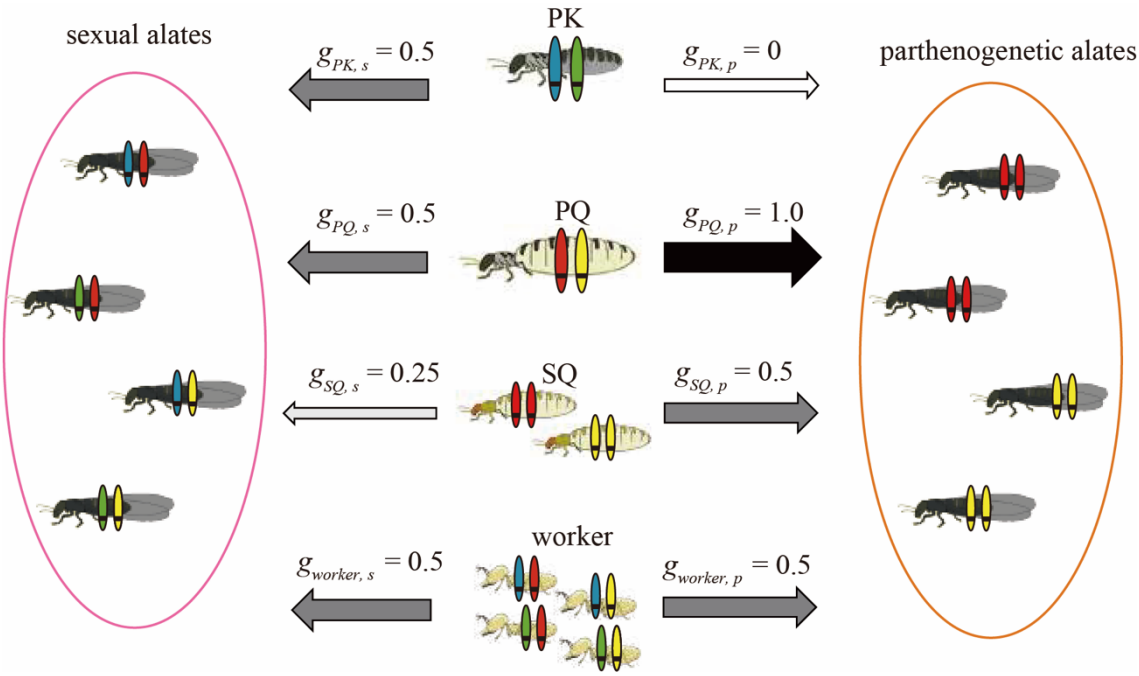

Figure 2.S5 A kin conflict over parthenogenetic alate production. PK: primary king, PQ: primary queen, and SQ: secondary queen. $g_{caste,\,s}$ and $g_{caste,\,p}$ indicate relatedness of sexual (s) and asexual (p) alates from each caste (PK, PQ, SQ, and worker), respectively. *wp*: the relative fitness of the parthenogenetic female alates to sexual female alates. Conditions for the preference for parthenogenetic alates production over sexual alates production are shown in parentheses.

2.6 References

1. Ardlie, K. G. 1998. Putting the brake on drive: meiotic drive of *t* haplotypes in natural populations of mice. Trends in Genetics 14:189–193.
2. Burt, A., and R. Trivers. 2006. Genes in Conflict: the Biology of Selfish Genetic Elements. Harvard University Press, Cambridge, MA.
3. Coltman, D. W., W. D. Bowen, and J. M. Wright. 1998. Birth weight and neonatal survival of harbour seal pups are positively correlated with genetic variation measured by microsatellites. Proceedings of the Royal Society of London. Series B: Biological Sciences 265:803–809.
4. Crow, J. F. 1979. Genes that violate Mendel's rules. Scientific American 240:134–43, 146.
5. Dawe, R. K., E. G. Lowry, J. I. Gent, M. C. Stitzer, K. W. Swentowsky, D. M. Higgins, J. Ross-Ibarra, et al. 2018. A Kinesin-14 motor activates neocentromeres to promote meiotic drive in maize. Cell 173:839-850.e18.
6. DeHeer, C. J., and E. L. Vargo. 2006. An indirect test of inbreeding depression in the termites *Reticulitermes flavipes* and *Reticulitermes virginicus*. Behavioral Ecology and Sociobiology 59:753–761.
7. Dronnet, S., A. G. Bagneres, T. R. Juba, and E. L. Vargo. 2004. Polymorphic microsatellite loci in the European subterranean termite, *Reticulitermes santonensis* Feytaud. Molecular Ecology Notes 4:127–129.
8. Dyer, K. A., B. Charlesworth, and J. Jaenike. 2007. Chromosome-wide linkage disequilibrium as a consequence of meiotic drive. Proceedings of the National Academy of Sciences of the United States of America 104:1587–1592.
9. Engelstädter, J. 2008. Constraints on the evolution of asexual reproduction. Bioessays 30:1138–1150.
10. Hall, D. W., and R. K. Dawe. 2018. Modeling the evolution of female meiotic drive in maize. G3: Genes, Genomes. Genetics 8:123–130.
11. Hardin, G. 1968. The tragedy of the commons. Science 162:1243–1244.
12. Hellemans, S., K. Dolejšová, J. Křivánek, D. Fournier, R. Hanus, and Y. Roisin.

2019. Widespread occurrence of asexual reproduction in higher termites of the *Termes* group (Termitidae: Termitinae). BMC Evolutionary Biology 19:1–14.

13. Hellemans, S., and Y. Roisin. 2020. Asexual queen succession in termites. Encyclopedia of Life Science 1:13–20.
14. Higgins, D. M., E. G. Lowry, L. B. Kanizay, P. W. Becraft, D. W. Hall, and R. K. Dawe. 2018. Fitness costs and variation in transmission distortion associated with the abnormal chromosome 10 meiotic drive system in maize. Genetics 208:297–305.
15. Hölldobler, B., and E. O. Wilson. 2009. The Superorganism: the Beauty, Elegance, and Strangeness of Insect Societies. W. W. Norton & Company, New York.
16. Husseneder, C., D. M. Simms, and D. R. Ring. 2006. Genetic diversity and genotypic differentiation between the sexes in swarm aggregations decrease inbreeding in the Formosan subterranean termite. Insectes Sociaux 53:212–219.
17. Kearney, M. R., M. E. Jasper, V. L. White, I. J. Aitkenhead, M. J. Blacket, J. D. Kong, S. L. Chown, et al. 2022. Parthenogenesis without costs in a grasshopper with hybrid origins. Science 376:1110–1114.
18. Konovalov, D. A., C. Manning, and M. T. Henshaw. 2004. KINGROUP: a program for pedigree relationship reconstruction and kin group assignments using genetic markers. Molecular Ecology Notes 4:779–782.
19. Kusaka, A., and K. Matsuura. 2017. Allee effect in termite colony formation: influence of alate density and flight timing on pairing success and survivorship. Insectes Sociaux 65:17–24.
20. Larner, W., T. Price, L. Holman, and N. Wedell. 2019. An X-linked meiotic drive allele has strong, recessive fitness costs in female *Drosophila pseudoobscura*. Proceedings of the Royal Society B: Biological Sciences 286:20192038.
21. Matsuura, K. 2017. Evolution of the asexual queen succession system and its underlying mechanisms in termites. The Journal of Experimental Biology 220:63–72.
22. Matsuura, K. 2020. Genomic imprinting and evolution of insect societies. Population Ecology 62:38–52.

23. Matsuura, K., M. Fujimoto, and K. Goka. 2004. Sexual and asexual colony foundation and the mechanism of facultative parthenogenesis in the termite *Reticulitermes speratus* (Isoptera, Rhinotermitidae). Insectes Sociaux 51:325–332.
24. Matsuura, K., N. Mizumoto, K. Kobayashi, T. Nozaki, T. Fujita, T. Yashiro, T. Fuchikawa, et al. 2018. A genomic imprinting model of termite caste determination: not genetic but epigenetic inheritance influences offspring caste fate. The American Naturalist 191:677–690.
25. Matsuura, K., and T. Nishida. 2001. Comparison of colony foundation success between sexual pairs and female asexual units in the termite *Reticulitermes speratus* (Isoptera: Rhinotermitidae). Population Ecology 43:119–124.
26. Matsuura, K., E. L. Vargo, K. Kawatsu, P. E. Labadie, H. Nakano, T. Yashiro, and K. Tsuji. 2009. Queen succession through asexual reproduction in termites. Science 323:1687.
27. Okasha, S. 2006. Evolution and the Levels of Selection. Clarendon Press, Oxford.
28. R Core Team. 2020. R: A language and environment for statistical computing. R foundation for statistical computing 1:409.
29. Ratnieks, F. L. W., K. R. Foster, and T. Wenseleers. 2006. Conflict resolution in insect societies. Annual Review of Entomology 51:581–608.
30. Rhoades, M. M. 1942. Preferential segregation in maize. Genetics 27:395–407.
31. Szathmáry, E., and J. M. Smith. 1995. The major evolutionary transitions. Nature 374:227–232.
32. Tamaki, C., M. Takata, and K. Matsuura. 2021. The lose-to-win strategy of the weak: intraspecific parasitism via egg abduction in a termite. Biology Letters 17:20210540.
33. Templeton, A. R. 1982. The prophecies of parthenogenesis. Pages 75–102 *in* H. Dingle and J. P. Hegmann, eds. Evolution and Genetics of Life Histories. Springer Varlag, New York.
34. Trivers, R. L., and H. Hare. 1976. Haploidploidy and the evolution of the social insect. Science 191:249–263.
35. Tsutsui, N. D., A. V. Suarez, D. A. Holway, and T. J. Case. 2000. Reduced genetic

variation and the success of an invasive species. Proceedings of the National Academy of Sciences of the United States of America 97:5948–5953.

36. Vargo, E. L. 2000. Polymorphism at trinucleotide microsatellite loci in the subterranean termite *Reticulitermes flavipes*. Molecular Ecology 9:817–820.
37. Walsh, P. S., D. A. Metzger, and R. Higuchi. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques 10:506–513.
38. Wenseleers, T., and F. L. W. Ratnieks. 2004. Tragedy of the commons in *Melipona* bees. Proceedings of the Royal Society B: Biological Sciences 271:S310–S312.
39. Wilson, D. S. 1975. A theory of group selection. Proceedings of the National Academy of Sciences of the United States of America 72:143–146.
40. Wilson, D. S. 1997. Altruism and organism: disentangling the themes of multilevel selection theory. The American Naturalist 50:S122–S134.
41. Yashiro, T., N. Lo, K. Kobayashi, T. Nozaki, T. Fuchikawa, N. Mizumoto, Y. Namba, et al. 2018. Loss of males from mixed-sex societies in termites. BMC Biology 16:1–18.
42. Yashiro, T., and K. Matsuura. 2014. Termite queens close the sperm gates of eggs to switch from sexual to asexual reproduction. Proceedings of the National Academy of Sciences of the United States of America 111:17212–17217.
43. Yashiro, T., Y.-K. Tea, C. Van Der Wal, T. Nozaki, N. Mizumoto, S. Hellemans, K. Matsuura, et al. 2021. Enhanced heterozygosity from male meiotic chromosome chains is superseded by hybrid female asexuality in termites. Proceedings of the National Academy of Sciences of the United States of America 118:e2009533118.
44. Zoberi, M. H. 1995. *Metarhizium anisopliae*, a fungal pathogen of *Reticulitermes flavipes* (Isoptera: Rhinotermitidae). Mycologia 87:354–359.

Chapter 3: The inheritance of parthenogenetically reproductive capacity relies on the maternal inheritance in termites

3.1 Introduction

The asexual queen succession (AQS) represents a minority system that offers a unique lens into the evolutionary mechanisms underlying termite reproductive strategies (Matsuura 2017). The ecological success of species employing AQS, such as *Reticulitermes speratus*, hinges on a delicate balance between asexual and sexual reproduction, optimizing genetic diversity and colony adaptability (Matsuura et al. 2009; Vargo et al. 2012). The intricacies of how termites inheriting AQS carry the capacity for parthenogenesis (the ability of females to produce offspring without male fertilization) remain an enigma, veiled by the complex interplay of genetic, environmental, and developmental factors. Genetic factors play a pivotal role, with evidence indicating that parthenogenesis may be regulated by specific genes or gene clusters selected through evolutionary processes for their adaptive advantages (Simon et al. 2003). Environmental factors also exert influence, as the challenge of mate scarcity can trigger parthenogenesis, ensuring species survival. Developmental mechanisms link parthenogenesis to the organism's ontogeny, where eggs can develop independently of fertilization (Stouthamer et al. 1999). The heritability of this mode of reproduction is affirmed, and it is contingent on the genetic machinations driving the trait. Notably, in species like *Reticulitermes speratus*, the parthenogenetic capability

seems to be a maternal inheritance, resulting in progeny that are genetic replicas or highly like the mother. However, there is still no evidence to demonstrate this trait because the paternal side carries an equal genome to the mother.

In this study, we used two cross types of unfertilized ergatoid queens from hybrid colonies between AQS termite *R. speratus* and non-AQS termite *R. amaminus* to produce asexual eggs and recorded the hatching numbers. Because the asexual eggs only carry half of the AQS species' genome, paternal or maternal, respectively, the result can indicate the sex-link parental influence on the hatching capacity of unfertilized eggs.

3.2 Materials and methods

3.2.1 Foundation of hybrid colonies

Two colonies containing alates for each species (*R. amamianus* and *R. speratus*) were collected in Kagoshima and Kyoto, Japan, in 2020 and used in hybrid experiments. The two *R. amamianus* colonies were labeled RaA and RaB, and the *R. speratus* colonies were labeled RsA and RsB. All alates were extracted from the logs, removed their wings, and sexed by sternite morphology (Zimet and Stuart 1982). Then, 720 individuals for each sex and colony were randomly selected and assigned to one of the following crosses (male × female): mRa × fRa (mRaA × fRaB, mRaB × fRaA), mRa × fRs (mRaA × fRsA, mRaB × fRsA, mRaA × fRsB, and mRaB × fRsB), mRs × fRa (mRsB × fRaA, mRsA × fRaB, mRsB × fRaB, mRsA × fRaA), and mRs × fRs (mRsA × fRsB, mRsB × fRsA). One hundred and twenty pairs were set for each (see Dataset 3.S1 for details).

Each pair was placed in a dish (34 mm diameter × 10 mm height) with brown-rotted pinewood mixed cellulose (BPC) medium (Mitaka et al. 2023) for six months and transferred to a larger plastic box (100 × 65 × 28 mm) filled with the BPC medium and a soil block (36 × 36 × 14 mm, W × D × H). They were maintained at 25 °C under dark conditions. We replenished the BPC medium whenever one-third was consumed to ensure a constant food supply. Two years after the founding of the colony, we transferred the colonies to even larger plastic boxes (194 × 104 × 26 mm, W × D × H) containing two soil blocks and two pieces of pine wood (45 × 45 × 10 mm, W × D × H).

At two and two and a half years after the foundation of colonies, all individuals from each colony were extracted. The numbers of eggs, larvae, workers, and nymphs were recorded. Soldiers were sexed by sternite morphology, and the counts for each sex were recorded.

3.2.2 Making ergatoid queens

We isolated 200 female workers from each type of colony, including mRa × fRa (mRaA × fRaB, mRaB × fRaA), mRa × fRs (mRaA × fRsA, mRaB × fRsA, mRaA × fRsB, and mRaB × fRsB), mRs × fRa (mRsB × fRaA, mRsA × fRaB, mRsB × fRaB, mRsA × fRaA), and mRs × fRs (mRsA × fRsB, mRsB × fRsA). We made six repeats in mRa × fRs and mRs × fRa pairs, four repeats in mRa × fRs pairs and mRs × fRs pairs.

3.2.3 Weekly observation in egg producing and hatching results

We observed each treatment colony weekly for 25 weeks and recorded the number of ergatoid eggs and F2 larvae (see Dataset 3.S1). We will isolate the fourth instar F_2 offspring from the colony set because it can molt to a neotenic nymphoid or ergatoid (Hayashi et al. 2007).

3.2.4 Egg-fostering experiment

We collected 80 eggs from mRs × fRa, mRa × fRs, mRa × fRa, and mRs × fRs colony sets. We then used 30 male workers from an independent colony to care for the 20 eggs over a period of 5 weeks.

3.2.5 Microsatellite analysis

We collected DNA from at least 8 F_2 individuals, all organoids and parental generation primary parents after 25 weeks from each 200 female workers treatment colonies for genotyping by four loci including *Rs15* (Dronnet et al. 2004), *Rf6-1*, *Rf24-2*, and *Rf21-1* (Vargo 2000). We extracted all DNA from the heads or antennas using 50 µL Chelex® solution (10% weight per volume; TE pH 8.0) and 0.5 µL proteinase K. After 3 hours incubation at 55 °C, samples were then heated at 95 °C for 15 min. Polymerase chain reaction (PCR) amplifications were performed. Primer *Rf6-1* was labeled by 6-FAM fluorescent tags, *Rf21-1* by VIC fluorescent tags, *Rf24-2* by NED fluorescent tags, and *Rs15* by PET fluorescent tags. We use a 10 µL PCR cocktail containing 1 µL of the DNA sample, 0.20 µL of 10 mM dNTP, 0.99 µL of 10 × PCR Buffer, 0.07 µL of 5 U/µL Taq DNA polymerase (New England Biolabs, Inc., Beverly, MA, USA), 1.15 µL of 5

μM multiplex primers and 6.59 μL of DW. Amplification consisted of initial denaturation at 95 °C for 3 min, then followed by 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 75 s, and an extension at 72 °C for 120s. The PCR products were mixed with 10 μL of Hi-Di formamide and 0.5 μL of GS-600 (LIZ) size standard. Sample detection was performed using an Applied BioSystems 3500 Genetic Analyzer. Raw data were analyzed using GeneMapper 5.0 software (Applied Biosystems, Inc., Foster City, CA, USA).

3.2.6 Statistical analysis

A Median Test was performed to compare the number of survival F2 individuals at the 25th week among all treatment types, that is, four combinations of parent species (mRa × fRa, mRa × fRs, mRs × fRa, and mRs × fRs), and Tukey's HSD was performed in an egg-fostering experiment. Mann-Whitney Test was applied from nymph ratio comparison between asexually produced F_2 individuals of mRa × fRs and mRs × fRs. A t-test was performed to compare allele frequency between maternal and paternal origin. All analyses were performed using the IBM SPSS 29.0 software. A significance value of $p < 0.05$ was considered to indicate statistical significance.

3.3 Results

After 25 weeks, we collected F_2 offspring individuals from 4 out of 6 mRa × fRs 200 female worker treatment sets and all four mRs × fRs colony sets. No F_2 individuals were produced in other colony sets. Almost all colony sets were found, ergatoid

neotenic queens. Beginning at the 18th week, eggs of mRa × fRs colony sets started to hatch. In the 20th week, eggs of mRs × fRs colony sets started to hatch. In the 25th week, F_2 individuals collected from mRa × fRs ergatoids were significantly more in mRs × fRa colony sets (Median Test, $\chi^2 = 14.44$, $p = 0.02$, Figure 3.1a). The eldest F2 individuals are the third instar, and most individuals are larva until the 25th week. In those third instar individuals, 28 nymphs and 169 workers were produced by mRa × fRs ergatoid queens, and eight nymphs and 0 workers were produced by mRs × fRs ergatoid queens.

