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RESEARCH ARTICLE

Regulation of neotenic differentiation through direct physical contact in the damp-wood termite *Hodotermopsis sjostedti*

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Abstract In cooperative societies such as those of ants, honey bees, and termites, the number of reproductives is often regulated by social interactions. In many termite species, helper individuals (i.e., larvae or workers) can potentially differentiate into a "neotenic" reproductive caste in the absence of reproductives. In some termite species, multiple neotenics coexist within a nest, often with female-biased sex ratios. However, although the presence of female neotenics can suppress neotenic differentiation of female workers, it is largely unknown how male neotenics affect the differentiation of female neotenics. Here, we show that male and female neotenics regulate the neotenic differentiation in a sex-specific manner in the damp-wood termite *Hodotermopsis sjostedti*, whose colonies are often headed by multiple male and female neotenics in the field. Our rearing experiments showed that the presence of female neotenics suppressed differentiation of female neotenics from fourth- to seventh-larvae, i.e., pseudergates (called as "workers" in this study), whereas male neotenics promoted the differentiation of female neotenics. Moreover, the results of rearing experiments that restricted physical contact between neotenics and workers suggested that these effects were not mediated by volatile chemicals, but rather by direct contact. We found that the male neotenics were frequently groomed by female workers, suggesting that these interactions promote the differentiation of female neotenics. Our results represent an empirical evidence that the neotenic differentiation from female and male workers is regulated by direct physical contact with the preexisting neotenics.

Keywords Caste differentiation · Grooming behavior · Pseudergate · Sex ratio · Social interaction · Supplementary reproductive

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Introduction

In cooperatively breeding animals, societies potentially face conflicts over reproduction (Ratnieks et al. 2006; Cant 2012). Understanding how the conflicts are resolved is a central question in evolutionary biology (Maynard Smith and Szathmáry 1995). Eusocial insect colonies represent sophisticated systems of interaction among colony members, including the reproductive division of labor (Wilson 1971; Bourke and Franks 1995; Bignell et al. 2011). In many eusocial insects, most colony members retain the potential to reproduce, but only one or a few individuals

play reproductive roles. Therefore, revealing how the number of reproductives is regulated is important for understanding the organization of such insect societies. One of the key mechanisms that inhibit helpers from reproducing involves chemicals produced by the reproductives, which control the physiological state of the helpers (Le Conte and Hefetz 2008; Kocher and Grozinger 2011).

In termites, one of the major social insect groups, many basal species (often called "lower" termites) have older juveniles (larvae), called "pseudergates" which are totipotent immature working stages having the potential to differentiate into all other castes (pseudergate *sensu lato*; Roisin and Korb 2011). These pseudergates can differentiate into "neotenics" (also called supplementary or replacement reproductives), i.e., a reproductive caste that lacks some imaginal characteristics such as wing development (Roisin and Korb 2011). Neotenics appear after the death of the primary reproductives (king and queen) and take over the reproductive roles within their natal colonies (Korb and Hartfelder 2008). In the neotenic-headed colonies, the differentiation of neotenics from female pseudergates is prevented by pheromones released by female reproductives (Lüscher 1961). However, despite the suppression of differentiation of female neotenics, multiple female neotenics are often found within single colonies, and the sex ratio of the neotenics is strongly female-biased (Howard and Haverty 1980; Lenz and Barrett 1982; Lenz 1985), suggesting the sex-specific regulation of reproductive-caste differentiation.

Lüscher (1961) proposed a model of the sex-specific regulation of reproductive-caste differentiation via pheromones based on the results of a series of rearing experiments in *Kalotermes flavicollis*: both female and male reproductives suppress neotenic differentiation from same-sex pseudergates, so that male reproductives promote the differentiation of female neotenics when female reproductives are absent in a nest. This suggests that male and female neotenics had opposite effects (i.e., promotion vs. inhibition) on the differentiation of female neotenics. Although neotenic-headed colonies of *K. flavicollis* typically comprise one pair of neotenics, many other species have multiple neotenics in their colonies (Lenz 1985). However, previous studies have ignored the effects of males in species with multiple neotenics.

