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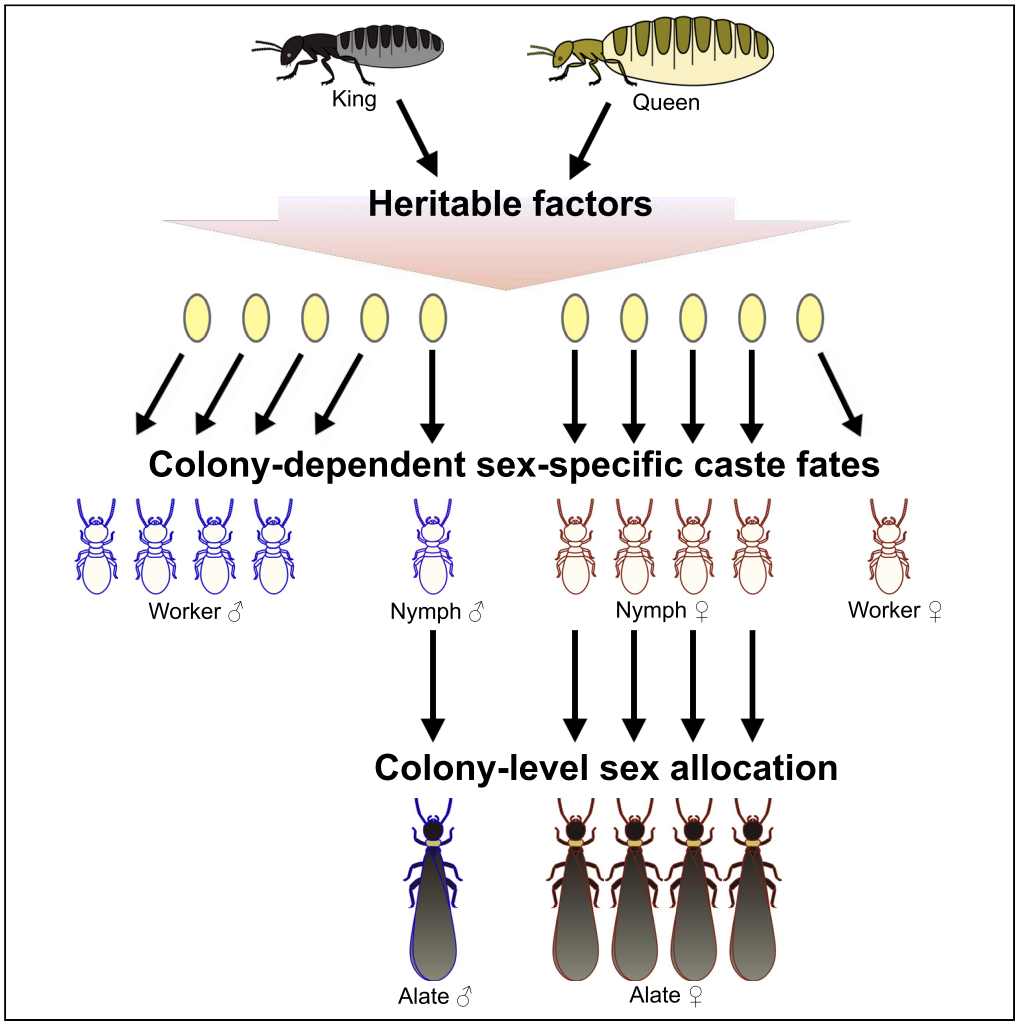

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Highlights

Caste fate is almost entirely determined before oviposition in termite field colonies

The colony-specific caste fates are reflected in the sex ratio of fertile dispersers

Heritable effects play a key role in the colony-level sex allocation

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Heritable effects on caste determination and colony-level sex allocation in termites under field conditions

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SUMMARY

The ecological success of social insects is attributed to the division of labor, where newly hatched offspring differentiate into either fertile progeny or functionally sterile worker castes. There is growing evidence for the heritable (genetic or epigenetic) effects on caste determination based on laboratory experiments. Here, we indirectly demonstrate that heritable factors have the principal role in caste determination and strongly affect colony-level production of both sexes of fertile dispersers (i.e., alates) in field colonies of the termite *Reticulitermes speratus*. An egg-fostering experiment suggests that the colony-dependent sex-specific caste fates were almost entirely determined before oviposition. Our investigation of field colonies revealed that such colony-dependent sex-specific caste fates result in the intercolonial variation in the numerical sex ratio of differentiated fertile offspring and, eventually, that of alates. This study contributes to better understanding the mechanisms underlying the division of labor and life-history traits in social insects.

INTRODUCTION

Reproductive division of labor is fundamental to the ecological and evolutionary success of social insects.[1–4] In their societies, offspring differentiate into either reproductive or functionally sterile castes,[3,5–7] and thus the caste fate for each sex determines the initial number of male and female reproductives. Environmental factors such as the nutrients and pheromones experienced during the larval stage play a principal role in caste determination in many species.[8–14] However, recent studies based on laboratory experiments have also detected heritable parental effects (genetic or epigenetic effects) on caste determination, that is, the genetic or epigenetic variation in the offspring influences their caste fate.[15,16] To elucidate the evolutionary significance of heritable effects on the caste determination system, there is an urgent need for studies investigating whether heritable factors influence caste determination and colony-level traits such as sex allocation into male and female fertile dispersers in field colonies.