Egg hatching ratios comparison shows that eggs in mRs × fRs colony sets hatched significantly more than other sets, in mRa × fRs (hatching ratios from 0 to 0.55, mean is 0.175) colony sets hatched no more than mRs × fRa (hatching ratios from 0 to 0.1, mean is 0.0375) colony sets (Tukey's HSD tests, $p < 0.001$, Figure 3.1b).

Genotyping results exhibit that in the F2 offspring individuals produced by ergatoid queens derived from worker of mRaB × fRsB via asexual reproduction inherited only *R. speratus* genes in four loci (Figure 3.2).

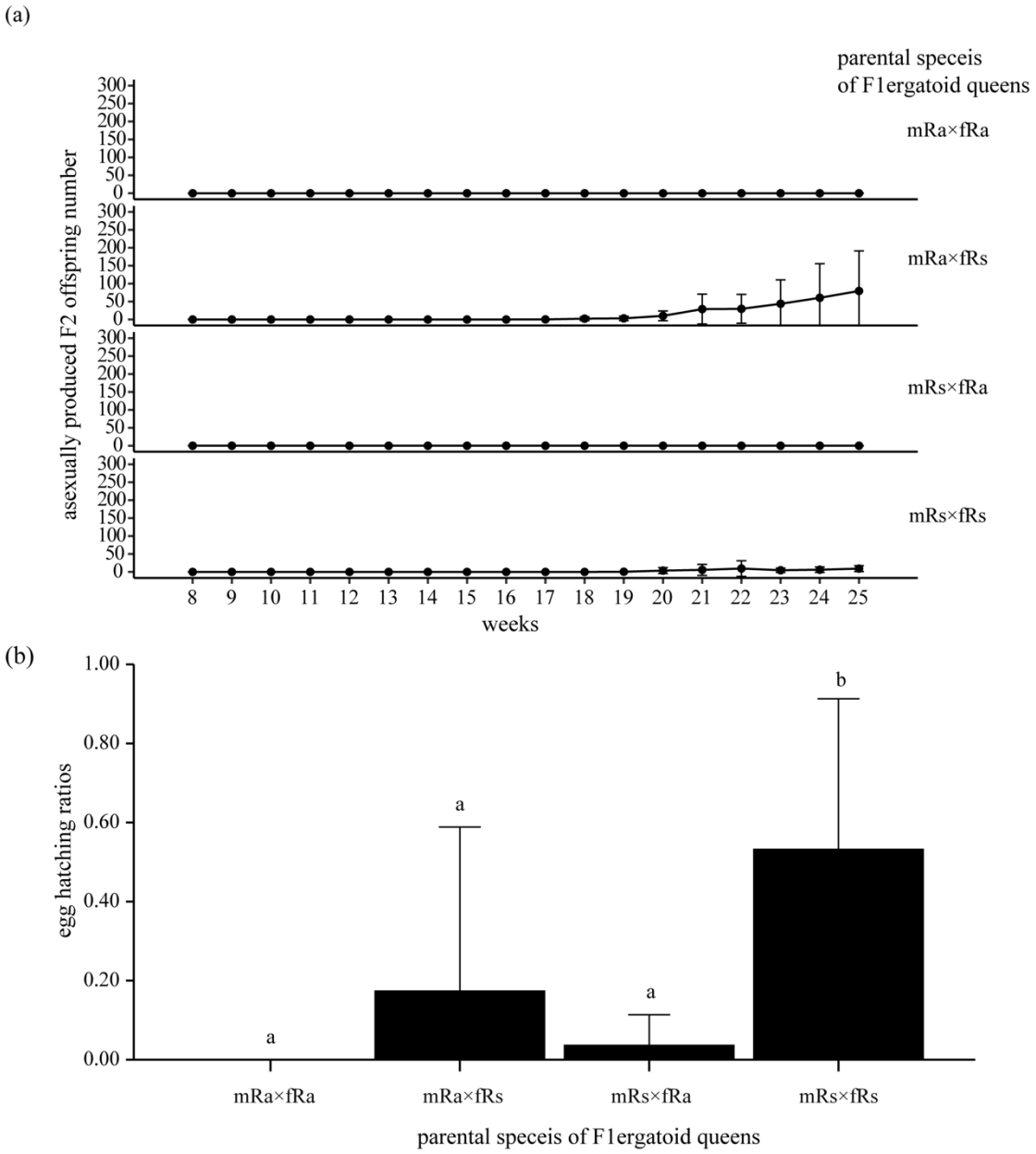

Figure 3.1 Comparison of the number of F2 offspring in each colony produced by ergatoid queens differentiated from workers of *R. speratus*, *R. amamianus*, and *R. speratus* × *R. amamianus* hybrid colonies, observed every week for 25 weeks. (a) The number of hatched and surviving larvae in each combination of parent species. Dots and bars exhibit mean and 95 % confidence intervals, respectively. (b) Comparison of hatching results of parthenogenic eggs produced by ergatoid queens differentiated from workers of four combinations of parents (i.e., mRa × fRs means the primary king is from *R. amamianus*, the primary queen is from *R. speratus*). Different letters indicate

significant differences (Tukey's HSD tests, $p < 0.001$).

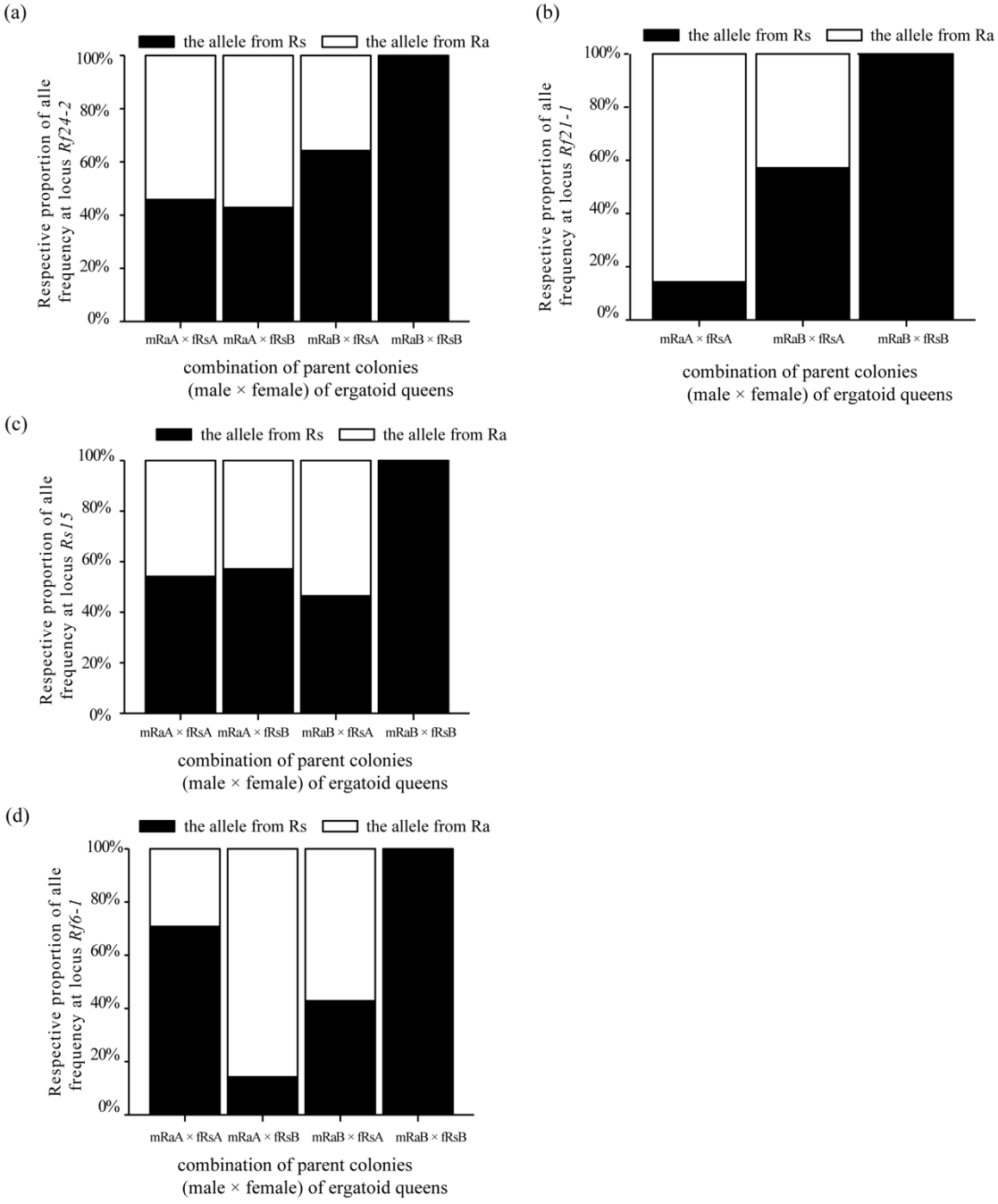

Figure 3.2 Respective proportion of allele frequency from *R. speratus* and *R. amamianus*. Ra: *R. amamianus.* Rs: *R. speratus.* Respective proportion of allele frequency from different origin of parental generation in the asexually produced F2 offspring at loci (a) *Rf24-2,* (b) *Rf21-1,* (c)*Rfs15, and* (d) *Rf6-1.* The black bars exhibit

the mean frequency of *R. speratus* alleles, and the white bars indicate the mean frequency of *R. amamianus* alleles. The combination of parent colonies (male × female) shows the species of primary king and queen (i.e., mRaA × fRsA means the primary king is *R. amamianus* colony *A*, and the primary queen is *R. speratus* colony B).

3.4 Discussion

In this study, we identified that asexually reproductive capacity inheritance relies on maternal inheritance in AQS termite *R. speratus*. The large number and earlier hatched of F2 asexually produced offspring from mRa × fRs ergatoid queens indicate that the maternal factors decide the offspring's asexual reproductive ability (Figure 3.1a). Even though the integrated hatching ratios of eggs asexually produced from mRa × fRs ergatoid queens are pretty low (17.5%), it is still numerically higher either than mRs × fRa (3.75%) or mRa × fRa (0%). There are three potential reasons for explaining the low hatching ratios of eggs asexually produced by mRa × fRs ergatoid queens. Firstly, the hatching ratios of eggs asexually produced by mRs × fRs ergatoid queens are also lower (53.3%) than prospect (75%) (Nozaki et al. 2018). Secondly, the isolated eggs were far away from the acquired effect of queens, who are able to regulate the egg developmental results (Matsuura and Kobayashi 2010; Yamamoto and Matsuura 2011). Thirdly, since paternal factors can control the survival rates of the larva (Wu et al. 2023), and the eggs asexually produced by mRa × fRa ergatoid queens failed in hatching, the hatching ratios of egg asexually produced by mRa × fRs ergatoid queens were predictably low. Genotyping results showed that the asexual F2 offspring produced by ergatoid queens derived from worker of mRaB × fRsB inherited only *R. speratus* genes

in four loci (Figure 3.2). The meiotic drive observed in the asexual reproduction of this hybrid colony suggests the presence of selfish genetic elements in the parthenogenesis of *R. speratus* and highlights conflict over transmission to subsequent generations through parthenogenesis.

AQS species are unusual species that queens process both sexual and asexual reproductive capacities. Because there is a tiny probability that non-AQS species' asexual eggs are able to hatch (Nozaki et al. 2018), and also there are small differences between termites and their ancestors (Mizumoto et al. 2024), we can hypothesize that asexually reproductive capacity was gradually silenced in evolution. This study shows that the maternal genome is more likely to transfer the capacity to the next generation (Figure 3.1a). This result can partly support the hypothesis that the evolution process, hybrid, for instance, can be detrimental to asexually reproductive capacities (Ramsey and Schemske 2002).

3.5 References

1. Dronnet, S., A. G. Bagnères, T. R. Juba, and E. L. Vargo. 2004. Polymorphic microsatellite loci in the European subterranean termite, *Reticulitermes santonensis* Feytaud. Molecular Ecology Notes 4:127–129.
2. Hayashi, Y., N. Lo, H. Miyata, and O. Kitade. 2007. Sex-linked genetic influence on caste determination in a termite. Science 318:985–987.
3. Matsuura, K. 2017. Evolution of the asexual queen succession system and its underlying mechanisms in termites. The Journal of Experimental Biology 220:63–72.
4. Matsuura, K., and N. Kobayashi. 2010. Termite queens adjust egg size according

to colony development. Behavioral Ecology 21:1018–1023.

5. Matsuura, K., E. L. Vargo, K. Kawatsu, P. E. Labadie, H. Nakano, T. Yashiro, and K. Tsuji. 2009. Queen succession through asexual reproduction in termites. Science 323:1687.
6. Mitaka, Y., T. Akino, and K. Matsuura. 2023. Development of a standard medium for culturing the termite *Reticulitermes speratus*. Insectes Sociaux 70:265–274.
7. Mizumoto, N., S. Hellemans, M. S. Engel, T. Bourguignon, and A. Buček. 2024. Extinct and extant termites reveal the fidelity of behavior fossilization in amber. Proceedings of the National Academy of Sciences of the United States of America 121:e2308922121.
8. Nozaki, T., T. Yashiro, and K. Matsuura. 2018. Preadaptation for asexual queen succession: queen tychoparthenogenesis produces neotenic queens in the termite *Reticulitermes okinawanus*. Insectes Sociaux 65:225–231.
9. Ramsey, J., and D. W. Schemske. 2002. Neopolyploidy in flowering plants. Annual Review of Ecology, Evolution, and Systematics 33:589–639.
10. Simon, J.-C., F. Delmotte, C. Rispe, and T. Crease. 2003. Phylogenetic relationships between parthenogens and their sexual relatives: the possible routes to parthenogenesis in animals. Biological Journal of the Linnean Society. 79:151–163.
11. Stouthamer, R., J. A. J. Breeuwer, and G. D. D. Hurst. 1999. *Wolbachia Pipientis*: microbial manipulator of arthropod reproduction. Annual Review of Microbiology 53:71–102.
12. Vargo, E. L. 2000. Polymorphism at trinucleotide microsatellite loci in the subterranean termite *Reticulitermes flavipes*. Molecular Ecology 9:817–820.
13. Vargo, E. L., P. E. Labadie, and K. Matsuura. 2012. Asexual queen succession in the subterranean termite *Reticulitermes virginicus*. Proceedings of the Royal Society B: Biological Sciences 279:813–819.
14. Wu, Y., J. Chen, M. Takata, and K. Matsuura. 2023. Maternal determination of soldier proportion and paternal determination of soldier sex ratio in hybrid *Reticulitermes* (Isoptera: Rhinotermitidae) termite colonies. PloS One

18:e0293096.

15. Yamamoto, Y., and K. Matsuura. 2011. Queen pheromone regulates egg production in a termite. Biology Letters 7:727–729.
16. Zimet, M., and A. M. Stuart. 1982. Sexual dimorphism in the immature stages of the termite, *Reticulitermes flavipes* (Isoptera: Rhinotermitidae). Sociobiology 7:1–7.

Chapter 4: Sex-link parental effects on the caste fate of offspring in hybrid *Reticulitermes* (Isoptera: Rhinotermitidae) termite colonies

4.1 Introduction

In the ecological and evolutionary progression of social insects, division of labor plays an integral role, as progeny differentiate into reproductive (nymph) and functionally sterile castes (worker and soldier) (Beshers and Fewell 2001). Workers primarily work for foraging, tunneling, tending to eggs, and maintaining hygiene, whereas soldiers predominantly respond to colony defense, and reproduction is only performed by the king and queen (Zhou et al. 2006; Fefferman et al. 2007). As reviewed by Schwander

(Schwander et al. 2010), various environmental factors, such as temperature conditions, nutritional content and abundance, parental pheromones, and colony size, have been demonstrated to affect the production of reproductive castes. However, accumulating data over the past two decades has decisively refuted the once-prevailing notion that broods of social insects possess complete totipotency and that environmental factors are the exclusive determinants of caste allocation. This result demonstrated that the caste fate of offspring is influenced by the sex-antagonistic action of epigenetic factors inherited from the king and queen in termites (Matsuura et al. 2018). Therefore, parental effects encompassing transgenerational epigenetic components or behavioral factors, for instance, parent-to-offspring proctodeal trophallaxis, can play a critical role in determining the caste fate of the offspring (Maekawa et al. 2012; Nalepa 2015; Takata et al. 2023).

In this study, we investigated the sex-specific parental effects on caste fate using laboratory foundation hybrid colonies of *Reticulitermes* termites. Accumulation of evidence suggests that hybrid termite colonies can inherit phenotypic traits from both parental species (Lefebvre et al. 2008; Chouvenc et al. 2015; Patel et al. 2020). Soldiers can differentiate from fourth or fifth instar workers in mature *Reticulitermes* colonies. The soldier proportions within the nests are strategically regulated, as an excessive or inadequate number of soldiers can adversely impact colony performance (Matsuura 2002). According to Matsuura's theory, sexual size dimorphism (SSD) is often associated with a sex ratio bias in soldiers (Matsuura 2006) because larger workers are more likely to molt into soldiers, and in some termite species, females are larger than

males (Matsuura 2006; Bourguignon et al. 2012).

Consequently, species with a biased soldier sex ratio might recruit suitable phragmotic soldiers, improving their defensive capabilities. For example, *R. virginicus*, *R. speratus*, *R. flavipes*, and *R. kanmonensis* differ in soldier sex ratio: *R. speratus* soldier is biased toward females, feminized in *R. virginicus*. At the same time, it is equal in *R. kanmonensis and R. flavipes* (Matsuura 2006).

Here, we investigated the sex-linked effects of parents on the colony's social characteristics by using hybrid colonies of *R. speratus* (Rs) and *R. amamianus* (Ra). First, we collected the nest of these two species in the field and compared the soldier sex ratios in mature field colonies; we found that the soldier sex ratio of *R. speratus* colonies was skewed to females, while that of *R. amamianus* colonies was skewed toward males slightly. Therefore, we compared soldier sex ratio, soldier proportion, and number of workers among four pair types of parent species: mRa × fRa, mRa × fRs, mRs × fRa, and mRs × fRs (m: male, f: female). We performed two rounds (two seasons) of observations and data collection: first in colonies aged two years and second in the colonies aged two and a half years.

4.2 Materials and methods

4.2.1 Termite colonies sampling

Decayed logs containing *R. amamianus* or *R. speratus* colonies were collected in pine or Japanese cedar forests in Kagoshima, Hyogo, Kyoto, Shiga, and Hokkaido, Japan, from 2019–2021 (see Dataset 4.S1 for details). Twenty and 17 colonies of *R.*

amamianus and *R. speratus*, respectively, were used to investigate the soldier sex ratio. Additionally, two colonies containing alates for each species were collected in Kagoshima and Kyoto, Japan, in 2020 and used in hybrid experiments. Each colony was individually processed in the following experiments.

4.2.2 Soldier sex ratios in field colonies of *R. amamianus* and *R. speratus*

All soldiers were extracted from the logs, and up to 100 individuals were randomly selected from each colony. All soldiers were used if the total soldier number was less than 100 in the sampled log. The soldiers were sexed by sternite morphology (Zimet and Stuart 1982), and the number of each sex was recorded.