For the mechanisms that regulate the number of reproductives, the transmission mode of pheromones (i.e., volatile or non-volatile chemicals) is also important. If the pheromone is a non-volatile substance, pheromone transmission via direct physical contact is required to prevent non-reproductive helpers from differentiating into extra reproductives. On the other hand, volatile substances can spread over long distances without direct interaction and can exhibit colony-wide effects on reproductive differentiation. In termites, transmission modes differ among species, and these differences affect the regulation of caste differentiation (Sevala et al. 2000; Matsuura et al. 2010; Yaguchi et al. 2016). Liebig et al. (2009) proposed that direct contact was a likely candidate mechanism for regulation of neotenic differentiation in basal termites with relatively small colony sizes.

The damp-wood termite *Hodotermopsis sjostedti* is a basal species with the linear caste developmental pathway (Miura et al. 2000, 2004; Legendre et al. 2013). In this species, the older larvae (from fourth- to around seventh-instar larvae) function as pseudergates (false workers) that play worker roles but have potential to molt into neotenics, nymphs, and presoldiers (Miura et al. 2000, 2004; Oguchi et al. 2016). In the field, colonies are often headed by multiple male and female neotenics (Matsumoto and Hirono 1985; Miura et al. 2000), and the sex ratio of the neotenics is biased toward females (Hirono 1990; Osamu Kitade unpubl. data). However, the regulatory mechanism of neotenic differentiation has yet to be elucidated in this species. In this study, we used *H. sjostedti* to investigate the effects of male and female neotenics on the differentiation of additional neotenics under different conditions in artificial colonies: order-instar larvae functioning as pseudergates (workers) were reared with a pair of male and female neotenics, with a single neotenic of either sex, or without neotenics.
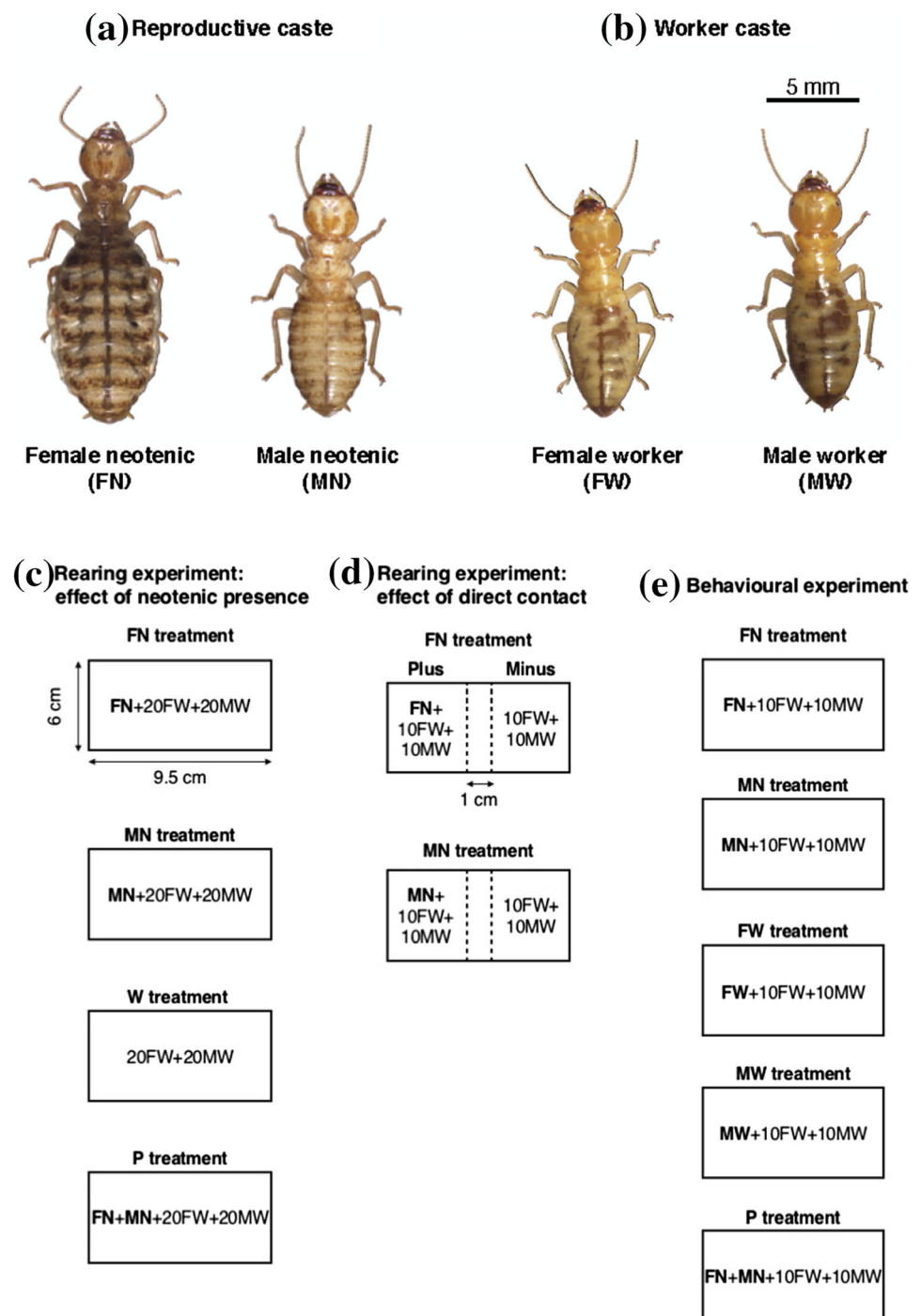
Materials and methods

