Neotenic Reproductives Influence Worker Caste Differentiation in the Termite Reticulitermes speratus (Isoptera; Rhinotermitidae)

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Abstract
Division of labor among castes in social insect colonies increases ergonomic efficiency and colony-level fitness, and has played a key role in the ecological success of social insects. Knowledge of the factors that regulate castes is important for understanding adaptive social organization. Our previous study on the termite Reticulitermes speratus demonstrated that the presence of a pair of nymphoid reproductives during development affected offspring caste ratios. In the present study, we investigated further the influence of individual neotenics on offspring caste ratios. Parthenogenetically-produced offspring were reared in worker-tended experimental colonies with the addition of different forms (nymphoid or ergatoid) and numbers of neotenics, and compared the caste ratios of the offspring between the different experimental treatments. We found that all offspring in worker-only tended colonies became nymphs, while a proportion of offspring in colonies with a single neotenic (with the exception of male ergatoids) differentiated into workers. These results show offspring caste ratios are influenced by the presence of single female ergatoids, single female and male nymphoids, while they remain unaffected by the presence of male ergatoids.

Introduction
A defining characteristic of social insect colonies is the presence of different castes. Each caste has specialized behavior and morphology, and carries out specific tasks for colony development. Such division of labor between castes accounts for the high ergonomic efficiency of insect societies and thus their ecological success (Oster & Wilson, 1978). Because ergonomic efficiency critically depends on caste composition of the colony (Oster & Wilson, 1978), colonies are expected to adjust caste ratios in changing environments and during different developmental stages of the colony life cycle (Wilson, 1971; Schmid-Hempel, 1992; Helms Cahan et al., 2010; Lecoutey et al., 2011). Mechanisms for controlling caste ratio are thus likely to evolve adaptively so that caste compositions can promptly reach their optima (Schmid-Hempel, 1992; Helms Cahan et al., 2010). Proximate factors that determine caste fates of individuals play an important role in the control of caste ratio (Hughes & Boomsma, 2008; Smith et al., 2008a), and are key to understanding adaptive social organization (Helms Cahan et al., 2010).

Proximate factors influencing caste determination have been the subject of many studies over several decades. Most of these studies demonstrated that environmental factors influence caste determination, and it became generally accepted that caste differentiation was controlled purely by environmental factors (Wilson, 1971; Wheeler, 2003). In termites, a major group of social insects, caste development is highly plastic and environmental signals are thought to be critical for caste determination (Wilson, 1971; Miura, 2001; Miura et al., 2003; Scharf et al., 2003; Scharf et al., 2005; Scharf et al., 2007; Matsuura et al., 2010; Hartke & Baer, 2011). In recent years, however, genetic influences on caste determination have been reported in some social insects (reviewed in Crozier & Schluns, 2008; Smith et al., 2008b; Schwander et al., 2010).
Our previous studies on the Japanese subterranean termites *Reticulitermes speratus* (Hayashi et al., 2007), *R. okinawanus*, *R. kannonensis*, and *R. yaeyamanus* (Kitade et al., 2011) provided evidence for genetic influences on caste determination of larvae. In *Reticulitermes*, each individual becomes either a nymph or a worker after two larval instar stages (Buchli, 1958; Shimizu, 1970; Takematsu, 1992). Nymphs have wing buds and are able to eventually develop into alates (adults), which can establish new colonies and become kings/queens. Workers are functionally sterile and never develop wing buds. Under some conditions (e.g. in the absence of reproductives under laboratory conditions), workers and nymphs can differentiate through special molts into neotenics, which are reproductive individuals retaining juvenile characteristics (Watanabe & Noda, 1991; Thorne et al., 1999; Miyata et al., 2004; Leniaud et al., 2011). Neotenics derived from workers are termed “ergatoids”, which are apterous, and those from nymphs are termed “nymphoids”, which have wing buds. Studies of natural colonies have shown multiple nymphoids are typically present, and that they play a key role in reproduction (Matsuura et al., 2009). Ergatoids, on the other hand, have not yet been found in extensive surveys of natural colonies (Matsuura et al., 2009; Matsuura et al., 2010), although there is some evidence that they may be present (Shimizu, 1970). In our previous study of *R. speratus* (Hayashi et al., 2007) we carried out crossing with four possible combinations of ergatoids and nymphoids, and examined caste and sex of their offspring, which were reared under uniform environmental conditions. There were extreme differences in the proportions of caste and sex between offspring from different combinations of parents, indicating that genetic factors influenced the developmental commitment to the worker or nymph caste. We also showed that almost all of the offspring produced through parthenogenesis developed into nymphs when they were reared only with tending workers, while 24% of parthenogenetically-produced offspring differentiated into workers when reared with an additional pair of nymphoids (Hayashi et al., 2007). This suggests that the existence of pairs of nymphoids can influence the developmental trajectory of larvae.