4.2.3 Foundation of hybrid colonies

All alates were extracted from the logs, had their wings removed, and were sexed by sternite morphology (Zimet and Stuart 1982). Then, 720 individuals for each sex and colony were randomly selected and assigned to one of the following crosses: mRa × fRa (mRaA × fRaB, mRaB × fRaA), mRa × fRs (mRaA × fRsA, mRaB × fRsA, mRaA × fRsB, and mRaB × fRsB), mRs × fRa (mRsB × fRaA, mRsA × fRaB, mRsB × fRaB, mRsA × fRaA), and mRs × fRs (mRsA × fRsB, mRsB × fRsA). One hundred and twenty pairs were set for each (see Dataset 4.S1 for details). Each pair was placed in a dish (34 mm diameter × 10 mm height) with brown-rotted pinewood mixed cellulose (BPC) medium (Mitaka et al. 2023) for six months and transferred to a larger plastic box (100 × 65 × 28 mm) filled with the BPC medium and a soil block (36 × 36 × 14

mm, W × D × H). They were maintained at 25 °C under dark conditions. We replenished the BPC medium whenever one-third was consumed to ensure a constant food supply. Two years after the founding of the colony, we transferred the colonies to even larger plastic boxes (194 × 104 × 26 mm, W × D × H) containing two soil blocks and two pieces of pine wood (45 × 45 × 10 mm, W × D × H).

At two and two and a half years after the foundation of colonies, all individuals from each colony were extracted. The numbers of eggs, larvae, workers, and nymphs were recorded. Soldiers were sexed by sternite morphology, and the counts for each sex were recorded.

4.2.4 Egg-fostering experiment

To investigate the sex-link parental effects on the offspring survival, we conducted an egg-fostering experiment and compared survival rates among combinations of parent species. We performed three replications in each combination of parent species (i.e., in mRs × fRs colonies, three colonies from each of mRsA × fRsB and mRsB × fRsA crosses were used for egg collection). Twenty eggs were randomly extracted from each colony and transferred into a dish (34 mm diameter × 10 mm height) with BPC medium and 50 male workers. The male workers were selected from a different colony of the same cross. The hatching period in *R. speratus* is approximately 35 days (Matsuura and Kobayashi 2007), so we recorded the number of larvae in each dish every week for ten weeks. The workers who tended to differentiate into neotenic kings or soldiers were removed immediately. If the offspring develop into N1s or W1s, the numbers of

individuals of each caste and sex were recorded. The caste and sex were identified by wing buds and sternite morphology, respectively (Zimet and Stuart 1982; Takata et al. 2020).

4.2.5 Statistical analysis

Exact binomial tests were applied to compare the observed numerical soldier sex ratio in field-collected colonies in *R. amamianus* and *R. speratus* against the null hypothesis, assuming that the numbers of males and females were equal. The total number of male and female soldiers in each species was used for the analyses.

Comparison of the numerical soldier sex ratio, soldier ratio, offspring survival rate, or offspring sex ratio among four combinations of parent species (mRa × fRa, mRa × fRs, mRs × fRa, and mRs × fRs) was performed with generalized linear models (GLMMs) with a binomial distribution. In the models, the objective variable was the numerical sex ratio of soldiers (number of males vs. females), soldier ratio (number of soldiers vs. workers), offspring survival rate (number of surviving vs. dead larvae), or offspring sex ratio (number of males vs. females), the explanatory variable was the combination of parent species. Colony ID was treated as a random factor. A comparison of the number of workers among the four combinations of parent species was performed with a GLMM and Poisson distribution. The model's objective variable was the number of workers, the explanatory variable was the combination of parent species, and colony ID was treated as a random factor. We also performed GLMMs, which included a species of king × queen interaction term instead of the combination of parent species to

account for sex and species-specific effects. Likelihood ratio tests (LRTs) were used to determine the statistical significance of each explanatory variable.

Exact binomial tests were applied to compare the observed numerical offspring sex ratio in each combination of parent species against the null hypothesis, assuming that the numbers of females and males were equal. The total number of male and female offspring in each combination of parent species was used for the analyses.

All analyses were performed by the software R v4.2.3 (R Core Team 2022), with the 'lme4' and 'car' packages. For the GLMMs and GLMs, Tukey's HSD tests using the 'glht' function in the 'multcomp' package (Hothorn et al. 2008) were conducted to test for differences among the combinations of parent species. A significance value of $p < 0.05$ was considered to indicate statistical significance.

4.3 Results

4.3.1 Comparison of soldier sex ratios

In the field-collected *R. amamianus* colonies, the mean numerical sex ratio of soldiers (proportion of females) was significantly skewed toward males (exact binomial test, 95% CI = 0.409–0.466, $p < 0.001$, Figure 4.1a), while in *R. speratus* it was significantly skewed toward females (exact binomial test, 95% CI = 0.590–0.634, $p < 0.001$).

The hybrid experiment showed that the species of the king had a significant effect on the soldier sex ratio (Tukey's HSD tests, $p < 0.05$, Figures 4.1b and 4.1c), while the species of the queen had no significant effect on it. There was no significant interaction between the species of king and queen (2-year-old colonies: GLMM, LRT: $\chi^2 = 0.728$,

$df = 1$, $p = 0.394$; 2.5-year-old colonies: GLMM, LRT: $\chi^2 = 0.435$, $df = 1$, $p = 0.510$).

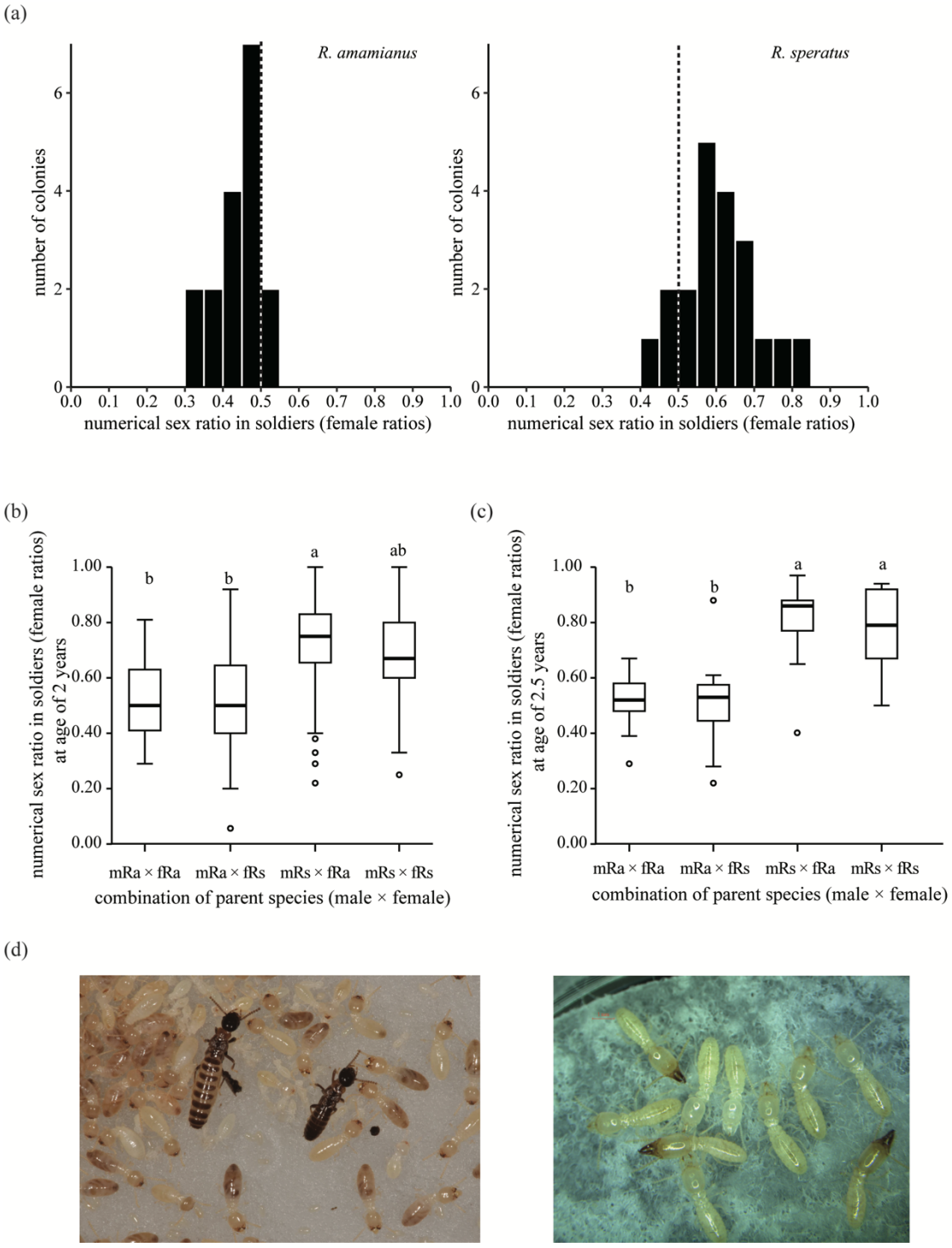

Figure 4.1 Comparison of soldier sex ratios in *R. speratus* and *R. amamianus* and their hybrid colonies. (a) Soldier sex ratios in *R. speratus* and *R. amamianus* are biased in nature. The dashed line exhibits an equal soldier sex ratio. (b) and (c) Soldier sex ratios

in 2- and 2.5-year-old colonies, respectively. The combination of parent species (male × female) shows the species of primary king and queen (i.e., mRa × fRs means the primary king is *R. amamianus*, and the primary queen is *R. speratus*). Box plots indicate the female sex ratios in each colony. Different letters indicate significant differences (Tukey's HSD tests, $p < 0.05$). (d) Photos of a 2.5-year incipient colony (left) and soldiers (right).

4.3.2 Comparison of soldier proportions

The species of the queen had a significant effect on the soldier proportions in 2-year-old colonies (Tukey's HSD tests, $p < 0.05$, Figure 4.2a), while the species of the king had no significant effect. There was no significant interaction between the species of king and queen (GLMM, LRT: $\chi^2 = 1.085$, $df = 1$, $p = 0.298$).

The pattern was similar in 2.5-year-old colonies, but there was a significant interaction effect between the species of king and queen (GLMM, LRT: $\chi^2 = 8.058$, $df = 1$, $p = 0.005$, Figure 4.2b). The queen species significantly affected the soldier proportions irrespective of the species of the king (Tukey's HSD tests, $p < 0.05$). In colonies where the queen is *R. amamianus*, the species of the king had a significant effect (Tukey's HSD tests, $p < 0.05$). In contrast, in colonies where the queen is *R. speratus*, the effect was not significant.

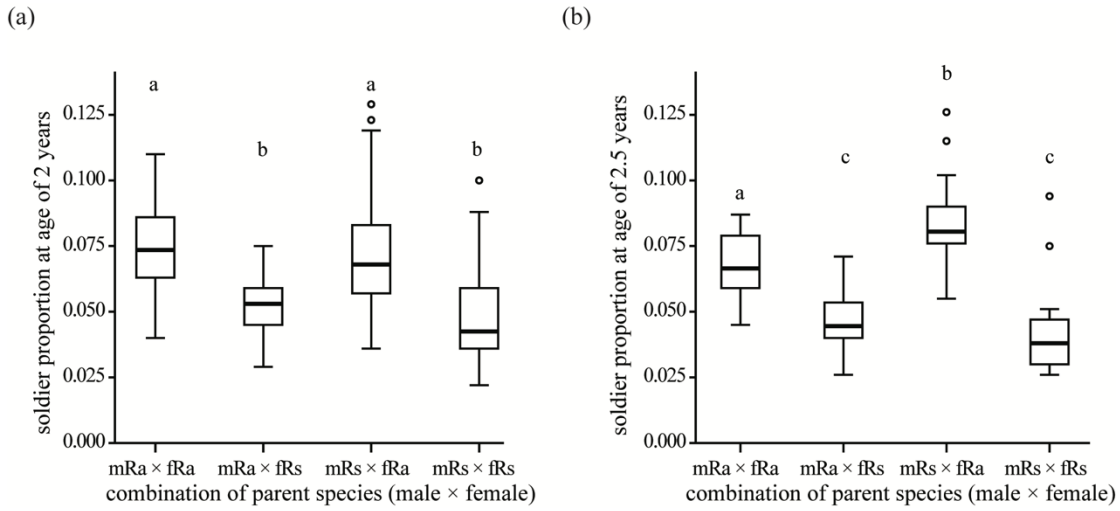

Figure 4.2 Comparison of soldier proportions among *R. amamianus*, *R. speratus*, and their hybrid colonies. (a) and (b) Soldier proportions in 2- and 2.5-year-old colonies, respectively. The combination of parent species (male × female) shows the species of primary king and queen (i.e., mRa × fRs means the primary king is from *R. amamianus*, and the primary queen is from *R. speratus*). Box plots exhibit soldier proportions in each colony. Different letters indicate significant differences (Tukey's HSD tests, $p < 0.05$).

4.3.3 Comparison of the number of workers in a colony

The species of the king had a significant effect on the number of workers in 2-year-old colonies (Tukey's HSD tests, $p < 0.05$, Figure 4.2a), while the species of the queen had no significant effect. There was no significant interaction between the species of king and queen (GLMM, $\chi^2 = 0.032$, $df = 1$, $p = 0.859$).

In 2.5-year-old colonies, there was a significant interaction between the species of king and queen (GLMM, LRT: $\chi^2 = 6.031$, $df = 1$, $p = 0.141$, Figure 4.2b). The species of the king had a significant effect on the number of workers irrespective of the species of the queen (Tukey's HSD tests, $p < 0.05$). In colonies where the king is *R. amamianus*,

the species of the queen had a significant effect on the proportion of soldiers (Tukey's HSD tests, $p < 0.05$), while in colonies where the king is *R. speratus*, the effect was not significant.

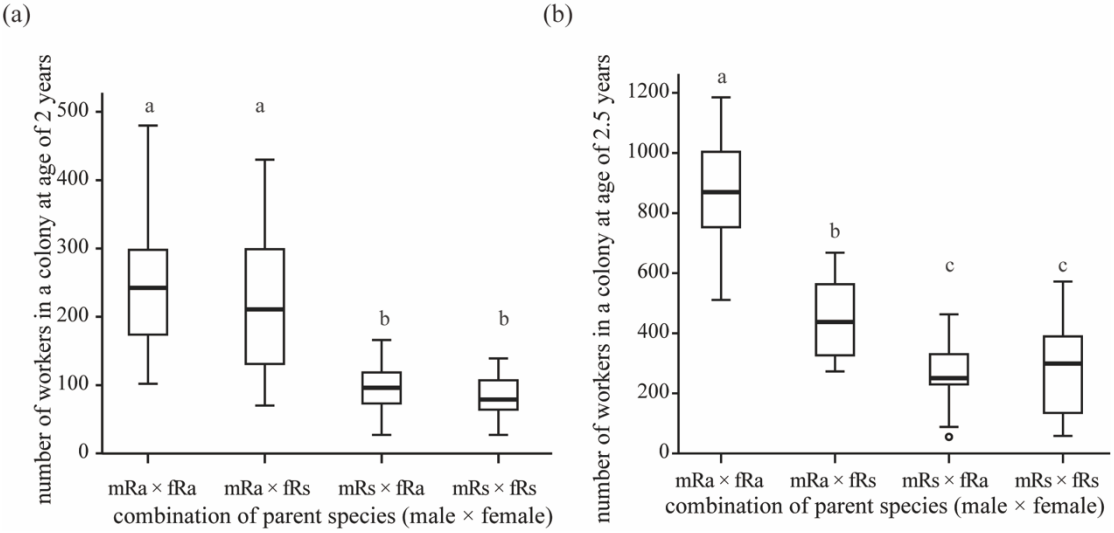

Figure 4.3 Comparison of worker numbers in *R. speratus*, *R. amamianus*, and their hybrid colonies. (a) and (b) The workers in 2- and 2.5-year-old colonies, respectively. The combination of parent species (male × female) shows the species of primary king and queen (i.e., mRa × fRs means the primary king is from *R. amamianus*, and the primary queen is from *R. speratus*). Plots exhibit the number of workers in each colony. Different letters indicate significant differences (Tukey's HSD tests, $p < 0.05$).

4.3.4 Comparison of offspring survival in the egg-fostering experiment

The number of larvae had reached a plateau 35 days after the start of the experiment (Figure 4.4a). The number of surviving larvae on the 35th day was used to compare the survival rate. There was no significant difference among mRa × fRa, mRa × fRs, and mRs × fRs colonies in the survival rate of fostered offspring except for mRs × fRa

combination (Tukey's HSD tests, $p < 0.05$, Figure 4.4b).

There was no significant difference in worker sex ratio among the colony types (Tukey's HSD tests, $p > 0.05$, Figure 4.4c). The exact binomial tests for each species showed that the larval sex ratio in mRa × fRa was significantly skewed toward females (exact binomial test, 95% CI = 0.503–0.712, $p = 0.045$), while there was no statistically significant bias in the larval sex ratio in mRa × fRs (exact binomial test, 95% CI = 0.466–0.619, $p = 0.287$), mRs × fRa (exact binomial test, 95% CI = 0.346–0.571, $p = 0.505$), and mRs × fRs (exact binomial test, 95% CI = 0.382–0. 588, $p = 0.839$). All the offspring were differentiated into workers, and no nymphs were found.

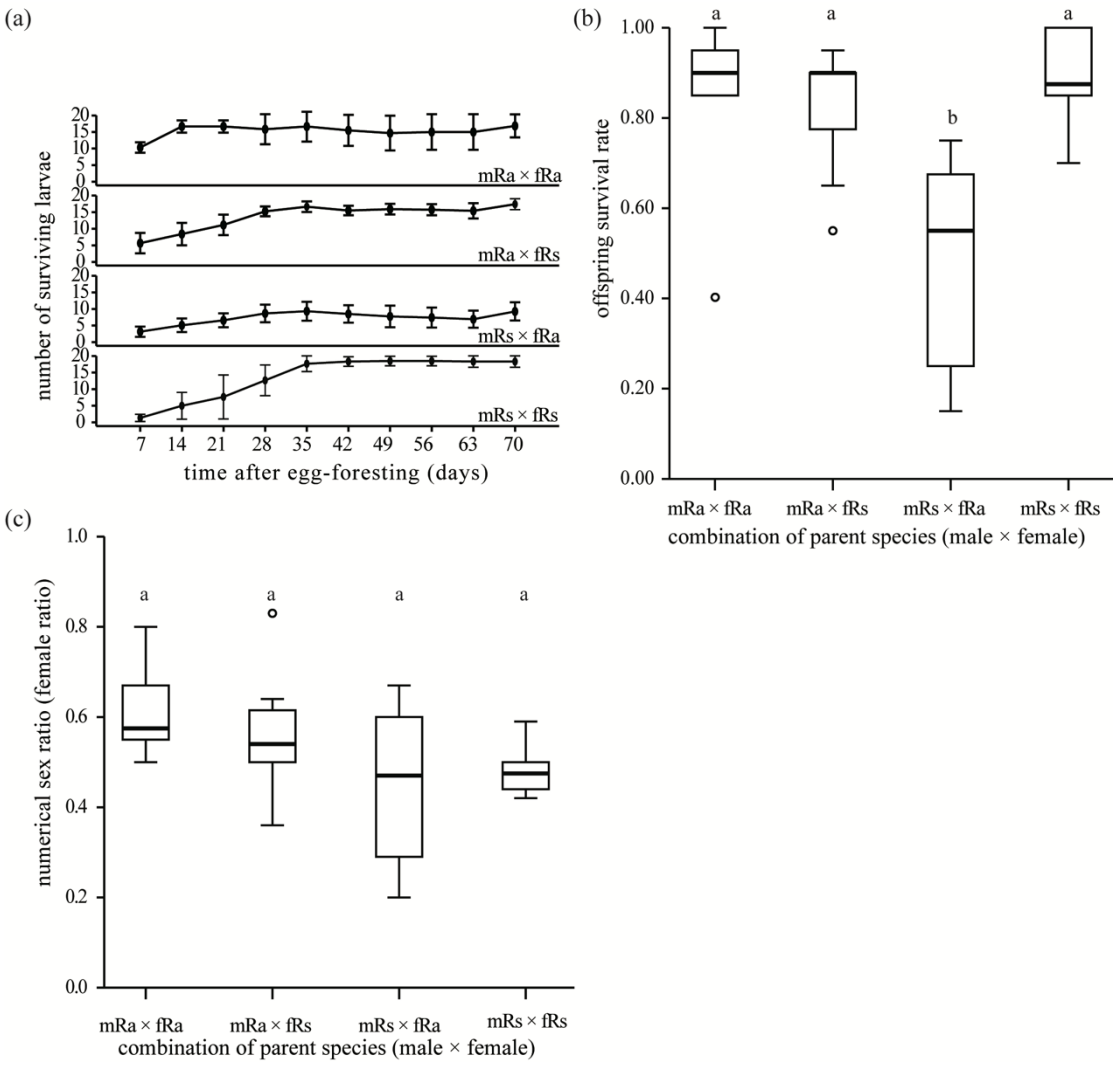

Figure 4.4 Comparison of offspring survival and sex ratios in *R. speratus*, *R. amamianus*, and their hybrid colonies. (a) Dynamics of number of hatched and surviving larvae in each combination of parent species. Dots and bars indicate mean and 95 % confidence interval, respectively. (b) and (c) Offspring survival rate and sex ratios on the 35th day after egg-fostering, respectively. The combination of parent species (male × female) shows the species of primary king and queen (i.e., mRa × fRs means the primary king is *R. amamianus*, and the primary queen is *R. speratus*). Box plots exhibit the number of workers in each colony. Different letters indicate significant differences (Tukey's HSD tests, $p < 0.05$).