Termite species

Colonies of *H. sjostedti* were collected in 2014 and 2015 on Yakushima Island, Kagoshima, Japan. The stock colonies were kept with nest logs in the laboratory, at approximately 25 °C under constant darkness. Moistened pine wood was occasionally supplied as food.

In many one-piece nesting termites, working immatures after third instar (i.e., pseudergates sensu lato) can differentiate into neotenics (Korb and Hartfelder 2008). Therefore, in this study, larvae from fourth to seventh instars were defined as pseudergates (hereafter called "workers", Fig. 1). The sex of workers was discriminated by the external morphology of the seventh abdominal sternite (Miura et al. 2000). We randomly selected workers from stock colonies.

Effects of neotenic presence on neotenic differentiation

To examine the effect of the presence of neotenics on the differentiation of both female and male workers, we established four types of experimental sub-colonies

Fig. 1 Castes used in this study and the experimental designs. **a** Female and male neotenics. **b** Female and male workers. **c** Four categories (pairings of neotenics and workers) were established as the experimental treatments; *FN* female neotenic, *MN* male neotenic, *FW* female worker, *MW* male worker, *W* a worker-only treatment without any neotenics, *P* a pair treatment with a pair of neotenics. **d** Plus and Minus for the FN and the MN treatment indicate worker categories in the arenas with and without a male/female neotenic. **e** Behavioral experiment to observe behavioral interactions, i.e., grooming behavior (color figure online)

(a) Reproductive caste

Female neotenic (FN)   Male neotenic (MN)

(b) Worker caste

5 mm

Female worker (FW)   Male worker (MW)

(c) Rearing experiment: effect of neotenic presence

FN treatment
6 cm | FN+20FW+20MW
9.5 cm

MN treatment
MN+20FW+20MW

W treatment
20FW+20MW

P treatment
FN+MN+20FW+20MW

(d) Rearing experiment: effect of direct contact

FN treatment
Plus   Minus
FN+ 10FW+ 10MW | 10FW+ 10MW
1 cm

MN treatment
MN+ 10FW+ 10MW | 10FW+ 10MW

(e) Behavioural experiment

FN treatment
FN+10FW+10MW

MN treatment
MN+10FW+10MW

FW treatment
FW+10FW+10MW

MW treatment
MW+10FW+10MW

P treatment
FN+MN+10FW+10MW

comprising 40 workers (20 female and 20 male workers that were randomly selected from stock colonies) with or without neotenics, which derived from three stock colonies (Fig. 1c): (1) a female neotenic (FN) treatment with 40 workers and a single female neotenic ($n = 7$), (2) a male neotenic (MN) treatment with 40 workers and a single male neotenic ($n = 7$), (3) a pair (P) treatment with 40 workers and a single pair of male and female neotenics ($n = 6$), and (4) a worker (W) treatment only with 40 workers ($n = 7$).