Termites are an ideal system for investigating the influence of heritable factors on caste determination and colony-level sex allocation. In contrast to the social Hymenoptera,[17] both males and females develop into nymphs (fertile progenies) or functionally sterile workers in the major termite groups.[3,5–7] Historically, termite caste fate is believed to be determined by the post-hatching environment.[10,12,13] However, in the termite *Reticulitermes speratus* and other related species, laboratory studies have shown that the caste fate is strongly affected by heritable factors related to the developmental origin of their parents.[18,19] In these species, workers are functionally sterile, but they retain the ability to differentiate into reproductives when experimentally isolated from fertile castes. Thus, both nymph- and worker-derived reproductives can be induced under laboratory conditions.[20,21] Crosses between nymph-derived reproductives produce only workers, but worker and nymph offspring are produced when at least one parent is a worker-derived reproductive because nymphs and workers are produced by normal sexual reproduction and share the same genetic background in *R. speratus*,[22] suggesting that a mechanism based on a difference in DNA sequence is not likely to be involved or has negligibly small effects in their caste determination. Instead, all known data can be explained by a mathematical model assuming that the transmission of epigenetic states via the gametes regulates caste fate.[23] To date, there is no information on heritable effects on caste determination in the field. Furthermore, since the caste fate in termites determines the initial number of male and female fertile progenies as mentioned previously, the caste determination process may play an important role in controlling colony-level sex allocation.

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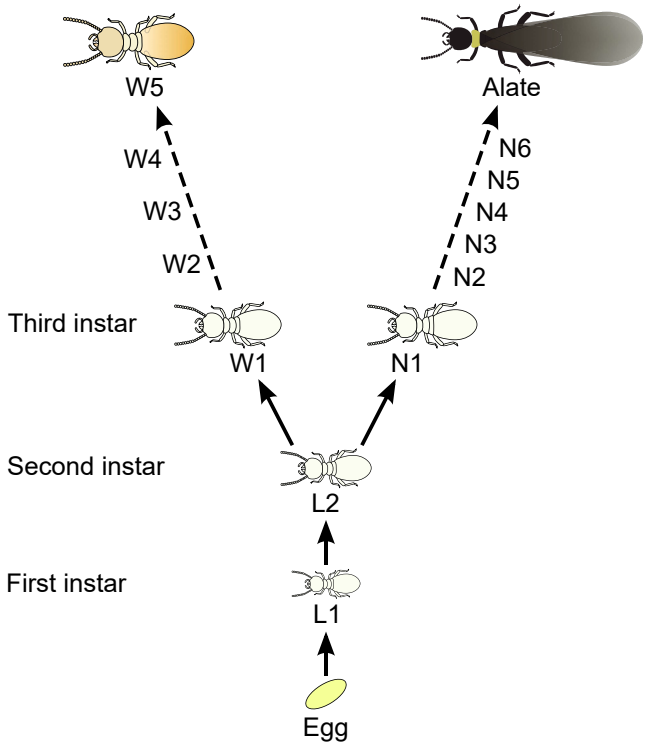

Figure 1. Schematic representation of caste developmental pathways in the termite *Reticulitermes speratus*

Termites are diploid and both sexes follow the same developmental pathways. After the second instar, larvae differentiate into either nymphs or workers. Nymphs develop into reproductives (alates) and disperse to establish new colonies. L1 and L2: first- and second-instar larvae; W1–W5: first- to fifth-instar workers; N1–N6: first- to sixth-instar nymphs. Arrows indicate molts; dotted arrows indicate multiple molts.

Herein, we investigated the influence of heritable and environmental factors on the caste determination and production of both sexes of fertile dispersers in field colonies of the subterranean termite *R. speratus*. In this species, colonies are typically headed by one primary king (PK) and some secondary queens (SQs) which are produced by parthenogenesis and have replaced the primary queen.[22,23] Sexually produced offspring differentiate into either nymph (N1) or worker (W1) at the third instar (Figure 1), while parthenogenetically produced offspring are destined to develop into secondary queens through the nymph stage.[18,22] Sexually produced nymphs develop into fertile dispersers, also known as alates,[22] and there is notable variation in colony-level sex allocation among colonies.[24] To evaluate the influences of heritable and environmental factors on larval caste fate under field conditions, we conducted an egg-fostering experiment and compared caste fate between the larvae developed in their natal colonies and uniform rearing condition. The strict genetic or epigenetic caste determination system predicts that larvae developed in uniform rearing conditions follow a colony-specific caste fate observed in their natal colony. Alternatively, the strict environmental caste determination system predicts that the larvae developed in uniform rearing condition exhibit the same caste fate irrespective of the caste fate observed in their natal colony. To test the possibility of mixing parthenogenetically produced eggs, we conducted genotyping of nymphs and workers of each sex in two representative colonies. Since we found that the heritable factors are the primary determinant of the caste fate in field colonies (Figure 2), we then investigated whether the variations in the caste fate influence alate sex ratio. We focused on three potential factors biasing the numerical sex ratio in alates (Figure 3): (A) sex ratio bias before caste differentiation (i.e., sex ratio of second-instar larvae, L2), (B) the sexual difference in caste fate (i.e., sex ratio of N1), and (C) sexual differences in survival rate during the N1-to-alate developmental period (see Figure 1 for details of developmental stages). To evaluate the impacts of these factors on the alate sex ratio, we investigated the sex ratio at three key developmental stages (L2, N1, and alate) and found that the sexual difference in caste developmental fate is the primary determinant of the alate sex ratio. Finally, since a bias in the numerical sex ratio of alates does not necessarily mean bias in resource allocation at the