In our previous study, however, the effects of individual neotenics were not surveyed. Thus, in this study, we focused on examining whether individual ergatoids and nymphoids influence proportion of worker caste among offspring. We reared eggs produced through parthenogenesis in treatments with workers plus a single female or male ergatoid or nymphoid (Fig 1). We also included a treatment with a pair of nymphoids (to replicate our previous study (Hayashi et al., 2007)), and a control treatment (with workers only). To examine variation of the influence on offspring caste ratio among different sexes and forms (nymphoid/ergatoid) of neotenics within a colony, we made replication of the experiment with neotenics collected from a single field colony.

Fig 1. Schematic diagram of the experiment. “E”, “N” and “W” represent ergatoid, nymphoid and worker, respectively. Two kinds of egg-source colonies were established: “ergatoid egg-source colonies”, which contained 3 female ergatoids and 200 workers, and “nymphoid egg-source colonies”, which contained 3 female nymphoids and 200 workers. Offspring-rearing colony W, ♂E, ♀E, ♂N, ♀N, and ♂N♀N consisted of 50 workers, a female ergatoid and 50 workers, a male ergatoid and 50 workers, a female nymphoid and 50 workers, a male nymphoid and 50 workers, and a female and a male nymphoid and 50 workers, respectively. Female neotenics in egg-source colonies were obtained from field colony A and the other individuals were from field colony B. Arrows show egg transfer from the egg-source colony to the offspring-rearing colony.

Materials and Methods

Termites

Two *R. speratus* colonies (A and B) were collected from Mito Shinrin Park, Mito, Ibaraki, Japan (36°25′N, 140°22′E) with their nest woods at 7 Jun and 25 July 2005 respectively. The colonies were transferred to the laboratory and maintained in large plastic boxes at room temperature until 1st Aug when experiments were commenced.

Experiments

To obtain parthenogenetic eggs for the experiments, we first made “ergatoid egg-source colonies” (n = 3) that consisted of three virgin ergatoids and 100 female workers, and “nymphoid egg-source colonies” (n = 3) that contained three virgin nymphoids and 100 female workers. Male workers were not used for the care of virgin neotenics. This is because they are able to differentiate into male ergatoids, which are difficult to distinguish morphologically from workers (Pichon et al., 2007; Fujita & Watanabe 2010), and male ergatoids may copulate with females and prevent us from obtaining parthenogenetic eggs. To obtain virgin ergatoids and nymphoids to be used for the egg-source colonies, we collected workers and nymphs from the field colony A. We then divided them...
by sex on the basis of sternite morphology, as described in *R. flavipes* (Thompson & Snyder, 1920) and prepared an artificial colony that consisted only of female workers and female nymphs. In the colony some workers and nymphs began to differentiate into nymphoids and ergatoids within a month, as reported in (Watanabe & Noda, 1991; Miyata et al., 2004). The female workers included with virgin neotenics were obtained from the field colony B.

We then made six types of offspring-rearing colonies: (1) colony W, containing 50 workers, (2) colony ♀E, containing a female ergatoid and 50 workers, (3) colony ♂E, containing a male ergatoid and 50 workers, (4) colony ♀N, containing a female nymphoid and 50 workers, (5) colony ♂N, containing a male nymphoid and 50 workers, and (6) colony ♀N♂N, containing a pair of nymphoids and 50 workers (Fig 1). Because our previous study revealed that a pair of nymphoids influenced the offspring caste differentiation, we set up colony ♀N♂N as a positive control for the experiment. Colony W was established as a negative control. The workers were randomly extracted from field colony B irrespective of their sex. The ergatoids and nymphoids of the offspring-rearing colonies were obtained from artificially orphaned colonies that consisted of workers and nymphs of both sexes from field colony B. Six replications were made for each of the six types of offspring-rearing colonies. Eggs produced in each of the ergatoid egg-source colonies were reared in three of them, while those of the nymphoid egg-source colonies were reared in the other three.