The 40 selected workers were marked with different enamel colors to identify their sex. Then each sub-colony was kept in a polystyrene case (9.5 cm × 6 cm × 2 cm) with enough food (a mixture of wood sawdust and cellulose powder) and water. We observed each sub-colony daily for 32 days. Because workers can molt into either an older-instar worker (progressive molt) or a neotenic (neotenic differentiation), the castes and sexes of molted individuals were recorded. Molted individuals were removed from the

sub-colonies to exclude unfavorable effects on the neotenic differentiation by the newly molted individuals. We compared the total number of molts and the proportion of neotenic differentiation against the total molts among the treatments. We excluded data from sub-colonies in which no workers molted (W treatment: 1/7; FN treatment: 2/7; MN treatment: 1/7).

Effects of direct contact on neotenic differentiation

To identify the mode of interactions among colony members that induce or inhibit the neotenic differentiation, experimental sub-colonies were set with the same polystyrene cases used in the first experiment, but with a barrier to prevent workers from contacting directly with a female or male neotenic (Fig. 1d). We used three stock colonies containing male and female neotenics. Each polystyrene case was divided into two arenas by double metal-mesh walls (mesh size $= \Phi 1$ mm). In one of the two arenas, either a female neotenic ($n = 9$) or a male neotenic ($n = 9$) was placed with 20 workers (10 males and 10 females), whereas the other arena contained only 20 workers (10 males and 10 females). The former treatment was defined as "FN plus" or "MN plus" treatment, and the latter treatment as the "FN minus" or "MN minus" treatment, respectively. As described above, the number of neotenics differentiated from male and female workers was recorded. Data from sub-colonies in which no workers molted during the observation were excluded from the analyses (MN minus: 1/9; FN plus: 1/9; FN minus: 1/9).

Behavioral observations

To reveal sex-biased behavioral interactions between neotenics and workers, five types of sub-colonies were established from five stock colonies containing various combinations of male and female neotenics and workers (Fig. 1e): (1) P treatment with 20 workers (randomly selected 10 female and 10 male workers) and a pair of male and female neotenics ($n = 6$), (2) FN treatment with 20 workers and a female neotenic ($n = 9$), (3) MN treatment with 20 workers and a male neotenic ($n = 9$), (4) FW treatment with 20 workers and an accompanying female worker ($n = 9$), and (5) MW treatment with 20 workers and an accompanying male worker ($n = 9$). Workers except for accompanying workers in the FW and MW treatments were marked with different enamel colors to distinguish their sexes. Then the sub-colonies were kept in the same cases as used in the above experiments, although the cases were lined with filter paper moistened with distilled water. On the day after colony establishment, observations were carried out to record the number of allogrooming behavior between workers and neotenics for 20 min. In the P treatment with a pair of neotenics, behavioral observations were made for 40 min (20 min per neotenic) to standardize the observation time per a neotenic. The frequency of allogrooming behavior from each sex of workers against neotenics was recorded, respectively. In this experiment, allogrooming behavior was defined as situations in which a worker groomed any body part of a neotenic. The comparisons between the condition with a single neotenic (Single condition) and that with a pair of reproductive (Pair condition) were also carried out.

Statistical analyses

Generalized linear mixed models (GLMMs) with a Poisson error structure were used to compare the frequency of allogrooming and molting between treatments. For the proportion of neotenic differentiation and the sex ratio, GLMM with binomial error structure was applied. The colony number was assigned as a random effect. If there was a statistical significance among treatments, the sequential Bonferroni correction to the pairwise comparison was performed to correct the p value ($\alpha < 0.05$). All statistical analyses were carried out using R 3.1.2 (R Core team 2014).

Results

Effects of neotenic existence on neotenic differentiation

We carried out rearing experiments to investigate how neotenic differentiation was regulated by the presence of male and female neotenics (Summarized data in Table S1). For female workers, the total number of molting events was significantly higher in the W and the MN treatment than in the FN treatment (Fig. 2a; W vs. MN: $\chi^2 = 1.89$, $p = 0.169$; W vs. FN: $\chi^2 = 5.147$, $p = 0.023$; MN vs. FN: $\chi^2 = 52.04$, $p < 0.001$). For male workers, however, there was no significant difference in the total number of molting events among the treatments (Fig. 2b; $\chi^2 = 5.025$, $p = 0.081$). In the paired (P) treatment, few workers differentiated into neotenics (1/20 female workers, 0/19 male workers). Therefore, we excluded data of P treatments from statistical analyses.