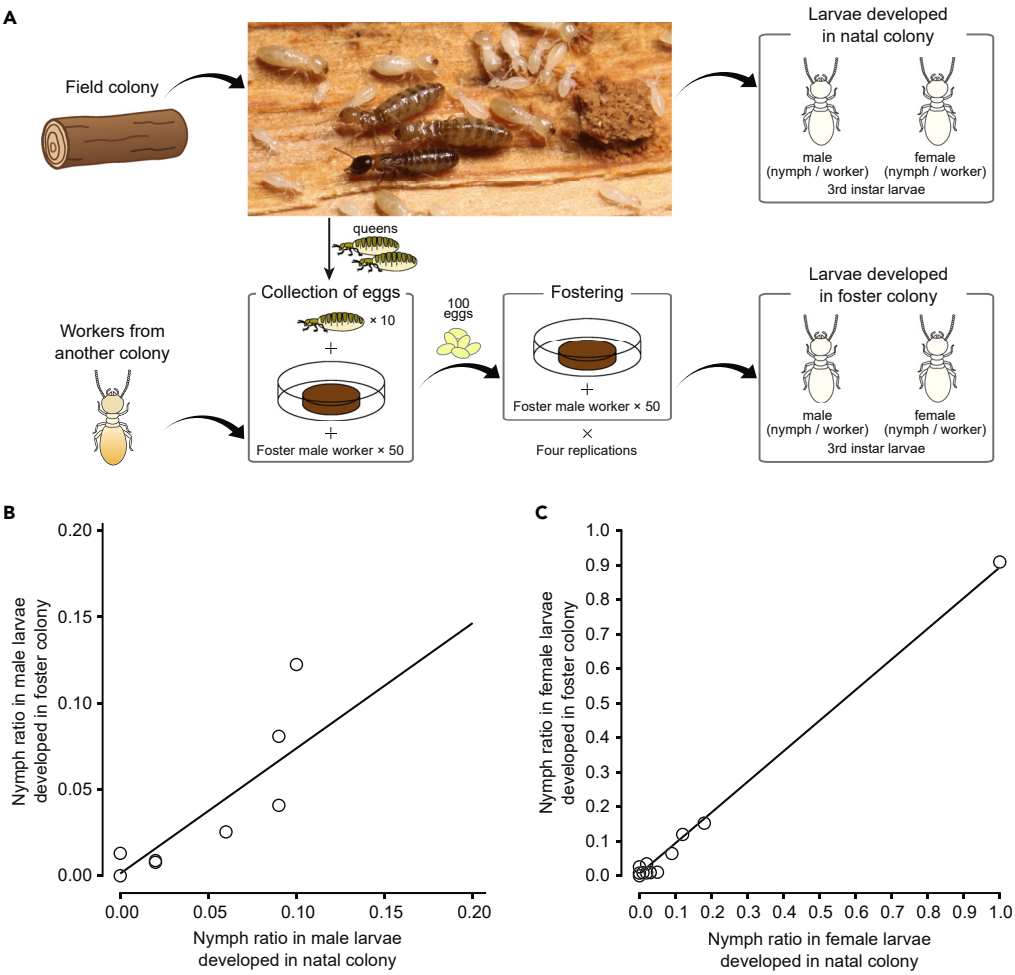

Figure 2. Comparison of caste fate in larvae developed in natal and foster colonies

(A) Schematic diagram of experimental procedure for the collection of larvae developed in natal and foster colonies. The nymph ratios for larvae developed in natal colonies were calculated by investigating 100 randomly selected individuals of third-instar larvae of each sex from each colony (n = 13 colonies). Then, queens were isolated from the colony, and eggs were obtained for the fostering experiment. The eggs were cared for by foster workers until they developed into third-instar larvae. All surviving larvae developed in the foster colonies were extracted and their nymph ratio was calculated for each sex. Correlation between the nymph ratio for larvae developed in natal and foster colonies for (B) males and (C) females.

colony level, we investigated the sexual difference in body weight in alates among field colonies with different alate sex ratios.