4.4 Discussion

Here, we show the sex-linked parental effects on the caste determination in *Reticulitermes* termites, where the king determines the soldier sex ratio and the number of workers, and the queen decides the soldier proportion. The closely related *R. amamianus* and *R. speratus* subterranean termites exhibited distinctions in population size, caste ratios, and sex allocation. Field colonies of *R. speratus* show a female-biased soldier sex ratio, whereas *R. amamianus* shows a slightly male-biased soldier sex ratio (Figure 4.1a). *Reticulitermes speratus* were observed to have a soldier sex ratio skew to females, whereas *R. amamianus* populations demonstrated a balanced ratio of soldiers by sex in incipient colonies. In *R. speratus*, the soldier sex ratio is more biased toward females as the colony size grows larger (Figure 4.S1b). As shown in Figure 4.1a, the soldier sex ratio bias in *R. amamianus* is smaller than that in *R. speratus*, even in mature field colonies. Therefore, the bias of the soldier sex ratio of *R. amamianus* incipient colonies may disappear because these colonies had not yet been large enough to exhibit such a bias. Understanding the soldiers proportions within *Reticulitermes* nests proved challenging due to the unknown nature of their nest structures, coupled with the unexplorable colony boundaries (Thorne et al. 1999). In the previous view, both environmental factors and colony age mainly affect the proportion of soldiers (Howard and Haverty 1981). However, it is nearly impossible to know the environment experienced by the colonies and their age in nature. In this study, by investing the lab-founded colonies, we found that *R. amamianus* possesses larger initial colony sizes (Figure 4.3) and a larger soldier proportion than *R. speratus* (Figure 4.2). Investigation of hybrid colonies indicates that the soldier sex ratio is consistent with the king's species

(Figures 4.1b and 4.1c). In contrast, the soldier proportion is exclusively consistent with the queen's species (Figure 4.2). The worker population size skews to the king's species (Figure 4.3). However, a significant difference in worker numbers between mRa × fRs and mRa × fRa (Figure 4.3b) indicates that the queen's genome also affects worker numbers. This research illustrated that the sex of a particular parent can wield an enhanced impact on colony characteristics, overshadowing the influence of the other parent's sex.

An assortment of elements may act as immediate determinants for the distinct effect of parental gender on colony features. These include inheritable factors like sex-associated genetic elements, cytoplasmic factors, cross-generational epigenetic elements, and socio-environmental considerations like parental behaviors and chemical interaction. The intergenerational dialog, exemplified by phenomena like trophallaxis, is crucial in setting the parameters for the soldier caste (Yaguchi et al. 2016). Nonetheless, the gender-based effects of parents on the ratio of soldier sex cannot be completely justified by the parents' socio-environmental impacts. Moreover, not only the soldier proportion but also the gender distribution among soldiers can oscillate based on the development phase of the colony (Figure 4.S1). Therefore, genetic factors alone cannot explain these results.

Heritable factors beyond DNA sequences can influence phenotypic traits in the offspring of various organisms (Youngson and Whitelaw 2008; Bonduriansky and Day 2009). A number of molecular mechanisms have been identified, such as the transmission of genomic imprinting (histone modification and DNA methylation),

cytoplasmic factors like hormones and nutrients, and small non-coding RNAs (Groothuis et al. 2005; Skvortsova et al. 2018). All these are transferred through the egg and sperm, even though hormones and nutrients are exclusively inherited from the egg (Adrian-Kalchhauser et al. 2020). Nevertheless, if the cytoplasmic substances were to control in vivo response mechanisms, the observed disparity in soldier sex ratio, although a consistent proportion of soldiers in mRs × fRa, mRa × fRa, mRa × fRs, and mRs × fRs, would be impossible. This is because both sexes of offspring have the same cytoplasmic substance from the same maternal genome. Instead, sex-link epigenetic factors inherited from parents might be implicated as immediate determinants of caste fate, as a mathematical model suggests (Matsuura et al. 2018). In *Reticulitermes* termites, it is known that epigenetic factors inherited from the king and queen can influence the caste fate determination of offspring (whether they follow the worker pathway or nymph pathway) (Matsuura et al. 2018), and there is a potential for these epigenetic factors to also regulate soldier differentiation.

Interestingly, the reproductive system and soldier sex ratio are related, as observed in species of the *Reticulitermes* genus. In the case of *R. virginicus* and *R. speratus*, which are AQS species (Matsuura et al. 2009; Vargo et al. 2012), the soldier sex ratio is biased to females, while in non-AQS species, there is no sex ratio bias (Matsuura 2006). Identifying epigenetic factors linked to the bias in soldier sex ratio will lead to a better understanding of the underlying reasons for this intriguing correlation. The current study does not directly investigate behaviors, heritable factors, and pheromones from the king and queen, respectively; either or both factors could be significant.

Therefore, further research is required to clarify the proximate causes of sex-link parental effects on colony characteristics.

4.5 Supplementary materials

4.5.1 Supplementary materials and methods

Decayed logs containing *R. speratus* colonies were collected in pine or Japanese cedar forests in Kyoto, Japan, in April and May 2018 and 2022 (see Dataset 4.S1 for details). The method of colony founding is the same as the one described in the hybrid experiment section in the main text. See Dataset 4.S2 for details on mating types and the number of replications.

In colonies founded in 2022, all individuals from each colony were extracted 0.5 years after the colony's foundation. In colonies established in 2018, the extraction was conducted 2.5 and 4.5 years after colony foundation. The weights of the kings and queens and ten workers from each colony were recorded. The numbers of eggs, larvae, workers, and nymphs were also recorded. Soldiers were sexed by sternite morphology, and the counts and total weights for each sex were recorded.

Exact binomial tests were applied to compare the observed numerical soldier sex ratio in 1-, 2.5-, and 4.5-year-old colonies of *R. speratus* against the null hypothesis, assuming that the numbers of males and females were equal. Comparison of the numerical soldier sex ratio or soldier ratio between 2.5- and 4.5-year-old colonies was performed with generalized linear models (GLMMs) with binomial distribution. In the models, the objective variable was the numerical sex ratio of soldiers (number of males

vs. females) or soldier ratio (number of soldiers vs. workers), the explanatory variable was the age of the colonies, and colony ID was treated as a random factor. A comparison of the number of workers between 2.5- and 4.5-year-old colonies was performed with a GLMM with a Poisson distribution. In the model, the objective variable was the number of workers, the explanatory variable was the age of the colonies, and colony ID was treated as a random factor. For the GLMMs, a likelihood ratio test (LRT) was used to determine the statistical significance of the explanatory variable. A significance value of $p < 0.05$ was considered to indicate statistical significance.

4.5.2 Supplementary results

4.5.2.1 Comparison of the soldier sex ratios

There was no statistically significant bias in the soldier sex ratio in the 1-year-old colonies (exact binomial test, 95% CI = 0.412–0.606, $p = 0.924$, Figure 4.S1a). The sex ratio was significantly skewed toward females in 2.5-year-old colonies (exact binomial test, 95% CI = 0.694–0.770, $p < 0.001$, Figure 4.S1b) and 4.5-year-old colonies (exact binomial test, 95% CI = 0.780–0.839, $p < 0.001$). The soldier sex ratio in 4.5-year-old colonies was significantly more biased towards females than that in 2.5-year-old colonies (GLMM, LRT: $\chi^2 = 10.117$, $df = 1$, $p = 0.001$).

4.5.2.2 Comparison of the soldier proportions

The soldier proportion in 4.5-year-old colonies was significantly lower than that in 2.5-year-old colonies (GLMM, LRT: $\chi^2 = 69.796$, $df = 1$, $p < 0.001$, Figure 4.S1c).

4.5.2.3 Comparison of the number of workers in a colony

There were significantly more workers in 4.5-year-old colonies than in 2.5-year-old colonies (GLMM, LRT: $\chi^2 = 5756.9$, $df = 1$, $p < 0.001$, Figure 4.S1d).

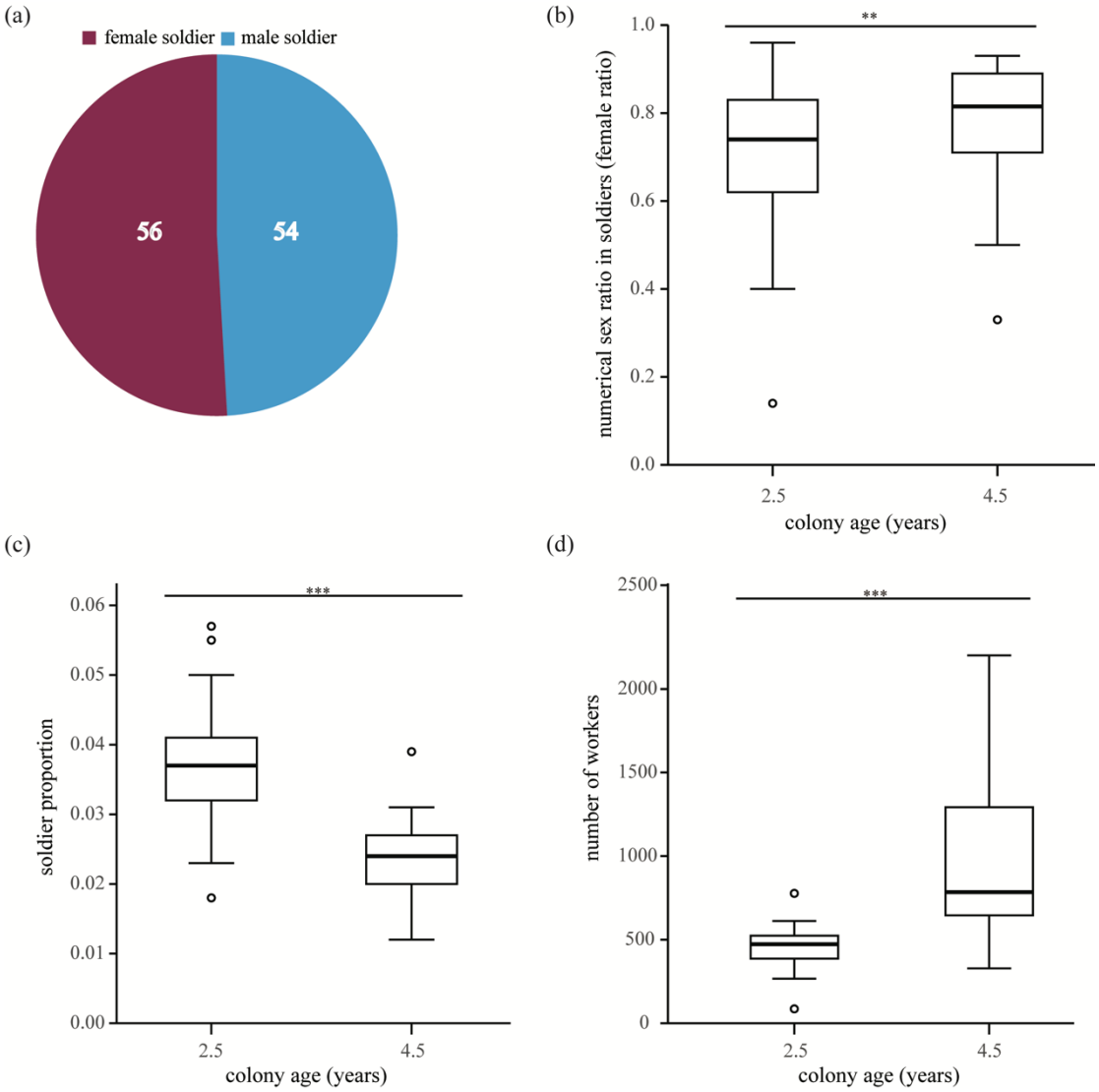

Figure 4.S1 Dynamics of caste and sex ratio in *R. speratus* colonies. (a) There was no bias in the sex ratio in the 0.5-year-old colonies. (b), (c), and (d) Comparison of soldier sex ratio, soldier proportion, and number of workers between 2.5- and 4.5-year-old colonies, respectively. Asterisks indicate significant differences (likelihood ratio test,

$^{**}p < 0.01$, $^{***}p < 0.001$).

4.6 References

1. Adrian-Kalchhauser, I., S. E. Sultan, L. N. S. Shama, H. Spence-Jones, S. Tiso, C. I. K. Valsecchi, and F. J. Weissing. 2020. Understanding non-genetic inheritance: insights from molecular-evolutionary crosstalk. Trends in Ecology & Evolution 35:1078–1089.
2. Beshers, S. N., and J. H. Fewell. 2001. Models of division of labor in social insects. Annual Review of Entomology 46:413–440.
3. Bonduriansky, R., and T. Day. 2009. Nongenetic inheritance and its evolutionary implications. Annual Review of Ecology, Evolution, and Systematics 40:103–125.
4. Bourguignon, T., Y. Hayashi, and T. Miura. 2012. Skewed soldier sex ratio in termites: testing the size-threshold hypothesis. Insectes Sociaux 59:557–563.
5. Chouvenc, T., E. E. Helmick, and N.-Y. Su. 2015. Hybridization of two major termite invaders as a consequence of human activity. PloS One 10:e0120745.
6. Fefferman, N. H., J. F. A. Traniello, R. B. Rosengaus, and D. V. Calleri II. 2007. Disease prevention and resistance in social insects: modeling the survival consequences of immunity, hygienic behavior, and colony organization. Behavioral Ecology and Sociobiology 61:565–577.
7. Groothuis, T. G. G., W. Müller, N. von Engelhardt, C. Carere, and C. Eising. 2005. Maternal hormones as a tool to adjust offspring phenotype in avian species. Neuroscience & Biobehavioral Reviews 29:329–352.
8. Hothorn, T., F. Bretz, and P. Westfall. 2008. Simultaneous inference in general parametric models. Biometrical Journal: Journal of Mathematical Methods in Biosciences 50:346–363.
9. Howard, R., and M. I. Haverty. 1981. Seasonal variation in caste proportions of field colonies of *Reticulitermes flavipes* (kollar). Environmental Entomology 10:546–549.

10. Lefebvre, T., N. Châline, D. Limousin, S. Dupont, and A.-G. Bagnères. 2008. From speciation to introgressive hybridization: the phylogeographic structure of an island subspecies of termite, *Reticulitermes lucifugus* corsicus. BMC Evolutionary Biology 8:38.
11. Maekawa, K., S. Nakamura, and D. Watanabe. 2012. Termite soldier differentiation in incipient colonies is related to parental proctodeal trophallactic behavior. Zoological Science 29:213–217.
12. Matsuura, K. 2002. Colony-level stabilization of soldier head width for head-plug defense in the termite *Reticulitermes speratus* (Isoptera : Rhinotermitidae). Behavioral Ecology and Sociobiology 51:172–179.
13. Matsuura, K. 2006. A novel hypothesis for the origin of the sexual division of labor in termites: which sex should be soldiers? Evolutionary Ecology 20:565–574.
14. Matsuura, K., and N. Kobayashi. 2007. Size, hatching rate, and hatching period of sexually and asexually produced eggs in the facultatively parthenogenetic termite *Reticulitermes speratus* (Isoptera: Rhinotermitidae). Applied Entomology and Zoology 42:241–246.
15. Matsuura, K., N. Mizumoto, K. Kobayashi, T. Nozaki, T. Fujita, T. Yashiro, T. Fuchikawa, et al. 2018. A genomic imprinting model of termite caste determination: not genetic but epigenetic inheritance influences offspring caste fate. The American Naturalist 191:677–690.
16. Matsuura, K., E. L. Vargo, K. Kawatsu, P. E. Labadie, H. Nakano, T. Yashiro, and K. Tsuji. 2009. Queen succession through asexual reproduction in termites. Science 323:1687.
17. Mitaka, Y., T. Akino, and K. Matsuura. 2023. Development of a standard medium for culturing the termite *Reticulitermes speratus*. Insectes Sociaux 70:265–274.
18. Nalepa, C. A. 2015. Origin of termite eusociality: trophallaxis integrates the social, nutritional, and microbial environments. Ecological Entomology 40:323–335.
19. Patel, J. S., S.-B. Lee, T. Chouvenc, and N.-Y. Su. 2020. Inferring termite colony size using wood consumption in subterranean termites (Blattodea: Rhinotermitidae) in laboratory-rearing conditions. Journal of Economic Entomology 113:905–910.

20. R Core Team. 2022. R: a language and environment for statistical computing. R foundation for statistical computing.
21. Schwander, T., N. Lo, M. Beekman, B. P. Oldroyd, and L. Keller. 2010. Nature versus nurture in social insect caste differentiation. Trends in Ecology & Evolution 25:275–282.
22. Skvortsova, K., N. Iovino, and O. Bogdanović. 2018. Functions and mechanisms of epigenetic inheritance in animals. Nature Reviews Molecular Cell biology 19:774–790.
23. Takata, M., T. Inagaki, T. Ishibashi, E. Tasaki, and K. Matsuura. 2020. A non-invasive method for sexing first and second instar larvae of termites using external morphology. Insectes Sociaux 67:487–493.
24. Takata, M., S. Nagai, T. Inagaki, Y. Ohkubo, E. Tasaki, and K. Matsuura. 2023. Heritable effects on caste determination and colony-level sex allocation in termites under natural conditions. iScience 26:106207.
25. Thorne, B. L., J. F. A. Traniello, E. S. Adams, and M. Bulmer. 1999. Reproductive dynamics and colony structure of subterranean termites of the genus *Reticulitermes* (Isoptera Rhinotermitidae): a review of the evidence from behavioral, ecological, and genetic studies. Ethology Ecology & Evolution 11:149–169.
26. Vargo, E. L., P. E. Labadie, and K. Matsuura. 2012. Asexual queen succession in the subterranean termite *Reticulitermes virginicus*. Proceedings of the Royal Society B: Biological Sciences 279:813–819.
27. Yaguchi, H., T. Inoue, K. Sasaki, and K. Maekawa. 2016. Dopamine regulates termite soldier differentiation through trophallactic behaviours. Royal Society Open Science 3:150574.
28. Youngson, N. A., and E. Whitelaw. 2008. Transgenerational epigenetic effects. Annual Review of Genomics and Human Genetics 9:233–257.
29. Zhou, X., F. M. Oi, and M. E. Scharf. 2006. Social exploitation of hexamerin: RNAi reveals a major caste-regulatory factor in termites. Proceedings of the National Academy of Sciences of the United States of America 103:4499–4504.
30. Zimet, M., and A. M. Stuart. 1982. Sexual dimorphism in the immature stages of

the termite, *Reticulitermes flavipes* (Isoptera: Rhinotermitidae). Sociobiology 7:1–7.