In the MN treatment, almost all female workers molted differentiated into neotenics, so that the proportion of neotenic differentiation (the number of neotenic differentiations divided by the total number of molting events) was significantly higher than in the W and the FN treatments (Fig. 2c; W vs. MN: $\chi^2 = 22.69$, $p < 0.001$; FN vs. MN: $\chi^2 = 57.674$, $p < 0.001$). Also, the proportion in the W treatment was significantly higher than that in the FN treatment (Fig. 2c; W vs. FN: $\chi^2 = 19.09$, $p < 0.001$). In

Fig. 2 Total number of molts and frequency of neotenic differentiation in the various treatments (Fig. 1c). **a** Number of molts from female workers. **b** Number of molts form male workers. **c** Proportions of neotenic differentiation from female workers (the number of neotenic differentiations divided by the total number of molting events). **d** Proportions of neotenic differentiation from male workers. Different alphabets indicate significant differences (paired-difference likelihood-ratio test with sequential Bonferroni correction, $\alpha < 0.05$). The paired (P) treatment was omitted since fewer than 6% of individuals underwent moltings
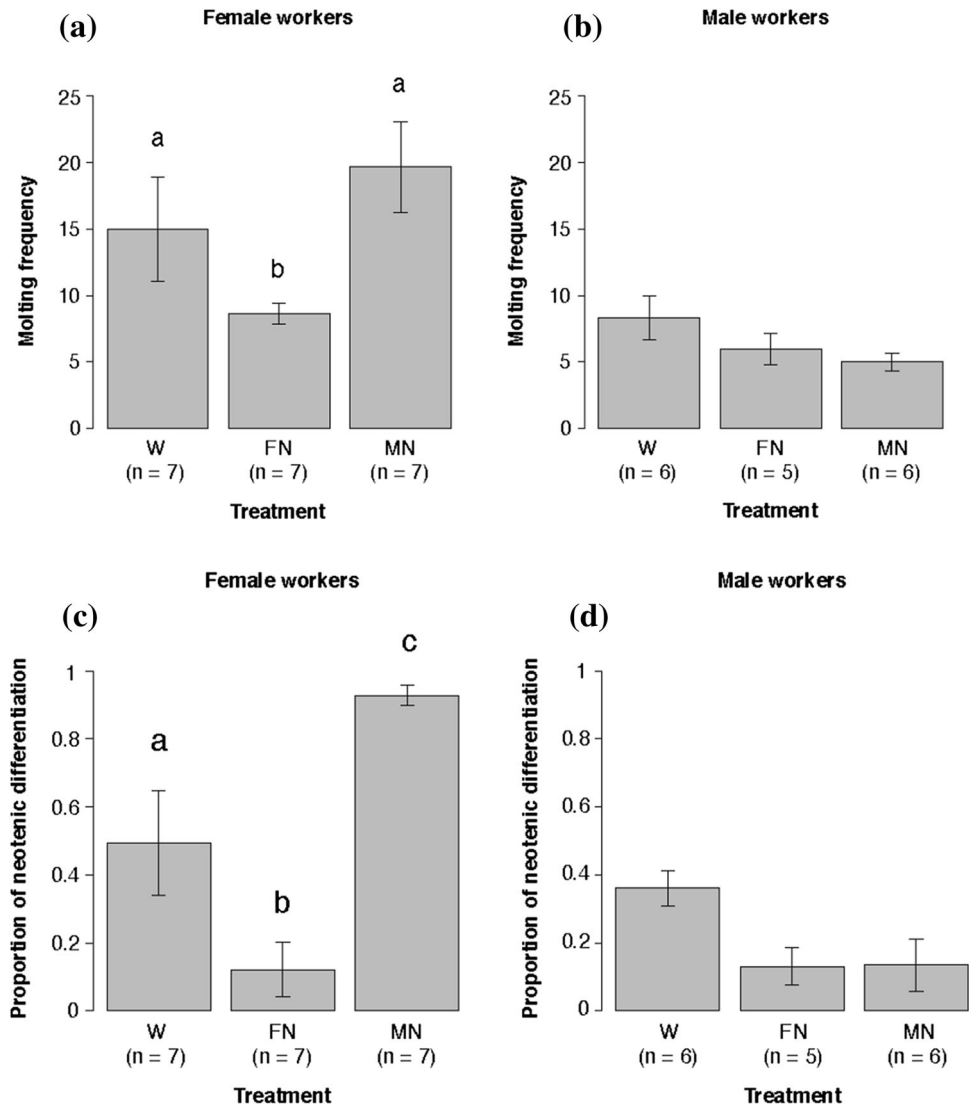
contrast, the proportions of male workers that differentiated into neotenics did not significantly differ among the treatments (Fig. 2d; $\chi^2 = 3.504$, $p = 0.173$).