RESULTS

Egg-fostering experiment

The egg-fostering experiment revealed that regardless of the offspring being produced via crosses between PKs and SQs and raised in standardized environmental conditions, the fostered larva followed the same colony-specific caste fate as their natal colony (Figure 2). The nymph ratio of larvae developed in the natal colony for both sexes best explained the variation of the nymph ratio of larvae developed in a foster colony (male: *AIC* = 35.36, *BIC* = 36.49, Figure 2B; female: *AIC* = 54.81, *BIC* = 55.94, Figure 2C). The foster workers' colony origin explained the nymph ratio variation slightly better than the null model (male: *AIC* = 87.0, *BIC* = 87.65; null model *AIC* = 88.69, *BIC* = 89.82; female: *AIC* = 348.00, *BIC* = 349.13; null model *AIC* = 493.61, *BIC* = 494.17). Although one colony had an extremely high nymph ratio in females (Figure 2C), the qualitative results did not change when we omitted this outlier colony (nymph ratio of larvae developed

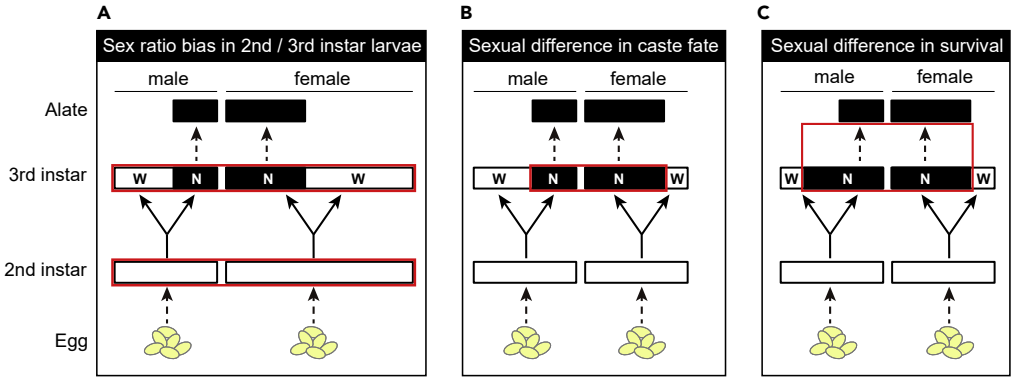

Figure 3. Three potential scenarios explaining sex ratio biases in alates

(A–C) Intercolonial variation in alate sex ratio can be explained by (A) sex ratio bias before caste differentiation (i.e., second instar), (B) the sexual difference in caste fate (i.e., nymph sex ratio at third instar), and (C) sexual differences in survival rate during the nymph-to-alate developmental period. The width of the bar indicates the number of individuals. Red boxes indicate the period of development determining alate sex ratio in each scenario. The results of this study support scenario B.

in the natal colony: *AIC* = 48.28, *BIC* = 49.25; social origin of foster workers: *AIC* = 70.83, *BIC* = 71.80; null model: *AIC* = 101.41, *BIC* = 101.92). Differences in the social environment—natal vs. foster colony—did not have a significant effect on the nymph ratio (male: GLMM, $\chi^2 = 1.220$, *df* = 1, p = 0.269; female: GLMM, $\chi^2 = 3.582$, *df* = 1, p = 0.058). No sex ratio bias was detected in the fostered third-instar individuals from all 13 colonies (see Table S1). Microsatellite genotyping showed that nymphs and workers were produced via normal sexual reproduction between field-collected PKs and SQs irrespective of the nymph sex ratio (see Tables S2 and S3).

Comparison of larval and alate numerical sex ratios

The proportion of offspring that developed into nymphs differed among sexes and colonies. The sex ratio of alates in the field colonies reflected the sexual difference in caste developmental fate (Figure 4) as a scenario shown in Figure 3B. The sex ratio of the N1s was the best predictor of the alate sex ratio (*AIC* = 125.17, *BIC* = 125.77, Figure 4A), with the model including the sex ratios for larval stages before (*AIC* = 349.71, *BIC* = 350.31, Figure 4B) and after (*AIC* = 358.17, *BIC* = 358.78, Figure 4C) caste determination explaining variation in the alate sex ratio slightly better than the null model (*AIC* = 372.54, *BIC* = 372.84). There was no significant change in the sex ratio from the N1s to the alates (GLMM, $\chi^2 = 1.399$, *df* = 1, p = 0.237). The sex ratio for the L2s and all third-instar individuals (sum of N1s and W1s) was not significantly biased in most of the colonies, except for the sex ratio of all third-instar larvae in two colonies.

Relationship between alate sex ratio and their sexual difference in biomass

Our field survey revealed that the difference in the numerical sex ratio of alates reflects in colony-level sex allocation. The sexual difference in alate body weight was constant among colonies and was not significantly affected by the alate sex ratio in each colony (GLM, $\chi^2 = 0.159$, *df* = 1, p = 0.690, Figure 5A). The mean numerical sex ratio of alates (proportion of males) was 0.445 $\pm$ 0.014 (mean $\pm$ SE, range 0.10–0.74, *N* = 100 colonies comprising 10,000 individuals, Figure 5B), indicating that it was significantly skewed toward females (exact binomial test, 95% CI = 0.436–0.456, p < 0.001). When adjusted for biomass, the proportional investment was more female biased (0.421 $\pm$ 0.013, range 0.09–0.72, *N* = 100 colonies comprising 10,000 individuals, two-tailed paired *t*-test, *t* = −6.058, *df* = 99, p < 0.001, Figure 5C), this is because female alates were heavier than males from the same nest (GLMM, $\chi^2 = 784.49$, *df* = 1, p < 0.001, Figure 5A).