Chapter 5: Conclusions

This thesis will deliver new empirical data highlighting several potential reproductive strategies and conflict dynamics in termite colonies. This thesis seeks to assess 1) distinguish the potential conflicts within colonies and 2) demonstrate the reproductive strategies by observing and analyzing the dynamics of the colony structure of termites. Obtaining a deep understanding of the evolutionary process of reproductive division of labor in termites will allow for refining research in the biology of eusocial organisms. The results are summarized as follows:

In Chapter 2, an overflow of dysfunctional parthenogenetic alates due to the overproduction of asexual offspring resulting from competition over queen succession among different clonal types was determined through microsatellite analysis and fieldwork. The queens from 23 field colonies of *Reticulitermes speratus* are genotyped. Our investigations have determined that as colonies mature, there is a notable decrease in genetic variation among the queens. Furthermore, a comparative study of alates and primary reproductives from newly formed colonies revealed that parthenogenetically produced offspring, although numerous, tend to become alates with significantly reduced body sizes and substantially lower survival rates than those produced sexually. These findings suggest that while making a more significant number of parthenogenetic eggs early on may provide an edge in securing the queen position, it entails considerable drawbacks for the overall health of the colony. This research underscores the complex evolutionary forces at play between individual-level advantages and colony-level selective pressures in the parthenogenesis exhibited by queens.

In Chapter 3, we demonstrate that the inheritance of asexually reproductive capacity in *R. speratus* relies on maternal inheritance by investigating hybrid ergatoid queens (worker-derived) asexual egg hatching results between *R. speratus* (Rs) and non-asexually reproductive species *R. amaminus* (Ra). Results of the asexual hybrid ergatoid queens fostering experiment reveal that mRa × fRs ergatoid queens can produce large numbers and early hatched asexually-produced eggs. Still, the mRs × fRa ergatoid queens only produced large amounts of asexually non-hatching eggs. Genotyping results show that the asexual F2 offspring inherited more *R. speratus* (maternal) genes than *R. amaminus* (paternal). These results indicate a sexual conflict attributed to kin selection that the maternal genome can transfer more maternal genes to offspring by asexual reproduction.

In Chapter 4, we analyze and contrast soldier sex ratios, soldier proportions, and population sizes across four mating configurations in *R. amamianus* (abbreviated as Ra) and *R. speratus* (Rs), which include combinations of male and female from both species (mRa × fRa, mRa × fRs, mRs × fRa, mRs × fRs). Our findings reveal that the sex ratio of soldiers and the overall population size of hybrid colonies tend to reflect the paternal species. In contrast, the proportion of soldiers aligns more closely with the maternal species. The survival rates of progeny from interspecific crosses were notably higher when male *R. amamianus* (mRa) were crossed with female *R. speratus* (fRs) than the converse. These outcomes suggest that the paternal genotype predominantly influences soldier sex ratios, egg-hatching success, and, by extension, the population sizes of colonies. At the same time, maternal-specific factors largely determine the proportion

of soldiers.

In conclusion, the findings from this thesis exhibit an intragroup conflict among various clone types of queens, leading to a group-level cost. Besides, this study shows that asexual reproductive capacity is attributed to maternal inheritance. Furthermore, the findings from this thesis convey significantly different roles carried by king and queen, attributing to colonies' eusociality building and growth. This work is the first to demonstrate the sex-link parental effect on offspring caste determination and colony growth in termites. Additionally, this study is also the pioneer in the description of the bifurcated life cycle between king and queen in AQS termites and verified Matsuura's hypothesis in 2009: primary kings keep bodily immortality, and primary queens keep genetic long-living in the AQS termite *Reticulitermes* species. Finally, in technical speaking, this thesis underwent eight years and developed a new, more effective method for incipient termite colony fostering.

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Appendix 1

An r-selection strategy in termite colonies following the death of kings: producing large quantities of low-quality reproductive offspring

1.1 Introduction

Asexual queen succession (AQS) species exhibit a distinct reproductive pattern, wherein workers, soldiers, and alates are generated through sexual reproduction, while secondary queens (SQ) are produced via thelytoky (Matsuura et al. 2009, 2018; Matsuura 2020). Previous studies have found that genetic factors may influence caste differentiation in termites as much as environmental factors. For example, excess maternal genomic imprinting will result in more offspring differentiating into neotenic queens rather than workers (Matsuura et al. 2018). It is unclear whether the maternal genomic imprinting function is affected when there is a sudden change in the nest environment, such as the unexpected death of the primary king (PK) or the transformation from a PK to a secondary king (SK). Studying the reciprocal effects between genomic imprinting and environmental pressure will contribute to a better understanding of the caste evolution in social insects.

Matsuura et al. (2018) analyzed the royals of 114 field-mature nesting colonies of *Reticulitermes speratus* and discovered that among 162 kings collected from these mature colonies, 104 were primary, and 58 were nymphoid. Interestingly, no ergatoid kings were found in the field. Out of 6824 queens, 6812 (99.82%) were nymphoid, six were primary (alate-derived), and only six (0.088%) were ergatoid (Matsuura et al. 2018). The mature colony royals were primarily organized in four patterns: PK & SQ, PK, PQ & SQ, PK, SK & SQ, and SK & SQ, with PKs present in 91.23% of the colonies.

No PQ & SK or PQ, SK & SQ-controlled colonies were identified, indicating that no field-mature colonies exhibited PQ survival without PKs (Matsuura et al. 2009, 2018). This observation suggests that, beyond sperm provision, the king may have an undiscovered yet crucial role in colony development.

Recent studies have focused on understanding termite colonies as superorganisms (Kennedy et al. 2017; Boomsma and Gawne 2018; Bernadou et al. 2021; Kramer et al. 2022; Miura et al. 2022). Within this framework, each caste in a termite colony is considered analogous to a distinct cell, tissue, or system based on its specialized function (Boomsma and Gawne 2018; Bernadou et al. 2021; Miura et al. 2022). During laboratory cultivation of termites, our team observed a gradual or sudden decline in colony size and an abnormal shift in the different caste termite proportions following the death of a specific individual, the PK, within two years, ultimately resulting in colony collapse (unpublished observations). This phenomenon is reminiscent of abnormal cell proliferation in organisms due to the absence of tumor suppressor genes, which can ultimately lead to cancer. Given this observation, we hypothesize that the PK may be analogous to the tumor suppressor gene in controlling excessive cell growth and proliferation and preventing tumor formation in superorganisms. The present study aims to explore this hypothesis and elucidate the underlying mechanisms involved.

The subterranean termite *R. speratus*, commonly found in Japan (Matsuura et al. 2018) and certain cities in northern China (Park and Shin 2005), is among the most extensively researched termite species concerning reproductive systems and strategies (Matsuura and Nishida 2001; Matsuura et al. 2009; Kobayashi et al. 2013; Matsuura 2017). This study investigates the king's significant role in colony growth. We examined mature colonies in the field and cultivated two-year colonies in the laboratory, controlling for different reproductive castes. The primary objective is to provide insight

into the king's impact on colony development, which may have significant implications for termite colony management and control.

S-1.2 Materials and methods

S-1.2.1 Termite colonies sampling

Reticulitermes speratus colonies were collected in pine or Japanese cedar forests in Hakodate, Kyoto, and Shiga, Japan, during the summers of 2018, 2019, 2020, 2021, and 2022. Reproductives from each colony were immediately preserved in 100% ethanol along with nestmate workers and nymphs in a vial for subsequent genetic analysis. Primary (alate-derived) and secondary (neotenic) reproductives were distinguished based on their fully melanized body color and the presence of wing scales. The sex of each specimen was determined by examining the configuration of caudal sternites under a stereoscope. Neotenic reproductives were further categorized as either nymphoid (nymph-derived) or ergatoid (worker-derived) based on the presence or absence of wing pads, according to Matsuura et al. (2009). Three PK & SQ, two PK, SK & SQ, and three SK & SQ-headed colonies were randomly chosen. From each colony, SQs and workers were randomly selected and subjected to microsatellite analysis.

S-1.2.2 Foundation of colonies

In the spring of 2017, 2018, and 2020, *R. speratus* nest wood with alate samples was collected in Kinki, Japan. After the alates emerged from the wood, they were separated into two groups by sex and housed in Petri dishes containing moist filter paper until wing shedding occurred. Individual dealates were randomly chosen from each colony and designated to either female-male pair.

Each pair was placed in a dish (34 mm diameter × 10 mm height) with brown-rotted pinewood mixed cellulose (BPC) medium (Mitaka et al. 2023). Colonies were then transferred to plastic boxes (100 × 65 × 28 mm, W × D × H) filled with BPC and a soil block (36 × 36 × 14 mm, W × D × H). BPC was replenished when approximately one-third was consumed. The colony foundation method was modified from Matsuura and Nishida (Matsuura and Nishida 2001). Two years post-colony formation, colonies were carefully dismantled, and all members were extracted using an aspirator and forceps. Reproductive (kings and queens), soldiers, workers, nymphs, larvae, and eggs from each colony were placed in a moist, non-woven cloth in a 90-mm Petri dish and counted. Ten colonies with primary kings (PK) and primary queens (PQ) surviving, six with deceased PK and surviving PQ, and six with deceased PK and PQ were randomly chosen for offspring caste composition analysis. Nymphs and workers from three PK-dead and PQ-surviving colonies, workers and nymphs, and secondary queens (SQs) from two PK and PQ-deceased colonies were used for subsequent microsatellite analysis.

S-1.2.3 Microsatellite analysis

Termite DNA was extracted using a modified Chelex extraction protocol (Walsh et al. 1991). Total DNA was extracted from termite heads or thoraxes by adding 20 µL Chelex solution (10% w/v; TE pH 8.0) and 0.2 µL proteinase K. Samples were incubated at 55 °C for three h, followed by heating at 95 °C for 15 min. PCR amplifications were conducted in a multiplex format using primers *Rf6-1*, *Rf21-1*, *Rf24-2* (Vargo 2000), and *Rs15*(Dronnet et al. 2004). *Rf6-1* was labeled with 6-FAM fluorescent tags, *Rf21-1* with VIC fluorescent tags, *Rf24-2* with NED fluorescent tags, and *Rs15* with PET fluorescent tags. A 10 µL PCR cocktail was prepared containing 1 µL DNA sample, 0.20 µL 10

mM dNTP, 0.99 μL 10× PCR Buffer, 0.07 μL 5 U/μL Taq DNA polymerase (New England Biolabs, Inc., Beverly, MA, USA), 1.15 μL 5 μM multiplex primers, and 6.59 μL DW. Amplification conditions included an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 75 s, and extension at 72 °C for 2 min. PCR products were combined with 10 μL Hi-Di formamide and 0.3 μL GS-600 (LIZ) size standard and analyzed on an Applied BioSystems 3500 Genetic Analyzer. Raw data were processed using GeneMapper 5.0 software (Applied Biosystems, Inc., Foster City, CA, USA).

S-1.3 Results

S-1.3.1 Parthenogenetically-produced and sexually-produced offspring in field and Lab. colonies

Colonies headed by PK, SK, and SQ, as well as colonies headed by PK and SQ (Figure S-1.1), all the SQ in the colonies were produced by parthenogenesis (Figure S-1.1). In colonies headed by SK and SQ (PK absence), the SQs were produced partly or entirely from sexual reproduction (Figure S-1.1). Given the lack of PK's genotype in those colonies, it is impossible to distinguish whether the homozygosity SQs were produced by parthenogenetic reproduction or inbreeding. All the SK were derived only from male nymphs (nymphoid, nymph-derived). All the workers in the colonies were produced by sexual reproduction.

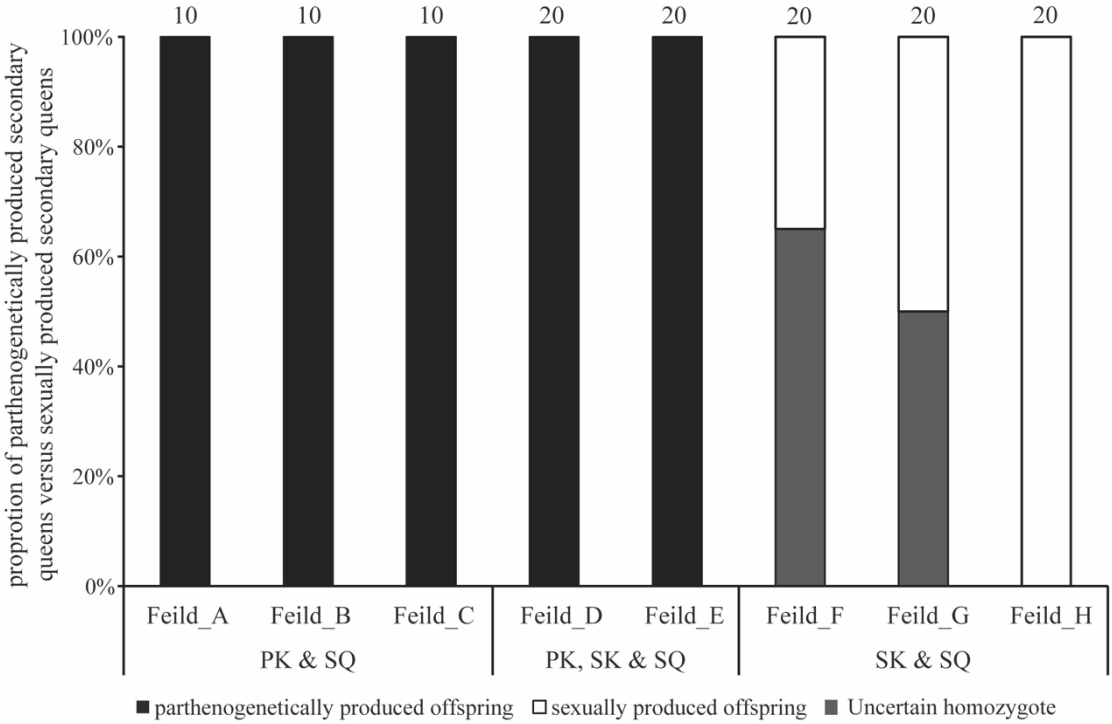
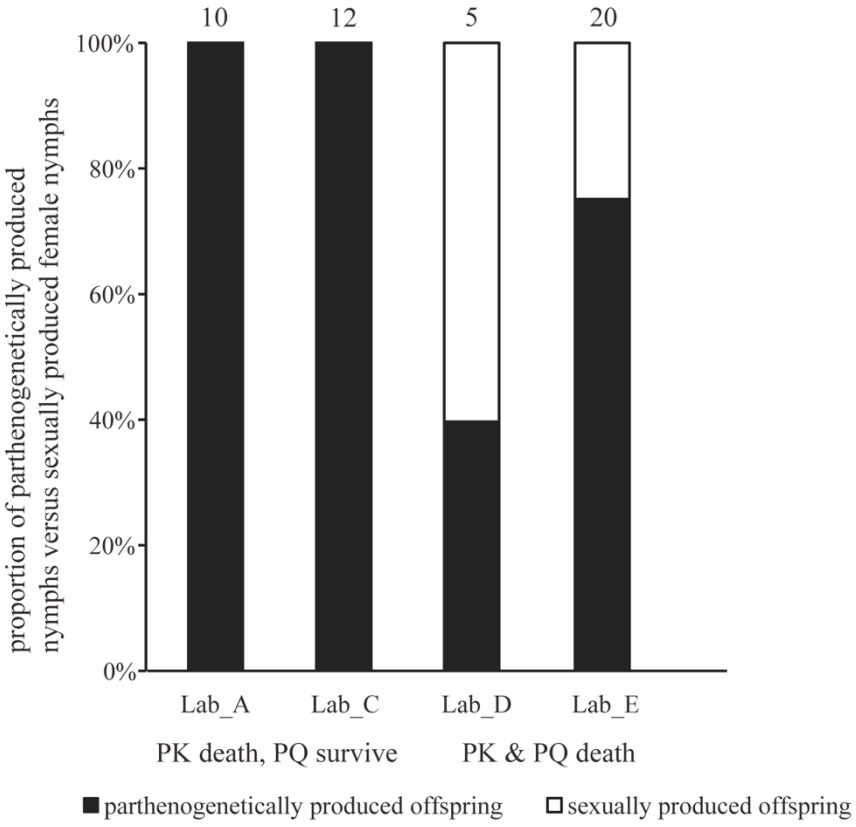


Figure S-1.1 The proportion of parthenogenetically produced secondary queens. PK: primary king, PQ: primary queen, SK: secondary king, and SQ: secondary queen. SQs are parthenogenesis in colonies headed by the PK (including those headed by PK and SQs and those headed by PK, SKs, and SQs). They are at least partly sexually produced in colonies headed by SKs and SQs.

During the colony founding stage, colonies in which PK died but PQ survived (Figure S-1.2a), like those in which both PK and PQ survived (Figure S-1.2a), all the nymphs and the SQ were derived only from parthenogenesis (Figure S-1.2a). Colonies in which both PK and PQ survived had difficulty giving rise to nymphs or SQs at the incipient colony (Figure S-1.2a). Nymphs and SQs increased significantly in colonies with PK death and PQ survival (Figure S-1.2a). In colonies in which both PK and PQ died, the nymphs and the SQs were derived partly from parthenogenesis and partly from sexual reproduction (Figure S-1.2a. During the colony-finding stage, colonies in which PK and PQ died (Figure S-1.2b), like those in which both PK and PQ survived (Figure

S-1.2b), all the workers were derived only from sexual reproduction. In colonies where PK died but PQ survived, all the workers were derived almost from parthenogenesis (Figure S-1.2b).