In the W treatment, the total number of molting events did not significantly differ between female and male workers ($\chi^2 = 0.2834$, $p = 0.5945$), although the proportion of neotenic differentiation was significantly different between them ($\chi^2 = 7.9435$, $p = 0.005$).

Effects of direct contact on neotenic differentiation

In the rearing experiments in which each polystyrene case is partitioned into two arenas (Summarized data in Table S2), female workers in the MN plus treatment (the arena with a male neotenic) differentiated into female neotenics significantly more frequently than those in the MN minus treatment (the arena without a male neotenic; Fig. 3a; $\chi^2 = 4.455$, $p = 0.035$). In contrast, the proportion of

female workers that differentiated into neotenics in the FN plus treatment (the arena with a female neotenic) was significantly lower than that in the FN minus treatment (the arena without a female neotenic; Fig. 3b; $\chi^2 = 6.04$, $p = 0.014$). However, for the proportion of neotenic differentiation of male workers, there were no significant differences between FN minus and FN plus and between MN minus and MN plus treatments (Fig. S1; FN plus vs. FN minus: $\chi^2 = 0.015$, $p = 0.904$; MN plus vs. MN minus: $\chi^2 = 0.658$, $p = 0.417$).

Behavioral observations

As the results of observations on grooming behavior, both female and male neotenics were groomed more frequently than workers (Fig. 4a; FN vs. FW: $\chi^2 = 245.14$, $p < 0.001$; FN vs MW: $\chi^2 = 222.66$, $p < 0.001$, $p = 0.023$; MN vs. FW: $\chi^2 = 109.78$, $p < 0.001$; MN vs. MW: $\chi^2 = 94.142$,
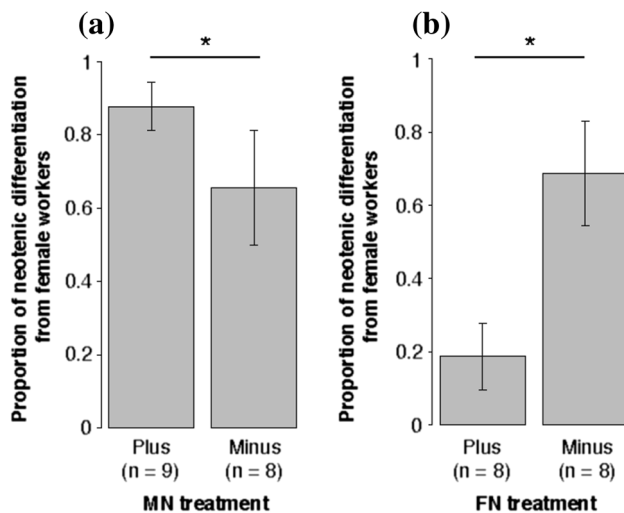
Fig. 3 Proportions of neotenic differentiation from female workers in the experiments using two-arena cases to determine the effects of contact with neotenics (Fig. 1d, see "Materials and methods"); **a** male neotenic (MN) and **b** female neotenic (FN) treatments. Plus and minus, respectively, indicate arenas with and without neotenics. Asterisks indicate significant differences between the treatments (sequential Bonferroni correction; $p < 0.05$). Values are mean ± SE

$p < 0.001$). There was no significant difference between the FW and MW treatments (Fig. 4a; FW vs. MW: $\chi^2 = 0.681$, $p = 0.41$). When focusing on grooming behavior of workers to neotenics, female neotenics were groomed significantly more frequently than male neotenics in the FN and MN treatments (Single condition), in which a single female or male neotenic was accompanied by workers (Fig. 4a; $\chi^2 = 29.897$, $p < 0.001$). This tendency was also observed in the Pair condition, in which a pair of neotenics were accompanied by workers (Fig. 4b; $\chi^2 = 27.021$, $p < 0.001$). When focusing on the sexes of grooming and groomed individuals, male neotenics were groomed more frequently by female workers in the Single condition (i.e., MN treatment) than in the Pair condition (Fig. 4c; $\chi^2 = 8.182$, $p = 0.004$). In contrast, the frequency of allogrooming of male workers against female neotenics did not significantly differ between the two conditions with a single neotenic or a pair of neotenics (Fig. 4d; $\chi^2 = 1.473$, $p = 0.225$).