From the egg-source colonies, we collected well-developed eggs that had exhausted their yolk and transferred them equally into the six types of offspring-rearing colonies. Some *Reticulitermes* species, including *R. speratus*, do not distinctively eliminate eggs of the other congeneric species (Matsura et al., 2007), and also in this study eggs of the neotenics (derived from field colony A) were piled up and reared by the non-nestmate workers (from field colony B). Because the positive control (i.e., colony ♀N♂N) was set up based on the result of our previous study (Hayashi et al., 2007), in which eggs were transferred into colonies comprised of workers derived from a different field colony, we followed the method of the previous study and reared eggs with workers from a different colony also in the present experiment. We reared the offspring until they reached the third instar, and then identified their caste on the basis of wing bud development. Young eggs laid by the neotenics in the offspring-rearing colonies, which could be easily discriminated from the well-developed eggs transferred from egg-source colonies, were carefully removed. All of the above experimental treatments were carried out once every three days.

All rearing experiments were carried out at 25°C under constant darkness. The termites were fed with a mixture of cellulose powder and sawdust of *Quercus* woods (mixed food). We provided arbitrary amount of the mixed food when termites almost exhausted it. We continued the experiments for 360 days.

We determined the castes of offspring at the third instar on the basis of presence/absence of wing buds. A small number of offspring (10 individuals) in the offspring-rearing colonies with neotenics were morphologically intermediate with respect to their wing buds; these were reared up to fourth or fifth instar to determine their caste, and 8 of 10 offspring were classified as nymph caste. We examined the number of offspring that differentiated into workers and nymphs, for each type of offspring-rearing colony, and then calculated proportions of each caste to the total number of third-instar offspring. Survival rates of offspring were calculated by dividing the number of offspring that developed to the third instar by the number of eggs that were transferred into offspring-rearing colonies.

### Statistical analyses

For testing difference in survival rates between offspring produced by nymphoids and those by ergatoids of the egg-source colonies, logistic regression analysis was carried out. Offspring survival status (i.e., death or survival), which is considered to follow the binomial distribution, was set as the dependent variable, and types of offspring-rearing colonies and forms of the mother neotenics in egg-source colonies as the independent variables. Interaction between those two dependent variables was not significant in the preliminary logistic regression analysis and we used the model without the interaction for the logistic regression.

Effect of the forms of the mother neotenics in egg-source colonies and forms and sexes of neotenics in the offspring-rearing colonies on offspring caste ratio was analyzed by logistic regression analysis. In the analysis, offspring caste (nymph or worker) was set as the dependent variable, and types of offspring-rearing colonies and forms (ergatoid/nymphoid) of the mother neotenics in egg-source colonies as the independent variables. In the preliminary logistic regression analysis with the interaction term, because there were no significant effects of the interaction between the independent variables, we used the model without the interaction. We applied the logistic regression using the penalized maximum likelihood estimation (Firth, 1993; Kosmidis, 2007), because no offspring differentiated into workers in some offspring-rearing colonies and thus the logistic regression analysis included some zero-cells in the contingency table of offspring caste status, which causes bias of the maximum likelihood estimates (Allison et al., 2004).