DISCUSSION

Understanding the mechanism controlling caste determination is a central issue in the biology of social insects. Our egg-fostering experiment suggests that the colony-dependent sex-specific caste fates are almost entirely determined before oviposition in field colonies (Figure 2). Despite completely different food and environmental conditions exposed to the field colonies, both sexes of offspring from fostered eggs exhibited similar caste fates to that of in their natal colonies (Figures 2A and 2C). Therefore,

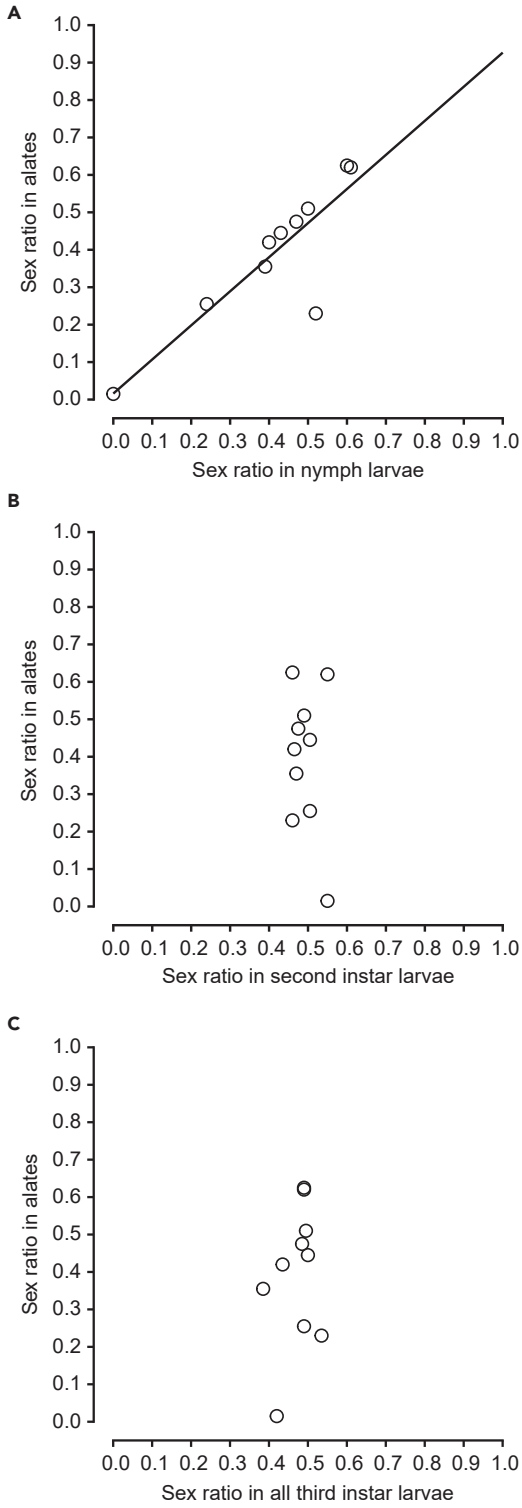

Figure 4. Comparison of sex ratios across different developmental stages

(A–C) Correlation between the numerical sex ratio in alates and that in (A) nymphs, (B) second-instar larvae, and (C) all third-instar larvae (sum of N1s and W1s). Plots indicate the male sex ratio in each colony, which was calculated by investigating 200 randomly selected individuals per developmental stage or caste, except nymphs (100 individuals; n = 10 colonies).

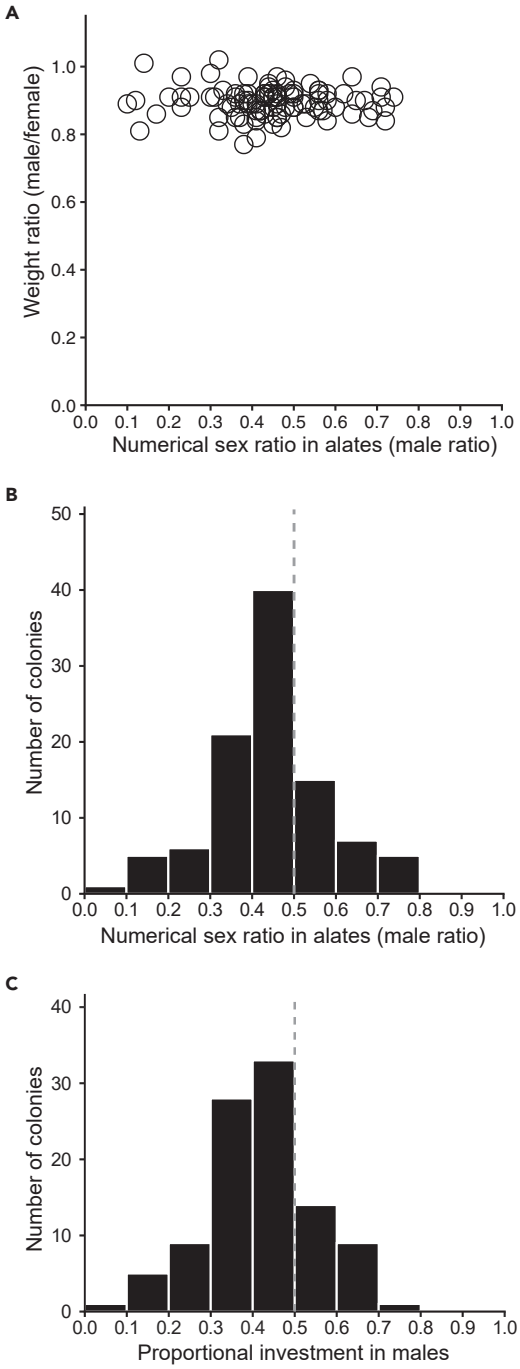

Figure 5. Numerical sex ratio in alates defining colony-level sex allocation

(A) Sexual difference in alate body weight among colonies with various alate sex ratios. The weight ratio (male/female) in alates is plotted as a function of the numerical sex ratio in alates across colonies. The sexual difference in alate biomass is constant among colonies.