(a)


10
12
5
20
100%
80%
60%
40%
20%
0%
proportion of parthenogenetically produced nymphs versus sexually produced female nymphs
Lab_A
Lab_C
Lab_D
Lab_E
PK death, PQ survive
PK & PQ death
parthenogenetically produced offspring
sexually produced offspring

(b)

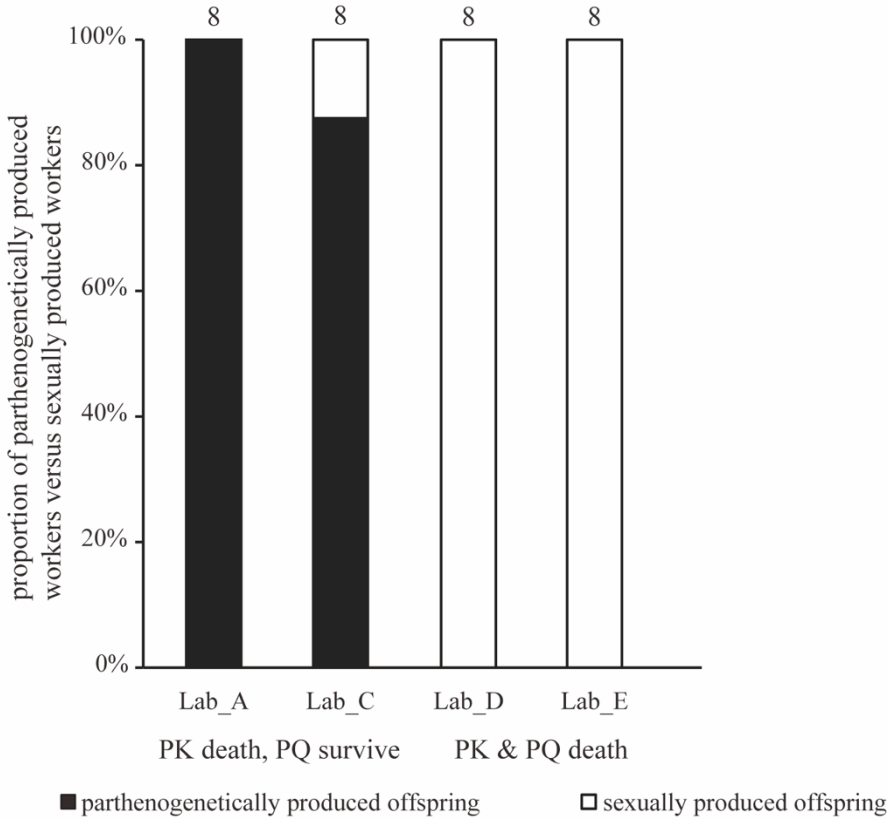
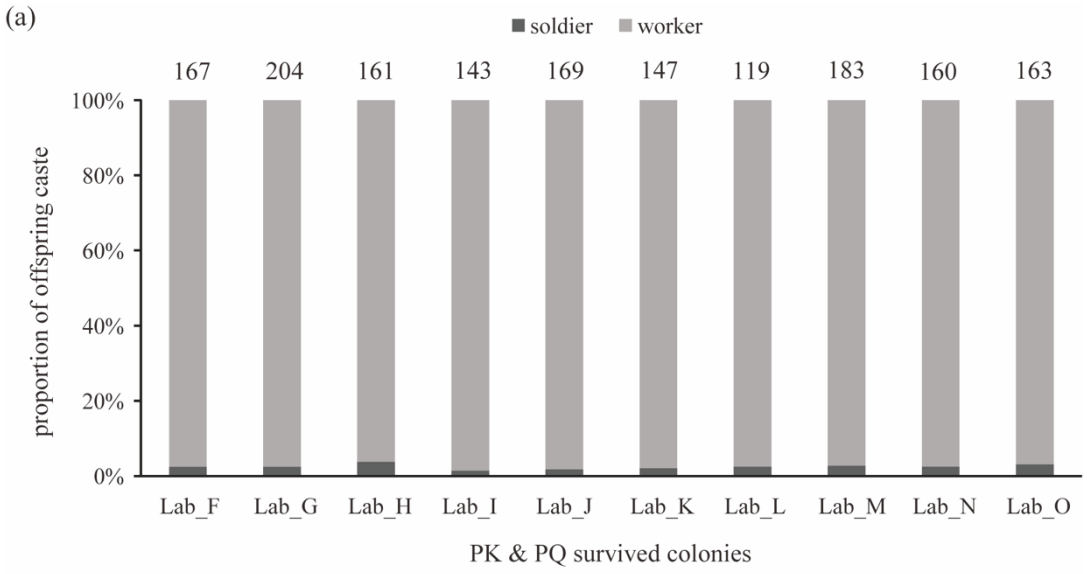
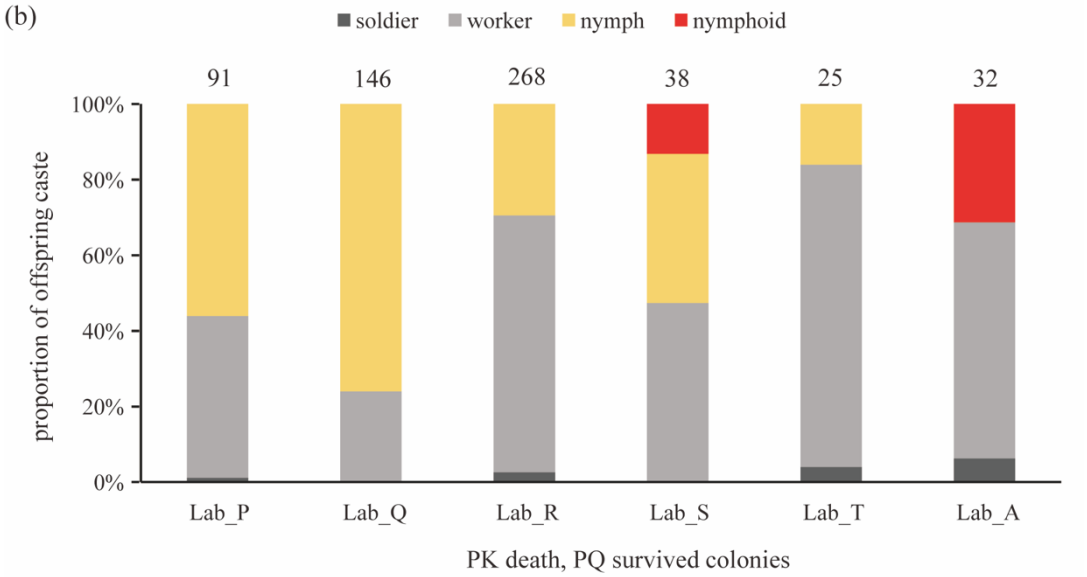

8
8
8
8
100%
80%
60%
40%
20%
0%
proportion of parthenogenetically produced workers versus sexually produced workers
Lab_A
Lab_C
Lab_D
Lab_E
PK death, PQ survive
PK & PQ death
parthenogenetically produced offspring
sexually produced offspring

Figure S-1.2 Respective proportion of parthenogenetically produced offspring. Parthenogenetically and sexually produced offspring in (a) female nymphs and (b) workers.

S-1.3.2 Proportion of offspring castes in colonies with the presence of primary parents, solely absence of primary kings, and both absence

In colonies with the presence of primary parents, the offspring almost always consisted of a few soldiers (1.4%-3.7%) and many workers (Figure S-1.3a). Colonies with solely the absence of primary kings would have produced many female nymphs. Colonies with the absence of both primary parents and the presence of secondary queens would have produced many female nymphs and nymphoid queens (Figures S-1.3b and S-1.3c)


(a)
soldier
worker
167
204
161
143
169
147
119
183
160
163
100%
80%
60%
40%
20%
0%
proportion of offspring caste
Lab_F
Lab_G
Lab_H
Lab_I
Lab_J
Lab_K
Lab_L
Lab_M
Lab_N
Lab_O
PK & PQ survived colonies


(b)
soldier
worker
nymph
nymphoid
91
146
268
38
25
32
100%
80%
60%
40%
20%
0%
proportion of offspring caste
Lab_P
Lab_Q
Lab_R
Lab_S
Lab_T
Lab_A
PK death, PQ survived colonies

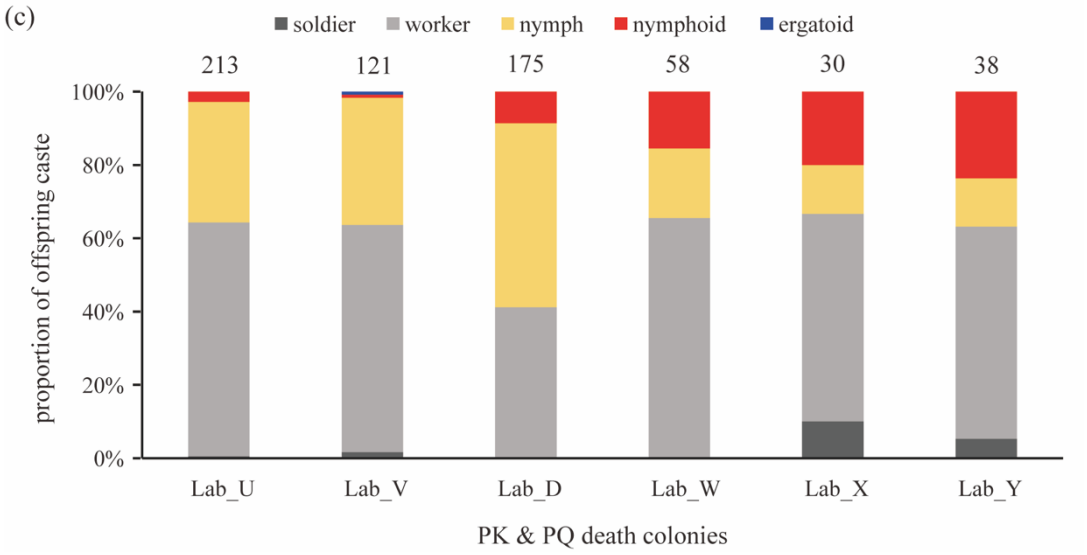

(c)
soldier
worker
nymph
nymphoid
ergatoid
213
121
175
58
30
38
100%
80%
60%
40%
20%
0%
proportion of offspring caste
Lab_U
Lab_V
Lab_D
Lab_W
Lab_X
Lab_Y
PK & PQ death colonies

Figure S-1.3 Respective proportions of offspring of different in colonies with the presence of primary parents, solely absence of primary kings, and absence of primary parents. PK: primary king, PQ: primary queen, SQ: secondary queen. (a) Both primary kings and queens survived colonies and only produced workers and soldiers in the early stage. (b) and (c) An excess number of female nymphs were present in PK or PK & PQ death colonies. The bars with different colors exhibit respective proportions of different castes of offspring per colony.

S-1.4 Discussion

In this study, the presence of primary kings is found to suppress abnormal asexual offspring production in colonies. This is reminiscent of other social insects such as ants, bees, and wasps, where the queen produces alternate reproductive castes through asexual reproduction while utilizing sexual reproduction for non-reproductive castes engaged in tasks such as production and defense (Guo et al. 2013; Lillico-Ouachour and Abouheif 2017). This reproductive strategy eliminates inferior genes for subsequent colony formation and ensures that the current colony exhibits exceptional resistance to risk. A similar phenomenon was observed in our experiment, with PK-controlled colonies generating homozygous SQ or nymphs as asexual reproductive offspring and heterozygous workers as sexual reproductive offspring (Figure S-1.1, Figure S-1.2a).

Intriguingly, upon the death of PK and the subsequent control of the cultivated two-year colonies by PQ alone, the worker population shifted from being entirely heterozygous to almost exclusively pure conspecifics (Figure S-1.2b). This observation suggests that PQ may favor producing all offspring via asexual reproduction. It is worth noting that, based on the genomic imprinting model, the offspring of asexual reproduction should be more inclined to develop into SQ rather than workers (Matsuura

et al. 2018). We speculate that environmental pressures within the nest cause the differentiation of offspring from asexual reproduction into workers. Specifically, an excessive number of SQ and nymphs within the colony disrupts the supply balance, leading to some of the asexually reproduced offspring having to differentiate into workers. However, relying solely on asexual reproduction would increase the genetic contribution of the queen in the offspring while reducing the genetic diversity in non-reproductive castes, ultimately resulting in a higher risk of apoptosis. Consequently, the suppression of PQ's production of asexual workers by PK is crucial for proper colony development. This finding may shed light on why, in termites, the king persists and cooperates with the queen long after the establishment of the colony (Matsuura et al. 2009). By suppressing asexual reproduction, PK ensures proper colony development and preserves genetic diversity, promoting colony stability and enhancing resistance to risk.

In termite colonies, PKs seem to play a unique role as tumor suppressor genes in superorganisms. It regulates the birth mode of workers to ensure proper colony development and controls the Proportion of progeny caste composition within the colony. No nymphs are produced in two-year-old colonies where PK and PQ are present (Figure S-1.3a). Following the death of PK, the sperm stored in the spermatheca of the PQ loses its viability after one week (unpublished observations). Once the sperm is depleted, the offspring produced by the PQ are exclusively asexual reproductive offspring, with a significant portion developing into nymphs or SQ. The proportion of nymphs or SQ within the colony increases significantly while the worker population declines considerably. Consequently, the colony's total population decreases gradually or abruptly (Figure S-1.3). The excessive differentiation of nymphs or SQ does not benefit colony development (Korb and Hartfelder 2008). This is because nymphs or SQ

require an appropriate number of workers to ensure their development. The presence of an abnormally large number of nymphs or SQ undoubtedly increases the burden on the colony's workers. When the workers can no longer support the high number of nymphs and SQs, the colony begins to collapse and eventually dies out. This phenomenon explains why mature colonies are frequently found in the field where PKs survive but not where PKs die and PQs remain. In summary, PK acts as a unique tumor suppressor gene in superorganisms, inhibiting the overproduction of asexual non-reproductive castes (like cancer cells) and maintaining a large population of sexual workers (analogous to somatic cells). This ensures the colony's smooth development and protection from decay.

S-1.5 References

1. Bernadou, A., B. H. Kramer, and J. Korb. 2021. Major evolutionary transitions in social insects, the importance of worker sterility and life history trade-offs. Frontiers in Ecology and Evolution 9:732907.
2. Boomsma, J. J., and R. Gawne. 2018. Superorganismality and caste differentiation as points of no return: how the major evolutionary transitions were lost in translation. Biological Reviews of the Cambridge Philosophical Society 93:28–54.
3. Dronnet, S., A. G. Bagnères, T. R. Juba, and E. L. Vargo. 2004. Polymorphic microsatellite loci in the European subterranean termite, *Reticulitermes santonensis* Feytaud. Molecular Ecology Notes 4:127–129.
4. Guo, X., S. Su, G. Skogerboe, S. Dai, W. Li, Z. Li, F. Liu, et al. 2013. Recipe for a busy bee: microRNAs in honey bee caste determination. PloS One 8:e81661.
5. Kennedy, P., G. Baron, B. Qiu, D. Freitak, H. Helanterä, E. R. Hunt, F. Manfredini, et al. 2017. Deconstructing superorganisms and societies to address big questions in biology. Trends in Ecology & Evolution 32:861–872.
6. Kobayashi, K., E. Hasegawa, Y. Yamamoto, K. Kawatsu, E. L. Vargo, J. Yoshimura,

and K. Matsuura. 2013. Sex ratio biases in termites provide evidence for kin selection. Nature Communications 4:2048.

7. Korb, J., and K. Hartfelder. 2008. Life history and development - a framework for understanding developmental plasticity in lower termites. Biological Reviews of the Cambridge Philosophical Society 83:295–313.
8. Kramer, B. H., G. S. van Doorn, B. M. S. Arani, and I. Pen. 2022. Eusociality and the evolution of aging in superorganisms. The American Naturalist 200:63–80.
9. Lillico-Ouachour, A., and E. Abouheif. 2017. Regulation, development, and evolution of caste ratios in the hyperdiverse ant genus *Pheidole*. Current Opinion in Insect Science 19:43–51.
10. Matsuura, K. 2017. Evolution of the asexual queen succession system and its underlying mechanisms in termites. The Journal of Experimental Biology 220:63–72.
11. Matsuura, K. 2020. Genomic imprinting and evolution of insect societies. Population Ecology 62:38–52.
12. Matsuura, K., N. Mizumoto, K. Kobayashi, T. Nozaki, T. Fujita, T. Yashiro, T. Fuchikawa, et al. 2018. A genomic imprinting model of termite caste determination: not genetic but epigenetic inheritance influences offspring caste fate. The American Naturalist 191:677–690.
13. Matsuura, K., and T. Nishida. 2001. Comparison of colony foundation success between sexual pairs and female asexual units in the termite *Reticulitermes speratus* (Isoptera: Rhinotermitidae). Population Ecology 43:119–124.
14. Matsuura, K., E. L. Vargo, K. Kawatsu, P. E. Labadie, H. Nakano, T. Yashiro, and K. Tsuji. 2009. Queen succession through asexual reproduction in termites. Science 323:1687.
15. Mitaka, Y., T. Akino, and K. Matsuura. 2023. Development of a standard medium for culturing the termite *Reticulitermes speratus*. Insectes Sociaux 70:265–274.
16. Miura, T., K. Oguchi, H. Yamaguchi, M. Nakamura, D. Sato, K. Kobayashi, N. Kutsukake, et al. 2022. Understanding of superorganisms: collective behavior, differentiation and social organization. Artificial Life and Robotics 27:204–212.

17. Park, I.-K., and S.-C. Shin. 2005. Fumigant activity of plant essential oils and components from garlic (*Allium sativum*) and clove bud (*Eugenia caryophyllata*) oils against the Japanese termite (*Reticulitermes speratus* Kolbe). Journal of Agricultural and Food Chemistry 53:4388–4392.
18. Vargo, E. L. 2000. Polymorphism at trinucleotide microsatellite loci in the subterranean termite *Reticulitermes flavipes*. Molecular Ecology 9:817–820.
19. Walsh, P. S., D. A. Metzger, and R. Higuchi. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques 10:506–513.

Appendix 2

Identifying the starting timepoint of nymph-producing in *Reticulitermes* termite colonies

S-2.1 Introduction

Despite natural selection predicting that organisms should act selfishly to avoid reducing their fitness by expending time and energy helping others, altruism continues to be prevalent, as individuals helping closely related recipients can enhance the average fitness of a population through kin selection (Foster et al. 2006; Kay et al. 2019). In social insects, altruism is characterized by non-reproductive individuals, often workers or soldiers, who relinquish their reproductive potential to aid their reproductive members (reproductives and nymphs), thereby supporting the survival and reproductive success of their kin (Hamilton 1964). In the colony foundation stage, male founders contribute to reproductive investment through direct nutrient transfer (trophallaxis) and labor (Shellman-Reeve 1990). For example, in *Reticulitermes* termite incipient colonies, a heavier primary king leads the queen to gain more weight (Matsuura and Nishida 2001) for efficient reproduction. Termite colonies always only produce workers in the earlier stage because nymphs aren't responding to maintaining the resource-limited young colonies but consuming resources. Therefore, we propose a novel hypothesis for the timing point of outset in nymph production in *Reticulitermes* termite colonies: a king's altruism leads to a sufficient worker population by promoting the queen's fecundity, ultimately triggering nymph production. To test the hypothesis, we further set and forest *R. speratus* colonies (monogamous pair) and *R. amamianus* - *R. speratus*

hybrid colonies (monogamous pair) annually to search for the timing point of nymph production. Then, each caste's population size and weights (and weights changing) were compared between colonies that would house nymphs and those that wouldn't.

Previous studies never report the timing point of outset in caste bifurcation by empirical data in *Reticulitermes* termite male-female colonies. Alternatively, the genetic caste determination model predicts ergatoid neotenic reproductives (worker-derived) are necessary for triggering caste bifurcation (Hayashi et al. 2007). Furthermore, the genomic imprinting caste determination model theory indicates that the genomic imprinting level gap can trigger the caste bifurcation (Matsuura *et al.*, 2018) The *Reticulitermes* termite exemplifies one of the most successful models of eusociality: most offspring of both sexes are functionally sterile and follow the wingless worker pathway (Light and Weesner 1955; Howard and Haverty 1980). This contrasts with a smaller fraction of offspring that become reproductive nymphs, characterized by wing buds, which eventually develop into alates (Roisin 2000). These two classes - worker and nymph - have the potential to transform into neotenic reproductives, ergatoid and nymphoid, respectively (Haverty and Howard 1981; Matsuura and Nishida 2001; Roisin 2001; Matsuura et al. 2002, 2004). In *R. speratus*, queens are known to produce neotenic queens via thelytokous parthenogenesis, driven by terminal fusion (Matsuura 2017; Matsuura et al. 2018). We here use the two species for the following reasons: 1) *R. speratus* are likely easier to produce nymphs because the female-female pairs (failed to survive in the field) can produce asexual nymphs in every earlier stage (Matsuura et al. 2018; Tamaki et al. 2021), 2) *R. amamianus* incipient colonies grow very fast as our

long period observation, thereby hybrid colonies may inherit *R. speratus*' trait of nymph producing and *R. amamianus*' trait of colony growth.