We carried out all of the logistic regression analyses in R2.13.1 (R Development Core Team, 2011). In the logistic regression analyses, to examine effects of types of the offspring-rearing colonies, colony W was set as the reference level and compared with the other colony types. For the logistic regression with penalized method for maximum likelihood estimation the R function 'brglm' (Kosmidis, 2008) was used.
Results

We obtained 184, 194, and 267 eggs from three replicates of egg-source colonies with parthenogenetic ergatoids, and 341, 232, and 253 eggs from those with parthenogenetic nymphoids. Number of eggs was not significantly different between nymphoids and ergatoids (Student's T test, t = -1.42, d. f. = 4, p = 0.23), although it is common in Reticulitermes that nymphoids have higher fecundity than ergatoids (Myles 1999). We divided those eggs almost equally into six types of offspring-rearing colonies. We obtained 101, 109 and 136 third-instar offspring from ergatoid egg-source colonies and 186, 119, and 133 from nymphoid egg-source colonies. The number of eggs reared and the number of offspring that developed to the third instar in each offspring-rearing colony are shown in Table 1.

Table 1. Number of eggs transferred from egg-source colonies to offspring-rearing colonies, number of offspring that reached third instar from the eggs and survival rate of offspring (mean ± SE).

<table>
<thead>
<tr>
<th>Offspring-rearing colony</th>
<th>N° of eggs</th>
<th>N° of third instar offspring</th>
<th>Survival rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>31, 32, 44</td>
<td>16, 16, 22</td>
<td>0.51 ± 0.005</td>
</tr>
<tr>
<td>♀E</td>
<td>57, 39, 42</td>
<td>25, 18, 22</td>
<td>0.47 ± 0.025</td>
</tr>
<tr>
<td>♂E</td>
<td>30, 32, 44</td>
<td>18, 22, 27</td>
<td>0.63 ± 0.027</td>
</tr>
<tr>
<td>♀N</td>
<td>57, 38, 42</td>
<td>34, 18, 23</td>
<td>0.54 ± 0.036</td>
</tr>
<tr>
<td>♂N</td>
<td>30, 32, 44</td>
<td>18, 18, 18</td>
<td>0.52 ± 0.058</td>
</tr>
<tr>
<td>♀N♂N</td>
<td>56, 38, 42</td>
<td>30, 16, 23</td>
<td>0.50 ± 0.040</td>
</tr>
<tr>
<td>♀E</td>
<td>31, 33, 45</td>
<td>14, 19, 22</td>
<td>0.51 ± 0.037</td>
</tr>
<tr>
<td>♂E</td>
<td>57, 39, 43</td>
<td>40, 25, 24</td>
<td>0.63 ± 0.042</td>
</tr>
<tr>
<td>♀N</td>
<td>31, 33, 45</td>
<td>16, 15, 18</td>
<td>0.46 ± 0.034</td>
</tr>
<tr>
<td>♂N</td>
<td>57, 39, 42</td>
<td>24, 19, 18</td>
<td>0.45 ± 0.021</td>
</tr>
<tr>
<td>♀N♂N</td>
<td>31, 32, 45</td>
<td>19, 19, 29</td>
<td>0.62 ± 0.015</td>
</tr>
<tr>
<td>♂N♂N</td>
<td>57, 39, 42</td>
<td>33, 23, 23</td>
<td>0.57 ± 0.013</td>
</tr>
</tbody>
</table>

The upper and bottom rows show offspring numbers derived from egg-source colonies with ergatoids and those with nymphoids, respectively. In each row, except for the column for survival rates, numbers of offspring of three replicates are shown. For calculating survival rates the numbers of eggs transferred from egg-source colonies to offspring-rearing colonies were divided by the number of offspring that reached the third instar from the egg stage (n = 3).

In W and ♀E colonies, all of the offspring differentiated into nymphs (Fig 2). In contrast, some offspring differentiated into workers in ♀E colonies [proportions of workers derived from ergatoid and nymphoid egg-source colonies (mean ± SE): 0.099 ± 0.065, and 0.160 ± 0.087, respectively], ♀N colonies (0.072 ± 0.011, and 0.070 ± 0.047), ♂N colonies (0.176 ± 0.081, 0.177 ± 0.070), and ♀N♂N colonies (0.128 ± 0.022, 0.258 ± 0.069). The result of the logistic regression analysis showed no significant effect of egg-providing neotenic (i.e. ergatoid vs nymphoid) on offspring caste ratios (Wald X2 = 1.002, p = 0.317). The offspring caste ratios were signifi-