(B and C) (B) Biased numerical sex ratio and (C) proportional investment in males in field colonies. The dashed line indicates an equal sex ratio. Numerical sex ratio is significantly skewed toward females (exact binomial test, $p < 0.001$), and the proportional investment is female biased (two-tailed paired t-test, $p < 0.001$).

post-hatching environments had a negligible effect on caste determination in field colonies in *R. speratus*. Furthermore, the caste determination process plays an important role in controlling colony-level sex allocation. As a consequence of caste differentiation, the initial number of both sexes of fertile progenies is determined as explained in Figure 3. In termites, there is little or no conflict over sex allocation between royals and workers due to the diplo-diploid and functionally monogamous reproductive system,[13,22,25–29] implying that with workers, there is no need to alter the sex ratio of fertile progenies after caste differentiation. Indeed, investigation of the caste and sex composition in the field colonies indicates that the colony-dependent sex-specific caste fates result in the intercolonial variation in the numerical sex ratio of alates, and there is almost no adjustment of their sex ratio after caste differentiation (Figure 4). The results suggest the principal role of heritable factors in caste determination and colony-level sex allocation which are key traits that shape fitness consequences of social organisms in field colonies.

Caste determination due to transgenerational non-genetic factors is the most plausible explanation for the previously reported data and results presented here. In *R. speratus*, a difference in parental developmental origin—not the genetic background—alters the offspring caste fate under experimental conditions[18,22,23] and the same cross of parents (i.e., PK and SQ) could generate various caste fates in field colonies (Figures 2B and 2C), suggesting that a mechanism based on a difference in DNA sequence is not likely to be involved or negligibly small in caste determination. Mixing of parthenogenetic daughters also cannot explain our results for caste fate in female offspring. Parthenogenetic daughters have the developmental propensity to become fertile progenies in termites with asexual queen succession (AQS) and even in non-AQS species.[15,18,22,23,30,31] However, our microsatellite genotyping showed that nymphs in the field colonies were produced by normal sexual reproduction irrespective of the nymph sex ratio (Tables S2 and S3). Moreover, the sex ratio for third-instar larvae was close to 1:1 in all colonies examined, including those where caste fate in females was extremely nymph biased (Table S1). This excludes the possibility of the mixing of parthenogenetic daughters leading to a female-biased sex ratio for every larval stage.

Heritable factors other than DNA sequences influence the offspring phenotype in various organisms.[32,33] Various molecular mechanisms have been reported, including the transmission of epigenetic states (DNA methylation and histone modification), small non-coding RNAs, and cytoplasmic factors (e.g., hormones and nutrients).[34,35] DNA methylation, histone modification, and non-coding RNAs are transmitted via both the sperm and egg, whereas hormones and nutrients are inherited only from the egg.[36] In *Pogonomyrmex* seed harvester ants, the quantity of a cytoplasmic substance (vitellogenin in eggs) mediates the intergenerational transmission of information.[37] Eggs that have the potential to differentiate into a queen are produced only by mothers that have experienced hibernation.[38] However, in termites, cytoplasmic factors are not likely to be the primary determinant of caste fate, because both maternal and paternal phenotypes have almost equal effects on offspring caste predisposition.[18,19] Instead, epigenetic factors transmitted from both parents may be involved as proximate factors of caste fate, as indicated by a mathematical model.[23] The present study does not directly test the heritable factors from both the king and queen, and it could be either or both; further studies are needed to elucidate proximate cause of heritable effects.

This study indicates roles of heritable factors in determining the caste fate and colony-level sex allocation in termites. Recent studies in other groups of social insects reported that the initial trigger for caste determination stems from heritable factors.[15,16] This study calls for studies to evaluate the impact of heritable and environmental effects on colony-level traits that shape fitness consequences of social organisms in the field. The present study also sheds light on the proximate mechanisms for caste determination which at this stage remains elusive. Transgenerational non-genetic factors are a promising target for future studies investigating molecular mechanisms determining caste fate. These studies will promote a further understanding of the mechanisms underlying the division of labor, life-history traits, and phenotypic plasticity of social insects.