Discussion

In this study, we examined the regulatory effects of female and male neotenics on the additional neotenic differentiation of workers in *H. sjostedti*. Rearing experiments and behavioral observations provided five noteworthy findings. (1) Female neotenics suppressed the neotenic differentiation from female workers (Fig. 2a). (2) Male neotenics promoted neotenic differentiation from female workers when
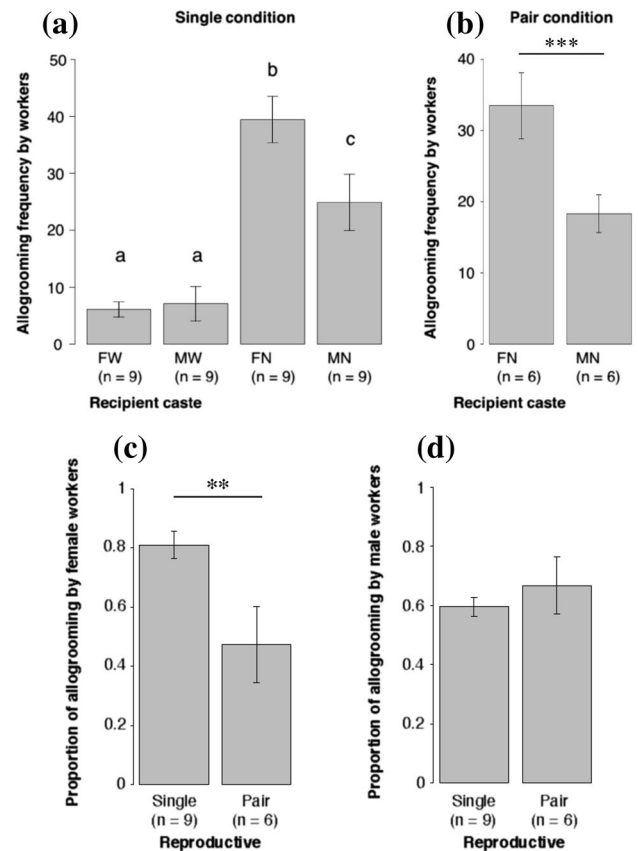

Fig. 4 Comparisons of the total number of allogrooming behaviors by workers in **a** the treatment with and without a single neotenic (single) and **b** in the treatments with a pair of neotenics (pair) (Fig. 1e). Different alphabets on values (mean ± SE) indicate significant differences (likelihood-ratio test with sequential Bonferroni correction; $p < 0.05$ for the single and $p < 0.001$ for the pair treatments). **c** Allogrooming frequency by female workers against a male neotenic. **d** Allogrooming frequency by male workers against a female neotenic. An asterisk indicates a significant difference between the single and pair treatments (paired-difference likelihood-ratio test with sequential Bonferroni correction; $p < 0.01$)

female neotenics were absent (Fig. 2c). (3) Female neotenics did not promote neotenic differentiation from male workers (Fig. 2d). (4) Male neotenics promoted the neotenic differentiation from female workers via direct contact (Fig. 3). (5) Male neotenics were groomed more frequently by female workers rather than by male workers when female neotenics were absent, although the frequency did not differ under the presence of both sexes of neotenics (Fig. 4).

In many termite species, the existence of sex- and caste-specific pheromones has been suggested (Liebig et al. 2009; Weil et al. 2009; Hanus et al. 2010; Matsuura et al. 2010; Hoffmann et al. 2014), and thus it is likely that pheromonal signals are involved in the sex-specific effects on caste differentiation. In *Reticulitermes speratus*, highly volatile chemicals suppressed neotenic differentiation from female workers (Matsuura et al. 2010). However, the present

experiments using the partitioned arenas strongly suggested that information on the presence of female neotenics was transmitted to workers, not by volatile chemical substances, but by direct contacts.