Discussion

Our previous study (Hayashi et al., 2007) showed that presence of a pair of female and male nymphoids resulted in increased worker rate of offspring that were produced parthenogenetically, when compared to offspring raised by workers alone. The current study demonstrates that presence
of single female ergatoids and single female and male nymphoids also increased worker rate among offspring, and on the other hand, that of single male ergatoids did not. In the field, hundreds to thousands of neotenics, with various sex ratios, were found from single nests of Reticulitermes and most of them were nymphoids (Howard & Haverty, 1980; Matsuura et al., 2009; Hu & Forschler, 2012). Thus, the result of the present study suggests that worker rates tend to be higher in the field colonies harboring a lot of neotenics. However, our study used neotenics derived from a single source colony. Further studies involving neotenics from additional colonies are required to determine whether our results represent a widespread feature of the neotenic effect on the caste differentiation of this species.

Two possible mechanisms are possible for the control of worker rate. First, the caste developmental trajectory to nymph caste would have been converted to that to worker caste in some larvae that were reared in ♀E, ♀N, ♂N, and ♀N♂N colonies, which result in increased worker rates in those colonies. This is supported by the fact that a few of the offspring from ♀E, ♀N, ♂N, and ♀N♂N colonies were morphologically intermediate between nymphs and workers (cf. “Experiments” in Materials and Methods): those intermediates had relatively smaller wing buds. In termites, morphologically intermediate individuals have also been found during artificial induction of caste differentiation with juvenile hormone analogs, which strongly induce workers to differentiate into presoldiers, (Miura et al., 2003; Tsuchiya et al., 2008; Watanabe & Maekawa, 2008).

The second possible mechanism is that larvae destined to be workers were eliminated by attending workers in W and ♂E colonies. However, elimination of worker-destined larvae is unlikely because survival rates of offspring were not significantly different between W colonies and the other colonies. If worker-destined larvae were eliminated in W colonies, survival rates of offspring from the late stages of the egg phase to third instar in W colonies would be significantly lower than the other colonies.

The survival rates from late egg stages to third instar were not significantly different between eggs produced parthenogenetically from nymphoids and ergatoids. In contrast, our previous study showed that survival rates from early stages of the egg phase to third instar were significantly lower in eggs parthenogenetically produced by ergatoids than in those produced parthenogenetically by nymphoids (Hayashi et al., 2007). These data suggest that lethal genetic effects in eggs produced by ergatoids (Hayashi et al., 2007) appeared only during the early stages of the egg phase.

One potential proximate cause for worker differentiation in ♀E, ♀N, ♂N, and ♀N♂N colonies is the transfer of substances between neotenics, workers and larvae via grooming, and stomodeal and proctodeal trophallaxis. Stomodeal trophallaxis is thought to be important in caste determination in other termite species (Lüscher, 1961; Korb et al., 2012). We observed stomodeal trophallaxis and grooming among workers, larvae, and all forms of neotenics with the exception of male ergatoids (data not shown). A recent study revealed the existence of queen-specific termite proteins (Hanus et al., 2010); similar substances might be responsible for the effects on caste differentiation seen in this study. Another potential cause of worker differentiation could be the action of volatile compounds. Recent studies on R. speratus revealed that volatile pheromones influence the development of ergatoids (Matsuura et al., 2010; Matsuura & Yamamoto, 2011). Further experiments are needed to reveal the roles of direct behavioral interactions and volatile pheromones in induction of worker differentiation.

Our results revealed that the egg-stage is relatively insensitive to the induction of worker differentiation by neotenics. Eggs were reared together with their parental neotenics until just before hatching, and only the eggs that were transferred to the W and ♀E developed solely into nymphs. Further studies are needed to determine which stages of development are crucial for the induction of worker vs nymph development by reproductive individuals.

In addition to nymph/worker caste ratio regulation demonstrated in the current and the previous study (Hayashi et al., 2007), neotenics are known to suppress differentiation of new neotenics through pheromones in R. speratus (Matsuura et al., 2010), and some other termite species (Castle, 1934; Lüscher, 1961). In natural colonies of Reticulitermes, occurrence of neotenics is common (Howard & Haverty, 1980; Matsuura et al., 2009). These facts suggest that neotenics are not only egg producers but also very important regulators of caste composition of the colonies in R. speratus.

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