We developed a novel foresting model by mimicking natural nutrient conditions to grow the incipient colonies. Young colonies in the field always have sufficient food supplements when monogamous paired alate dig into the log because the volume of the log is much larger than the volume of the young nest. Therefore, we keep furnishing food in colonies when food in colonies is consumed by 1/3. By establishing and observing those colonies, we conducted thorough investigations into the caste of offspring produced at various time points to pinpoint the timing of the commencement of nymph-production, nymph production, and a mathematical relationship linking the existence of nymphs with other cast members. This included factors such as parent phenotypes, the number of workers, larvae, and eggs. Through further exploration into the sex and genotypes of nymphs, we elucidated the patterns of sex allocation in the nymph caste on a colony level.

S-2.2 Materials and methods

S-2.2.1 Foundation of colonies

The termite colonies were collected several weeks before the swarming season, focusing on colonies that displayed nests consisting of alates within the wood. Following collection, these colonies were housed in laboratory-grade plastic containers, each measuring 25 cm × 35 cm × 20 cm. The temperature within the laboratory was consistently maintained at 20°C. The termite colonies were subjected to this controlled

environment for a minimum of five days post-collection to synchronize the off-light timing and achieve uniform observation conditions.

Our study collected distinct colonies of *R. speratus* (Rs) and *R. amamianus* (Ra) from different locations in Japan. We obtained three Rs colonies (RsA, RsB, RsC) from Yakushima Island and Kyoto in May 2022. All alates were extracted from the logs, had their wings removed, and were sexed by sternite morphology. Then, alates from these colonies were randomly selected and assigned to one of the following crosses: mRsA × fRsA, mRsB × fRsB, mRsC × fRsC, mRsA × fRsB, mRsB × fRsA, RsA × fRsC, mRsC × fRsA, mRsB × fRsC, and mRsC × fRsB. In addition, we gathered two Ra colonies (RaA, RaB) from Amami-Oshima-Island in March 2020 and multiple Rs colonies (RsD, RsE, RsF, RsF) from varied mountains in Kyoto from March to May 2020. Then alates from these colonies were randomly selected and assigned to one of the following crosses: mRsD × fRsE, mRsE × fRsD, mRsD × fRaA, mRsD × fRaB, mRsE × fRaA, mRsE × fRaB, mRaA × fRsD, mRaA × fRsE, mRaB × fRsD, mRaB × fRsE, mRaC × fRaD and mRaD× fRaC, mRsF × fRsF, mRsG × fRsG, mRsF × fRsG, and mRsG × fRsF. three Rs colonies (RsH, RsI, RsJ) from Shiga and Kawanishi in April and May 2018. Then alates from these colonies were randomly selected and assigned to one of the following crosses: mRsK × fRsL, mRsL × fRsK, mRsK × fRsN, mRsN. × fRsK, mRsN × fRsL, mRsL × fRsN, mRsM × fRsL. We obtained four Rs colonies (RsK, RsL, RsM, and RsN) from Shiga and Kyoto in April and May 2017. Then alates from these colonies were randomly selected and assigned to one of the following crosses: mRsL × fRsM; and mRsA × fRsA, mRsB × fRsB, mRsC × fRsC, mRsA × fRsB, mRsB × fRsA,

RsA × fRsC, mRsC × fRsA, mRsB × fRsC, and mRsC × fRsB. Before experimentation, colonies were transferred to a 25°C room with 80% humidity and lit conditions, inducing the emergence of alates for swarming. After landing, alates were sex-separated and temporarily housed in plastic containers for four hours.

All pairings were placed into 25 mm petri dishes containing sawdust-bait cookies for six months before being transferred to large transparent plastic cases (100 × 65 × 28 mm) filled with a mix of sawdust bait and soil blocks (36 × 36 × 14 mm). The sawdust bait was a mixture of brown rotten pine wood powder and cellulose powder at a weight ratio 9:1. To ensure a constant food source, we replenished the sawdust bait whenever a third was consumed.

The experimental process was conducted over two years. Initially, colonies were housed in compact environments, and after the stipulated period, we transitioned the colonies to expansive, transparent plastic enclosures. The measurements of these new accommodations were 194 mm in length, 104 mm in width, and 26 mm in depth. Each enclosure was prepared with two blocks of soil and a pair of pine wood chips, each with dimensions 45 × 45 × 10 mm. This setup was designed to provide an environment closely mimicking the colonies' natural habitat, promoting their growth and behavioral expression. This method ensured an effective and standardized establishment of each colony, leading to reliable and replicable outcomes. For a comprehensive record of the colony positions within the experimental setting, please refer to Dataset S-2.1.

We conducted our dissections of the colonies with the utmost precision, utilizing dissecting needles, medicine spoons, and brush pens each year in the spring and fall

seasons. We meticulously counted the population of each caste within the colonies (workers, nymphs, soldiers, larvae, eggs, and neotenic reproductives). We also weighed individuals from different castes: workers, soldiers (distinguishing between males and females), and reproductives. For sex separation, we employed the method proposed by Takata (Takata et al. 2020).

S-2.2.2 Microsatellite analysis

Our study leverages microsatellite analysis to determine whether the sample nymphs and workers exhibit parthenogenesis. We collected DNA from eight workers and offspring nymphs per colony and from termites used for incipient colonies, details in Dataset S-2.S3. The analysis included four loci: *Rs15* (Dronnet et al. 2004), *Rf24-2*, *Rf21-1*, and *Rf6-1*(Vargo 2000). Termite DNA was extracted using a modified Chelex extraction (Walsh et al. 1991). We extracted all DNA from the heads or antennas using 50 µL Chelex® solution (10% weight per volume; TE pH 8.0) and 0.5 µL proteinase K. After 3 hours incubation at 55 °C, samples were then heated at 95 °C for 15 min. Polymerase chain reaction (PCR) amplifications were performed. Primer *Rf6-1* was labeled by 6-FAM fluorescent tags, *Rf21-1* by VIC fluorescent tags, *Rf24-2* by NED fluorescent tags, and *Rs15* by PET fluorescent tags. We use a 10 µL PCR cocktail containing 1 µL of the DNA sample, 0.20 µL of 10 mM dNTP, 0.99 µL of 10 × PCR Buffer, 0.07 µL of 5 U/µL Taq DNA polymerase (New England Biolabs, Inc., Beverly, MA, USA), 1.15 µL of 5 µM multiplex primers and 6.59 µL of DW. Amplification consisted of initial denaturation at 95 °C for 3 min, then followed by 35 cycles

consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 75 s, and an extension at 72 °C for 120s. The PCR products were mixed with 10 μL of Hi-Di formamide and 0.5 μL of GS-600 (LIZ) size standard. Sample detection was performed using an Applied BioSystems 3500 Genetic Analyzer. Raw data were analyzed using GeneMapper 5.0 software (Applied Biosystems, Inc., Foster City, CA, USA).

S-2.2.4 Data analysis

To investigate the relationship between the number of nymphs and workers or primary kings' body weights half a year prior to nymph production, we utilized general linear models (GLMs); between the increased number of nymphs and PK body weight changing (primary kings' body weights at 2.5 years minus 2.0 years), we utilized GLMs in *R. speratus* colonies.

A comparison of the number of workers among the four combinations of parent species was performed using generalized linear models (GLMMs) with Poisson distribution. The model's objective variable was the number of workers, the explanatory variable was the combination of parent species, and colony ID was treated as a random factor. We utilized GLMs to investigate the relationship between the changing of primary kings' body weights (weights at the age of 2.5 years minus 2.0 years) and the number of workers or egg changings (number of workers or eggs at the age of 2.5 years minus 2.0 years). We use Tukey's HSD tests to compare the changes in primary kings' body weights among the four combinations of parent species.

In addition, we conducted weekly inspections of the colonies to verify the presence

or absence of alates and neotenic reproductives. These checks were performed externally, negating the need for additional colony dissections. Statistical analyses were conducted using IBM® SPSS® Statistics software (version 29.0). 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.

S-2.3 Results

S-2.3.1 Colonies began to produce nymph at the age of 2-2.5 years old

We noted varying presence of male and female nymphs by dissection of *R. speratus* colonies at the ages of 0.5, 2, 2.5, 4.5, and 5.5 years. In 108 sampled colonies at 0.5 years, we found two female nymphs in one colony. In 51 sampled colonies at two years, we discovered one male nymph in one colony. In 99 sampled colonies at 2.5 years, 66 female and five male nymphs were present in 45 colonies. For colonies aged 4.5 years, 15 out of 30 sampled colonies contained 15 female nymphs. Lastly, in 7 out of 9 colonies at 5.5 years, we found 18 female nymphs/nymphoids. All female nymphs were identified as short-winged, and alates were absent from these colonies (as depicted in Figure S-2.1a).

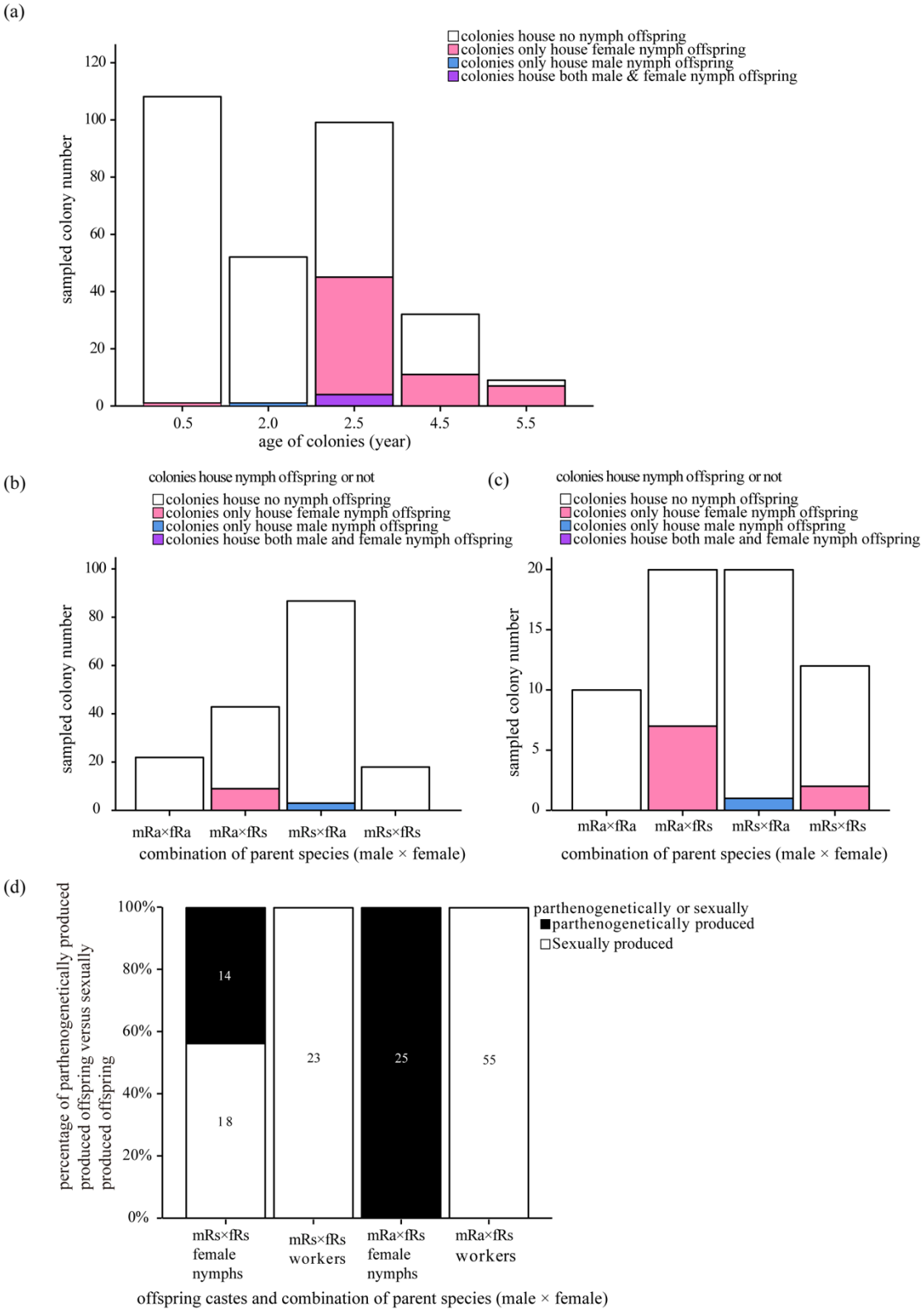


Figure S-2.1 Respective numbers of colonies with or without nymph at different ages, and parental species combination of colonies, and respective proportion of

parthenogenetically produced offspring. (a) Nymphs were present in colonies of various ages. (b) and (c) The number of colonies housed with nymph offspring among four parent combination colonies. (d) The proportions of parthenogenetically produced offspring in mRa × fRs and mRs × fRs colonies (i.e., mRa × fRs means the primary king is *R. amamianus*, the primary queen is *R. speratus*).

We also found only one nymphoid queen in 32 sampled colonies at 45 years, despite seeing 18 female nymphs in 16 colonies at 2.5 years. In colonies aged 5.5 years, nymphoid secondary queens were observed in 4 out of 9 sampled colonies.

We found three male nymphs in 3 out of 88 mRs × fRa colonies and 17 female nymphs in 9 out of 43 mRa × fRs colonies. Both mRs × fRs and mRa × fRa colonies, aged two years, lacked nymphs. At the same age, one male nymph was found in 1 out of 20 mRs × fRa colonies, 20 female nymphs were found in 5 out of 20 mRa × fRs colonies, and three female nymphs were found in 2 out of 12 mRs × fRs colonies. No nymphs were observed in the mRa × fRa colonies (as illustrated in Figures S-2.2b and 2.2c). Additionally, monthly observations from the colonies' exterior did not indicate any occurrences of alates.

S-2.3.2 Investigation on the populational size and weights or weights changings in different castes of individuals before and after the presence of nymph offspring

Upon investigating two sets of mRs × fRs colonies, with ages of 2 years and 2.5 years, respectively, our results indicate that the colonies aged at 2.5 years housed more nymphs as more workers found in colonies at the age of 2 years (GLM, LRT: $\chi 2 = 17.27$, $df = 1$, $p < 0.001$, Figure S-2.2b), heavier primary kings' body weights in colonies at

the age of 2 years (GLM, LRT: $\chi 2 = 14.61$, $df = 1$, $p < 0.001$, Figure S-2.2c). The number of nymphs produced by the colonies over the half year decreased with the increase of the primary kings' body weight (GLM, LRT: $\chi 2 = 4.159$, $df = 1$, $p = 0.041$, Figure S-2.2a).

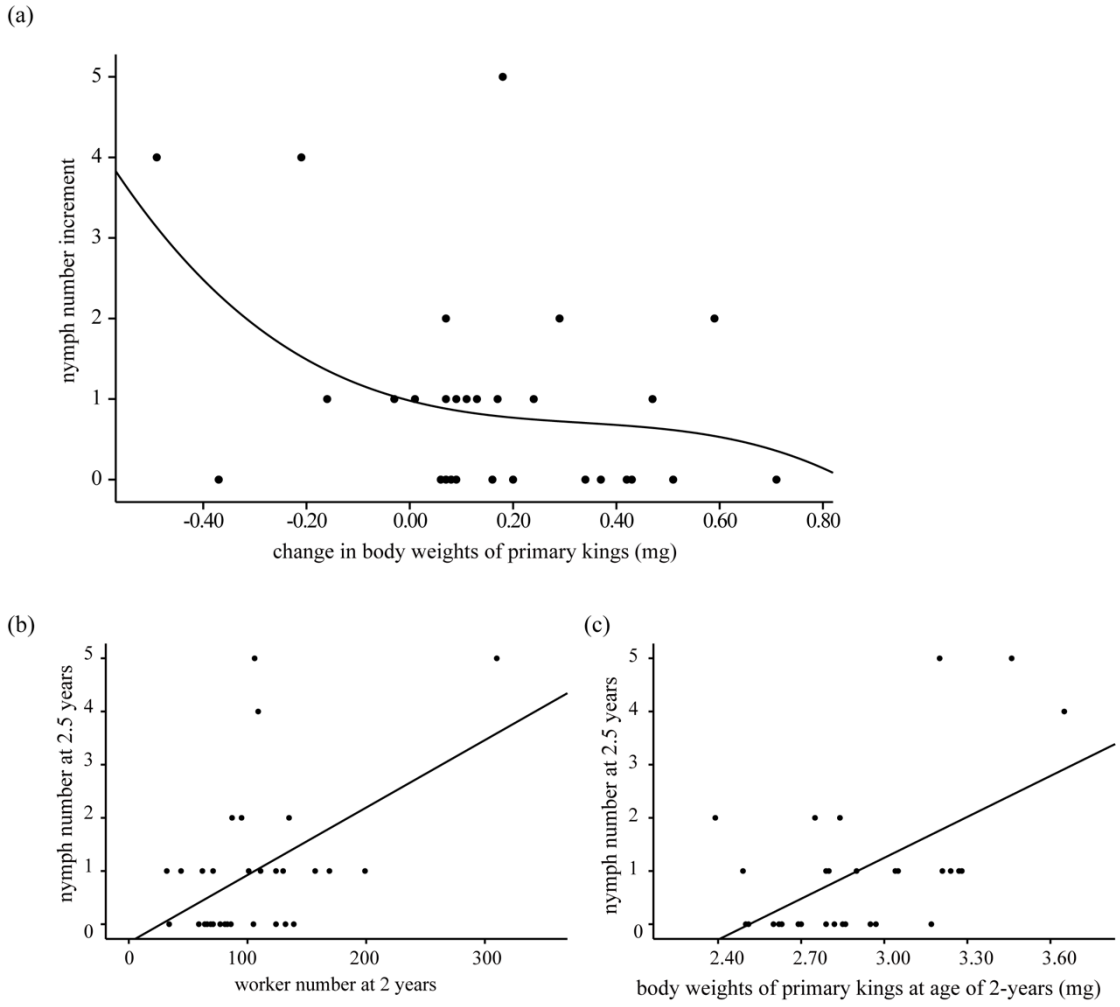


Figure S-2.2 Respective relationships between the yield of the nymph and the primary kings' body weights or worker population. (a) Relationship between changing primary kings' body weights and the number of nymphs housed by *R. speratus* colonies. The change in primary kings' body weights means primary kings' body weights are at the age of 2.5 years minus their weights at the age of 2 years. This method also describes

the changing nymph numbers. (b) Respective relationship between the number of nymphs housed by colonies aged at 2.5 years and their worker number aged at 2 years, and (c) their primary kings' body weights aged at 2 years.

Upon investigating colonies at the ages of 2 years and 2.5 years, respectively, primary kings' body weights changing in mRa × fRs colonies are significantly lower than the other three mating pairs (Tukey's HSD tests, $p < 0.05$, Figure S-2.3a). Furthermore, primary kings' changing body weights is also negatively associated with both a changing larva number (GLM, LRT: $\chi2 = 14.744$, $df = 1$, $p < 0.001$, Figure S-2.3b) and a changing egg number (GLM, LRT: $\chi2 = 4.846$, $df = 1$, $p = 0.028$, Figure S-2.3c).

The species of the king had a significant effect on the number of workers in 2-year-old colonies (Tukey's HSD tests, $p < 0.05$, Figure S-2.3d), while the species of the queen had no significant effect. There was no significant interaction between the species of king and queen (GLMM, $\chi2 = 0.032$, $df = 1$, $p = 0.859$).