However, it is also possible that the modes of pheromonal transmission depend on colony size. In *Reticulitermes*, colony size ranges from 51,505 to 363,512 (Howard et al. 1982; reviewed in Thorne et al. 1999), whereas *H. sjostedti* has a relatively small colony size, ranging from 345 to 20,558 (Osamu Kitade unpubl. data). In some primitive ant species with small colony sizes, the queen information is transferred to workers by direct contact (Gobin et al. 1999; Tsuji et al. 1999; Liebig et al. 2000; Dietemann et al. 2005), although, instead of direct contact, queen-derived eggs convey the queen information in large colonies (Endler et al. 2004). Therefore, the transmission modes of the presence of reproductives may have evolved independently in ants and termites, in response to colony sizes.

Workers groomed female neotenics more frequently than other nestmates, suggesting that the female neotenics attract them by some chemical substances (i.e., pheromones). In *Zootermopsis nevadensis*, reproductive-specific cuticular hydrocarbon profiles are suggested to induce these behaviors and thus strengthen social interactions (Liebig et al. 2009). Therefore, it is plausible that social communications mediated by non-volatile chemical profiles of neotenics could transmit information about their presence, leading to the inhibition of neotenic differentiation.

Our results showed that volatile substances released from male neotenics were not the candidate pheromone transmitted to workers. In our behavioral observations, male neotenics were frequently groomed by female workers under the absence of female neotenics, although this tendency greatly decreased when both male and female neotenics were present. These results correspond to the results of our rearing experiments (Fig. 2c), in which most female workers (about 90%) differentiated into neotenics in the situation with male neotenics and without female neotenics. Therefore, it is possible that female workers received some chemical substances from male neotenics, causing the physiological changes to differentiate into neotenics. In *Z. nevadensis*, the induction of presoldier differentiation was attributable to proctodeal trophallaxis from a primary female reproductive (Maekawa et al. 2012; Yaguchi et al. 2016). Thus, similar mechanisms could also be utilized for the induction of female neotenic differentiation.

Although the suppression of differentiation by female neotenics seems to be almost always effective regardless of the presence of male neotenics, the promotion by male neotenics was not effective under the presence of female neotenics. Our behavioral observations showed that the attraction of female workers to male neotenics dramatically decreased in the treatments with a pair of reproductives (P treatment; Fig. 4a). Taken together, it is suggested that the inducing effect of a male neotenic on the female neotenic differentiation is facultative; male neotenics do not release the induction pheromone, or workers do not react to the induction pheromones, under the presence of female neotenics. These imply that the presence of female neotenics affects the physiological states of male neotenics or female workers.

Previous field studies in the focal species reported a female-biased sex ratio in neotenic-headed colonies (Hirono 1990; Osamu Kitade unpubl. data). It is known, in termites, that the types of reproductive affect sex ratio in colonies (Roisin and Lenz 2002). Combining our results with nest structures (i.e., a colony can be divided into multiple nests), the female-biased sex ratio of neotenic-headed colonies in the focal species is suggested to be adaptive. Lenz (1985) proposed that nest structure is a factor determining the composition of neotenics within a termite colony. Under field conditions, mature colonies of *H. sjostedti* consist of multiple nest sites connected by underground trails (Matsumoto and Hirono 1985; Kitade et al. 2012).

We also revealed that the proportion of neotenic differentiation from female workers was higher than that from male workers in colonies without any neotenics (W treatments; Fig. 2). Therefore, the sexual differences in the ability of neotenic differentiation and the strong induction effect by male neotenics would together promote the neotenic differentiation from female workers in a nest lacking female neotenics. Male workers differentiated into neotenics much less frequently than female workers even in the W treatment that comprised workers only (Fig. 2c, d). If male neotenics actively stimulated female workers to differentiate into neotenics, an excess number of male neotenics could potentially reduce the colony efficiency such as the tragedy of commons in *Melipona* bees (Wenseleers and Ratnieks 2004). We speculate that a low proportion of neotenic differentiation from male workers might have a selective advantage at the colony level, although the relationship between the low proportion and the selective advantage should be clarified.

As for the physiological mechanisms underlying the regulation of neotenic differentiation, juvenile hormone is a key endocrine mediator that controls caste differentiation in termites (Nijhout and Wheeler 1982; Miura and Scharf 2011; Korb 2015). Therefore, further studies on the hormonal actions that mediate between social interactions and the induced neotenic differentiation will provide further insights into the sex-specific differentiation of neotenic reproductives in termites.

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