Limitation of the study

Our field survey and egg-fostering experiment found intercolonial variation in the caste fate of both sexes despite the offspring being produced via crosses between PKs and SQs and raised in controlled conditions. The molecular mechanisms for caste determination remain elusive. To uncover the cause of the variation in caste fate, further studies investigating the effect physiological conditions (e.g. age, development of sexual organs, and nutritional states) of a king and queen have on influencing offspring caste fate are necessary.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- QUANTIFICATION AND STATISTICAL ANALYSIS
  - The binomial linear model
  - Statistical analysis

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.106207.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.T.; methodology, M.T., Y.O., and K.M.; validation, M.T. and Y.O.; formal analysis, M.T. and Y.O.; investigation, M.T., S.N., and E.T.; resources, M.T. and K.M.; writing – original draft, M.T. and T.I.; visualization, M.T., Y.O., and K.M.; Supervision, M.T. and K.M.; project administration, M.T.; and funding acquisition, M.T. and K.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diversity, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Raw and analyzed data	This paper	Mendeley Data: https://doi.org/10.17632/mm824csy77.1
Experimental models: Organisms/strains		
Reticulitermes speratus	Wild-caught	N/A
Software and algorithms		
R ver. 3.3.3	R: A language and environment for statistical computing. R Foundation for Statistical Computing.	https://www.r-project.org

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Mamoru Takata (takata.mamoru.7z@kyoto-u.ac.jp).

Materials availability

Not applicable.

Data and code availability

- The dataset reported in this paper have been deposited at Mendeley and are publicly available as of the date of publication. The DOI is listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

METHOD DETAILS

Egg-fostering experiment

To identify heritable and environmental effects on larval caste fate under field conditions, we conducted an egg-fostering experiment and compared caste fate between larvae developed in foster and natal colonies in the subterranean termite *Reticulitermes speratus* (Figure 2A). Thirteen colonies that contained one PK, multiple SQs, W1s, and N1s were collected in oak/pine mixed forests in Kyoto, Shiga, and Fukui, Japan, from July to September 2020. Two additional colonies were collected to supply foster workers in Kyoto in July 2020. Within a week of collection, all termites were extracted from each piece of wood, and 100 of each sex of N1s and W1s (without distinguishing between the nymph and worker castes, since the castes at the developmental stage are only distinguishable under a microscope) were randomly selected from each colony (hereafter, larvae developed in the natal colony), and the number of individuals of each caste was recorded. In total, 2,600 larvae were investigated. The caste and sex were distinguished by the presence or absence of wing buds and sex-specific morphology of seventh and eighth sternites, respectively.[39–41]

To collect eggs, 10–80 SQs from each of the 13 colonies were separated into groups of ten, then were transferred into individual dishes (ca. 60 mm) lined with a moist unwoven cloth and 50 foster workers (including both sexes). Each of the 13 colonies was randomly assigned to one of two foster worker colonies, to have enough eggs for each natal and foster colony replication. After 48 h, 100 eggs laid by the secondary queens were transferred into dishes (ca. 30 mm) with mixed sawdust bait[42] and 50 male workers from previously assigned foster colonies. Four dishes were made for each of the 13 colonies; thus, 400 eggs were set in

each colony. Then, the dishes were maintained at 25°C under dark conditions. The dishes were checked weekly, and if N1s and/or W1s (hereafter, larvae developed in the foster colony) were found, the numbers of individuals of each caste and sex were recorded by observers who were naïve to the identity of the natal colonies. In total, 1,368 male and 1,362 female individuals were collected and used for analysis.

To confirm that nymphs and workers were produced by normal sexual reproduction between a PK and SQ pair, we performed genotyping of a PK, SQs, N1s, and W1s to identify their parents in representative colonies. Two field-collected representative colonies were randomly selected (the nymph sex ratios in colonies GA and GB were 0.51 and 0.72, respectively). One PK, four randomly selected SQs, and 10 of each sex of N1s and W1s individuals were analyzed. Total DNA was extracted using a modified Chelex extraction protocol.[43] The heads or antennae were digested in 20 μL of Chelex solution (10% w/v; TE pH 8.0) and 0.2 μL of proteinase K at 55°C for 3 h. After incubation, the samples were heated at 95°C for 15 min. Polymerase chain reaction (PCR) amplifications were performed in the multiplex to analyze four microsatellite loci (Rf21-1, Rf24-2, Rf6-1,[44] and Rs15[45]). Primers for Rf6-1, Rf21-1, Rf24-2, and Rs15 were labeled with 6-FAM, VIC, NED, and PET fluorescent tags, respectively. The 10-μL PCR cocktail contained 1 μL of template DNA, 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, Ipswich, MA, USA), 1.15 μL of 5 μM multiplex primers, and 6.59 μL of distilled water. Amplification consisted of 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. The PCR products were mixed with 10 μL of Hi-Di formamide and 0.3 μL of GeneScan 600 LIZ size standard. An Applied BioSystems 3500 Genetic Analyzer was used to perform sample detection. GeneMapper 5.0 software (Applied Biosystems, Foster City, CA, USA) was used to analyze raw data. We defined offspring carrying both paternal and maternal alleles as sexually produced, and ones carrying only maternal alleles as parthenogenetically produced.

Comparison of larval and alate numerical sex ratios

To investigate whether the variations in larval caste fate result in variation in colony-level sex allocation, we evaluated the influence of three potential factors affecting the numerical sex ratio of alates (Figure 3) by comparing the sex ratio of L2s and N1s to that of alates. To compare the sex ratio of L2s and N1s with that in alates in field colonies, 10 nests with L2s, W1s, N1s, and sixth-instar nymphs (N6: pre-alates), were collected in Kyoto, Shiga, and Fukui, Japan, from May to June of 2019–2020, just before the swarming season. Each nest was kept at 20°C under dark conditions until the N6s molted into alates. Then, all the termites in each colony were extracted from the wood. From each colony, 200 individuals of L2s, N1s and W1s mix (without distinguishing between their castes), and alates were randomly selected, and the number of each sex was recorded. For the N1s and W1s, the number in each caste (nymph/worker) was also recorded. Additional N1 individuals were collected until their total number reached 100, then the number of each sex was recorded. In total, 2,000 individuals for each developmental stage (L2s, N1s and W1s mix, and alates) and an additional 513 individuals of N1 were investigated. Observers were naïve to the colony information during the investigation.