In 2.5-year-old colonies, there was a significant interaction between the species of king and queen (GLMM, LRT: $\chi2 = 6.031$, $df = 1$, $p = 0.141$, Figure S-2.3e). The species of the king had a significant effect on the number of workers irrespective of the species of the queen (Tukey's HSD tests, $p < 0.05$). In colonies where the king is *R. amamianus*, the species of the queen had a significant effect on the worker number (Tukey's HSD tests, $p < 0.05$), while in colonies where the king is *R. speratus*, the effect was not significant.

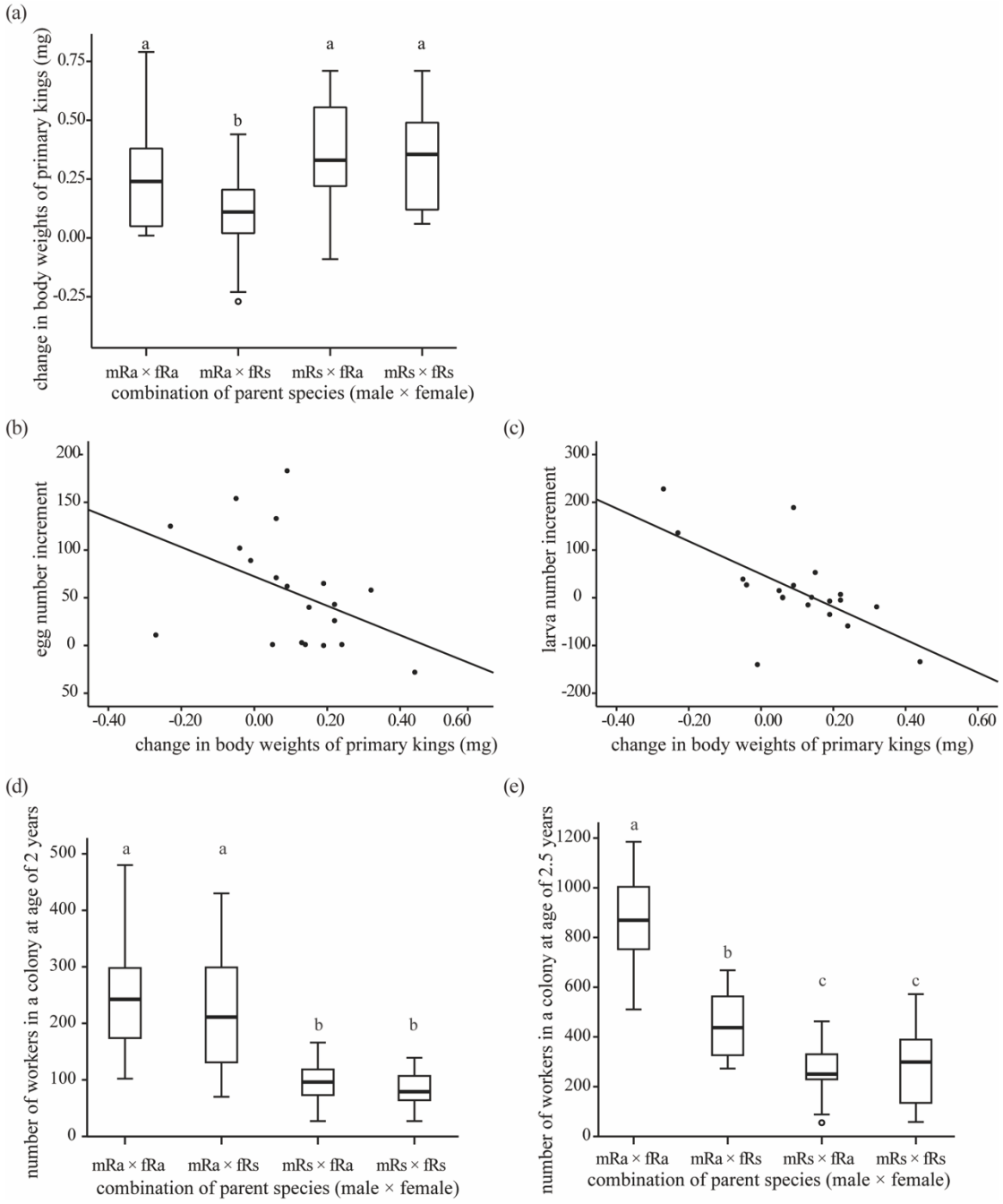


Figure S-2.3 Comparison of the worker number and primary kings' body weights among four combinations of parent species and correlations between primary kings' body weights changing with the larva and egg number changing. (a) Comparison of primary king weight changing among four combinations of parent species colonies. Plots indicate the primary kings' weights changing in each colony (Tukey's HSD tests, $p < 0.05$). The change in kings' body weights means primary kings' body weights are

at the age of 2.5 years, minus their weights at the age of 2 years. The correlations between the change in primary kings' body weights with the changes in number of (b) larva and (c) eggs. (d) and (e) Comparison of the number of workers in 2- and 2.5-year-old colonies among four combinations of parent species, respectively. Plots indicate the number of workers in each colony (Tukey's HSD tests, $p < 0.05$). The combination of parent species (male × female) shows the species of primary king and queen (i.e., mRa × fRs means the primary king is *R. amamianus*, and the primary queen is *R. speratus*). Different letters indicate significant differences.

S-2.3.3 Investigation in the proportion of parthenogenetically produced offspring

We have identified all potential alleles at the four loci by genotyping a sufficient number of individuals, whether alates or workers, from the colonies RsD, RsE, RsF, RsG, RaA, and RaB. Following this, we randomly collected 17 female nymphs from mRs × fRs colonies, 25 female nymphs, and 55 workers from mRa × fRs colonies, which were subjected to microsatellite analysis. Additionally, 15 female nymphs from mRs × fRs colonies aged 2.5 years were genotyped. In a further step, we randomly selected three colonies that housed female nymphs, including the 15 nymphs, for genotyping primary kings, and the workers.

Results from the microsatellite analysis suggest that 14 out of 32 sampled female nymphs from mRs × fRs colonies and 25 female nymphs from mRa × fRs colonies were homozygous at all four loci, and all alleles originated from maternal rather than paternal parents. Additionally, 23 workers from the Rs colonies and 55 workers from mRa × fRs colonies were found to be heterozygous (Figure S-2.1d).

S-2.4 Discussion

Understanding the mechanisms that control caste determination is a pivotal issue in the study of social insect biology. Experiments in our laboratory involving the establishment of colonies indicate that the genesis of offspring caste bifurcation for *R. speratus* begins at a colony age of 2 to 2.5 years in laboratory conditions, and mRa × fRs colonies produced nymphs earlier than two years. Notably, the nymphs are predominantly female (Figure S-2.1a). Besides, the nymphs from *R. speratus* colonies, including 43.75% parthenogenesis and 56.25 % sexual ones, but from mRa × fRs, are all parthenogenesis. *R. speratus* colonies housing more workers and heavier kings at the age of 2 years before producing nymphs and housing more nymphs as slow-weight growing kings indicated that producing nymphs based on colony size and cost resource of kings (Figure S-2.3). Therefore, kings' loss is essential to trigger caste bifurcation. That the King's loss correlates with the queen's potential benefit, which is supported by significance in mRa × fRs colonies because reproductive cast offspring are only parthenogenesis indicates the potential kin conflict because the queen can transfer all maternal genomes to reproductive caste offspring by asexual reproduction. Still, the king's genome is only transferred to helper castes.

Here, we suggest that the inherited components, especially heritable epigenetic factors, are intrinsic to causing that process (Takata et al. 2023). Previous research broadly accepted environmental factors as a critical influence on caste determination in social insects (Nijhout 2003; Miura 2019). However, all colonies in our experiment were exposed to the same laboratory conditions. Consequently, offspring's intrinsic factors, including heritable components, must inherently determine nymph production.

Given that all offspring within a termite colony share the same genetic background, and sexual nymphs can be produced without the presence of neotenic reproductives, it can be inferred that genetic factors based on variations in DNA sequence are not the driving force behind caste determination. Furthermore, our data implies that the initiation of nymph production correlates with robust worker populations and heavier colony members, suggesting a link to colony growth. Therefore, we propose that non-genetic factors linked to transgenerational transmission and colony growth are the most plausible explanations for caste determination, in accordance with both prior and current findings. Interestingly, our data does not support that the onset of parthenogenetic daughter production can explain caste fate in female offspring. Even though parthenogenetic daughters tend to develop into fertile progeny in termite species with asexual queen succession (AQS) and even in non-AQS species (Hayashi et al. 2007; Matsuura et al. 2018; Nozaki et al. 2018), our genotyping results indicate that female nymphs produced by 2.5-year-old *R. speratus* colonies can be both sexually (56.25%) and asexually (43.75%) reproduced (Figure S-2.2a).

A genomic imprinting model (GICD) proposed by Matsuura et al. 2018, wherein queen- and king-specific epigenetic marks antagonistically influence the sexual development of offspring, aligns well with our understanding of caste determination (Matsuura et al. 2018). These sex-specific epigenetic marks from parents can affect the offspring's phenotype through incomplete erasure of the marks in the germ line (Rice et al. 2016). The GICD model assumes that the expression of sex and growth regulatory genes in offspring is influenced by heritable yet unerased epigenetic marks.

Accumulation of these epi-marks relates to age, environment, and phenotype (Calvanese et al. 2009; Flatscher et al. 2012; Cencioni et al. 2013; Ecker et al. 2018; Yu et al. 2020). Our results suggest that a pronounced female bias in the sex of nymphs aligns with an increasing weight gap between parents. Also, though the primary queen and primary king are the same age, the primary queen is relatively older than the primary king due to the primary king's significantly longer lifespan (Matsuura 2017). Hence, the primary queen will likely accumulate more genomic imprinting strength than the primary king, leading to a female bias in nymphs.

This study provides the first empirical evidence for the onset of life cycle bifurcation in *Reticulitermes* termite colonies and identifies colony growth-linked heritable factors in offspring. It also introduces a novel methodology for cultivating *Reticulitermes* colonies, leading to a larger colony size than previous studies. This research offers insights into caste determination in social insects and enhances our understanding of colony age and developmental processes. Investigations into the molecular-level aspects of these growth-linked heritable factors are now underway. These studies promise to elucidate further the mechanisms underpinning labor division, life-history traits, and phenotypic plasticity in social insects.

S-2.5 Supplementary materials

S-2.5.1 Supplementary materials and methods and results

Upon investigating primary kings and primary queens body weights by age, the weights constantly increase by age (GLMM, LRT: primary king, $\chi 2 = 66.907$, $df = 1$, $p < 0.001$;

primary queen, $\chi 2 = 490.063$, $df = 1$, $p < 0.001$, Figure S-2.S1).

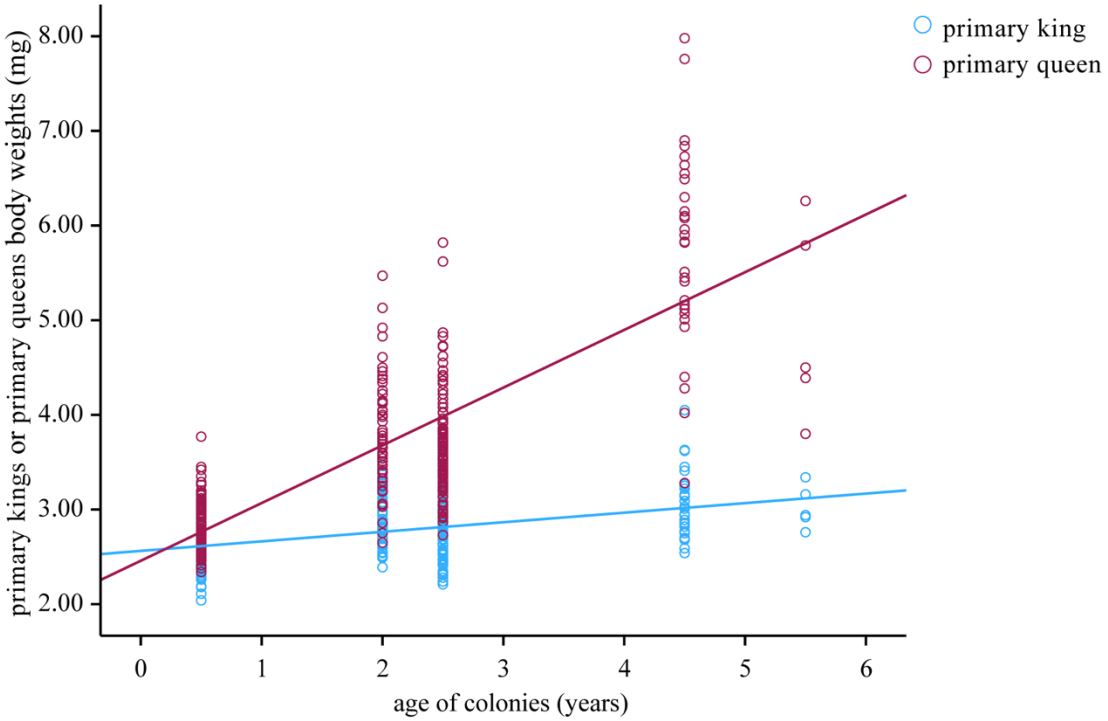


Figure S-2. S1 Dynamics of primary kings' body weights and primary queens' body weights by age. Respective relationship between colony age and primary queens' or kings' body weights. The red circle means the body weights of primary queens, and the blue circle means the weights of primary kings.

S-2.6 References

1. Calvanese, V., E. Lara, A. Kahn, and M. F. Fraga. 2009. The role of epigenetics in aging and age-related diseases. Ageing Research Reviews 8:268–276.
2. Cencioni, C., F. Spallotta, F. Martelli, S. Valente, A. Mai, A. M. Zeiher, and C. Gaetano. 2013. Oxidative stress and epigenetic regulation in ageing and age-related diseases. International Journal of Molecular Sciences 14:17643–17663.
3. Dronnet, S., A. G. Bagnères, T. R. Juba, and E. L. Vargo. 2004. Polymorphic

microsatellite loci in the European subterranean termite, *Reticulitermes santonensis* Feytaud. Molecular Ecology Notes 4:127–129.

4. Ecker, S., V. Pancaldi, A. Valencia, S. Beck, and D. S. Paul. 2018. Epigenetic and transcriptional variability shape phenotypic plasticity. Bioessays 40:1700148.
5. Flatscher, R., B. Frajman, P. Schönswetter, and O. Paun. 2012. Environmental heterogeneity and phenotypic divergence: can heritable epigenetic variation aid speciation? Genetics Research International 2012:698421.
6. Foster, K. R., T. Wenseleers, and F. L. W. Ratnieks. 2006. Kin selection is the key to altruism. Trends in Ecology & Evolution 21:57–60.
7. Hamilton, W. D. 1964. The genetical evolution of social behaviour. II. Journal of Theoretical Biology 7:17–52.
8. Haverty, M. I., and R. W. Howard. 1981. Production of soldiers and maintenance of soldier proportions by laboratory experimental groups of *Reticulitermes flavipes* (Kollar) and *Reticulitermes virginicus* (Banks) (Isoptera: Rhinotermitidae). Insectes Sociaux 28:32–39.
9. Hayashi, Y., N. Lo, H. Miyata, and O. Kitade. 2007. Sex-linked genetic influence on caste determination in a termite. Science 318:985–987.
10. Howard, R. W., and M. I. Haverty. 1980. Reproductives in mature colonies of *Reticulitermes flavipes*: abundance, sex-ratio, and association with soldiers. Environmental Entomology 9:458–460.
11. Kay, T., L. Lehmann, and L. Keller. 2019. Kin selection and altruism. Current Biology 29:R438–R442.
12. Light, S. F., and F. M. Weesner. 1955. The production and replacement of soldiers in incipient colonies of *Reticulitermes Hesperus* Banks. Insectes Sociaux 2:347–354.
13. Matsuura, K. 2017. Evolution of the asexual queen succession system and its underlying mechanisms in termites. The Journal of Experimental Biology 220:63–72.
14. Matsuura, K., M. Fujimoto, and K. Goka. 2004. Sexual and asexual colony foundation and the mechanism of facultative parthenogenesis in the termite

Reticulitermes speratus (Isoptera, Rhinotermitidae). Insectes Sociaux 51:325–332.

15. Matsuura, K., M. Fujimoto, K. Goka, and T. Nishida. 2002. Cooperative colony foundation by termite female pairs: altruism for survivorship in incipient colonies. Animal Behaviour 64:167–173.
16. Matsuura, K., N. Mizumoto, K. Kobayashi, T. Nozaki, T. Fujita, T. Yashiro, T. Fuchikawa, et al. 2018. A genomic imprinting model of termite caste determination: not genetic but epigenetic inheritance influences offspring caste fate. The American Naturalist 191:677–690.
17. Matsuura, K., and T. Nishida. 2001. Comparison of colony foundation success between sexual pairs and female asexual units in the termite *Reticulitermes speratus* (Isoptera: Rhinotermitidae). Population Ecology 43:119–124.
18. Miura, T. 2019. Juvenile hormone as a physiological regulator mediating phenotypic plasticity in pancrustaceans. Development, Growth & Differentiation 61:85–96.
19. Nijhout, H. F. 2003. Development and evolution of adaptive polyphenisms. Evolution & Development 5:9–18.
20. Nozaki, T., T. Yashiro, and K. Matsuura. 2018. Preadaptation for asexual queen succession: queen tychoparthenogenesis produces neotenic queens in the termite *Reticulitermes okinawanus*. Insectes Sociaux 65:225–231.
21. Rice, W. R., U. Friberg, and S. Gavrilets. 2016. Sexually antagonistic epigenetic marks that canalize sexually dimorphic development. Molecular Ecology 25:1812–1822.
22. Roisin, Y. 2000. Diversity and evolution of caste patterns. Pages 95–119 *in* T. Abe, D. E. Bignell, and M. Higashi, eds. Termites: Evolution, Sociality, Symbioses, Ecology. Kluwer Academic Publishers, Dordrecht.
23. Roisin, Y. 2001. Caste sex ratios, sex linkage, and reproductive strategies in termites. Insectes Sociaux 48:224–230.
24. Shellman-Reeve, J. 1990. Dynamics of biparental care in the dampwood termite, *Zootermopsis nevadensis* (Hagen): response to nitrogen availability. Behavioral Ecology and Sociobiology 26:389–397.

25. Takata, M., T. Inagaki, T. Ishibashi, E. Tasaki, and K. Matsuura. 2020. A non-invasive method for sexing first and second instar larvae of termites using external morphology. Insectes Sociaux 67:487–493.
26. Takata, M., S. Nagai, T. Inagaki, Y. Ohkubo, E. Tasaki, and K. Matsuura. 2023. Heritable effects on caste determination and colony-level sex allocation in termites under natural conditions. iScience 26:106207.
27. Tamaki, C., M. Takata, and K. Matsuura. 2021. The lose-to-win strategy of the weak: intraspecific parasitism via egg abduction in a termite. Biology Letters 17:20210540.
28. Vargo, E. L. 2000. Polymorphism at trinucleotide microsatellite loci in the subterranean termite *Reticulitermes flavipes*. Molecular Ecology 9:817–820.
29. Walsh, P. S., D. A. Metzger, and R. Higuchi. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques 10:506–513.
30. Yu, M., W. D. Hazelton, G. E. Luebeck, and W. M. Grady. 2020. Epigenetic aging: more than just a clock when it comes to cancer. Cancer Research 80:367–374.