Relationship between alate sex ratio and their sexual difference in biomass

To investigate whether the difference in the numerical sex ratio of alates is reflected in colony-level sex allocation, the sexual difference in body weight in alates in field colonies were investigated among colonies with different alate sex ratio. One hundred colonies with alates were collected in Kyoto, Shiga, and Fukui, Japan, from April to June of 2018–2019. All alates were extracted from the wood, then 100 individuals were randomly selected and the number of each sex was recorded. From the selected 100, 10 alates of each sex were randomly selected and their fresh body weight was recorded to the nearest 0.1 mg.

QUANTIFICATION AND STATISTICAL ANALYSIS

The binomial linear model

The binomial linear model for examining the linearity of the relationship between two variables is defined below. Suppose we have an objective variable y given T trials and an explanatory variable X. The binary linear model supposes that for $i = 1, 2, \ldots N$,

$$y_i \sim Binomial\,(T_i, p_i) \qquad \text{(Equation 1)}$$

$$p_i = beta * X_i + intercept, s.t.\ 0 < p < 1 \quad \text{(Equation 2)}$$

where Binomial (a, b) is the binomial distribution with trial number a and probability parameter b, and beta is the regression parameter. Note that the possible range of the parameters (beta, intercept) depends on datum X and the remaining parameter to satisfy $0 < p < 1$. We defined the log likelihood function of this model and obtained the maximum likelihood estimates (MLEs) of the parameters via numerical optimization ("optim" function in R). We used the Broyden–Fletcher–Goldfarb–Shanno algorithm whenever possible. When convergence was not attained during optimization, a simulated annealing algorithm was used instead. Before the actual data were analyzed, we confirmed that the usual asymptotic properties of the MLEs held for this model (e.g., normality and unbiasedness) using numerical simulations. Although numerical optimization is more difficult than the usual logit/probit model owing to complex restrictions on the parameter space, our approach is useful for analyzing the linear relationship between two ratios and still yields statistically reliable results.

Statistical analysis

The binomial linear models were applied for the comparison of caste fate between the larvae developed in the foster and natal colony in the egg-fostering experiment. The objective variable was the nymph ratio of male or female larvae developed in the foster colony, and the respective explanatory variable was the nymph ratio of male or female larvae developed in the natal colony or the social origin of the foster workers. To evaluate the impact of each factor on the objective variable, we compared the Akaike information criterion (*AIC*) and Bayesian information criterion (*BIC*) values of these models and the null model. We did not report p-values because theoretical rationale is lost when the post-model selection estimator is applied. Note that *AIC* and *BIC* values are identical to log-likelihoods up to a constant if the compared models have the same number of free parameters. Generalized linear mixed models (GLMMs) were run to investigate the effect of the social environment—natal vs. foster colony—on the offspring nymph ratio. Data for males and females were analyzed separately. The binomial objective variable was the nymph ratio; the post-hatching environment (natal vs. foster colony) was treated as an explanatory variable and the genetic origin of the larvae (colony identity) was treated as a random factor. An exact binomial test was applied to compare the observed numerical sex ratio for larvae developed in the foster colony against the null hypothesis assuming that the numbers of males and females were equal.

For the comparison of alate and larval numerical sex ratios in the different developmental stages in field colonies, we applied a binomial linear model. The binomial objective variable was the sex ratio for alates (the number of male subjects given the total number of subjects as the trial number) and the explanatory variable was the sex ratio (continuous) for second-instar larva (L2s), N1s, or all third-instar individuals (sum of N1s and W1s). To evaluate the impact of each factor on the objective variable, we compared the *AIC* and *BIC* values of these models and the null model. A GLMM was used to investigate the effect of caste—in N1s or alates—on the sex ratio. The binomial objective variable was the sex ratio; caste was treated as an explanatory variable and colony identity was treated as a random factor.

Generalized linear models (GLMs) were used to investigate the influence of the alate sex ratio in the colony on sexual differences in alate body weight. The body weight ratio (male/female) for individual alates was treated as a response variable assuming a Gaussian distribution; the numerical sex ratio for alates was treated as an explanatory variable. An exact binomial test was applied to compare the observed numerical sex ratio for field-collected alates against the null hypothesis assuming that the numbers of males and females were equal. A two-tailed paired *t*-test was used to compare the biomass of male and female alates. A GLMM was run to investigate the sexual difference in alate body weight. The fresh body weight of each alate was treated as a response variable assuming a Gaussian distribution; their sex was treated as an explanatory variable and the colony identity was treated as a random factor.

All statistical analyses were performed and graphs were generated using R v.3.3.3 software[46]; all data are available in the Supplementary Materials. For GLMMs and GLMs, the likelihood ratio test was used to determine the statistical significance of each explanatory variable. A significance value of $p < 0.05$ was considered to indicate statistical significance.