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**Sex-specific inhibition and stimulation of worker-reproductive transition in a termite**

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**Short Title:** Termite worker-reproductive transition

1 **Abstract**

2 In social insects, the postembryonic development of individuals exhibits strong phenotypic  
3 plasticity in response to environment, thus generating the caste system. Different from eusocial  
4 Hymenoptera, in which queens dominate reproduction and inhibit worker fertility, the primary  
5 reproductive caste in termites (kings and queens) can be replaced by neotenic reproductives  
6 derived from functionally sterile individuals. Feedback regulation of nestmate differentiation into  
7 reproductives has been suggested, but the sex-specificity remains inconclusive. In the eastern  
8 subterranean termite, *Reticulitermes flavipes*, we tested the hypothesis that neotenic  
9 reproductives regulate worker-reproductive transition in a sex-specific manner. With this *R.*  
10 *flavipes* system, we demonstrate a sex-specific regulatory mechanism with both inhibitory and  
11 stimulatory functions. Neotenic reproductives inhibit workers of the same sex from differentiating into  
12 additional reproductives, but stimulate workers of the opposite sex to undergo this transition.  
13 Furthermore, this process is not affected by the presence of soldiers. Our results highlight the  
14 extraordinary reproductive plasticity of termites in response to social cues, and provide insights  
15 into the regulation of reproductive division of labour in a hemimetabolous social insect.

16

17 **Keywords:** caste differentiation, developmental plasticity, ergatoid reproductive, *Reticulitermes*

18 *flavipes*

## 19 **Introduction**

20 Developmental plasticity plays an important role in the reproductive division of labour in social  
21 insects (Page & Amdam 2007). Caste differentiation in eusocial colonies is usually dependent on  
22 social stimuli as well as other environmental cues (Hartfelder & Engels 1998; Korb & Hartfelder  
23 2008). Although a fertilized egg is thought to be totipotent and able to develop into any caste,  
24 only a few individuals eventually become reproductives. For example, female honeybee larvae  
25 that are fed with royal jelly develop into queens, while others become workers (Kucharski et al.  
26 2008). The presence of queens in social Hymenoptera also inhibits worker reproduction by  
27 directly suppressing their ovarian development, or through policing behaviour (Le Conte & Hefetz  
28 2008).

29         As with most social insects, termites have caste systems resulting from developmental  
30 plasticity. In contrast to social Hymenoptera, hemimetabolous termites have both males and  
31 females for all castes. Termite colonies are typically founded by a pair of dispersing alates,  
32 which become the primary reproductives, i.e., kings and queens. In many “higher” termite genera  
33 (Termitidae) and most “lower” termite genera (all other termite families), workers and nymphs  
34 can differentiate into neotenic reproductives (ergatoids and nymphoids, respectively) and  
35 reproduce in the natal colony (Myles 1999; Roisin 2000; Roisin & Korb 2011). Neotenic  
36 reproduction is implicated to play a critical role in the early evolution of termite eusociality  
37 (Myles 1999). The fact that neotenic develop in response to orphaning (the absence of  
38 reproductives) has led to the prevailing hypothesis that fertile reproductives would inhibit sexual  
39 development (Long, Thorne & Breisch 2003; Matsuura et al. 2010; Moore 1974; Noirot 1990). A  
40 few studies, however, proposed the stimulatory effects of reproductive individuals on this  
41 process. For example, in *Mastotermes darwiniensis*, neotenic reproductives were produced in the

42 presence rather than the absence of neotenic, and female neotenic exhibited stronger  
43 stimulatory activities on workers of both sexes than males (Watson, Metcalf & Sewell 1975). In  
44 *Kaloterme flavicollis*, the production of female neotenic was promoted by the presence of a  
45 single male neotenic, while the stimulatory effect was not observed from female neotenic  
46 (Lüscher 1964). Although kings and queens can both be replaced by neotenic, the sex-  
47 specificity for either inhibition or potential stimulation is not conclusive in termites.

48 *Reticuliterme*, one of the most widely distributed termite genera in the world with  
49 substantial economic and ecological importance (Su, Scheffrahn & Cabrera 2001), is an ideal  
50 system to study developmental plasticity. *Reticuliterme* workers have three morphologically,  
51 behaviourally and functionally distinct developmental trajectories. They can undergo *status quo*  
52 moults and remain as workers, differentiate into pre-soldiers followed by an additional moult into  
53 soldiers, or develop into neotenic reproductives (i.e., ergatoids) (Lainé & Wright 2003; Zhou, Oi &  
54 Scharf 2006). Our preliminary study in the eastern subterranean termite *Reticuliterme flavipes*  
55 indicated that worker-reproductive transition was a lengthy process under orphaning condition  
56 (30-90 days). If one of the reproductives (e.g., queen) is lost, a stimulatory function from the  
57 remaining reproductive (e.g., king) that promotes the formation of neotenic reproductives of the  
58 missing sex (e.g., female ergatoid) would be beneficial to the colony. We hypothesized that  
59 worker-reproductive transition is regulated in a sex-specific manner in *R. flavipes*. Specifically,  
60 reproductive individuals inhibit same-sex workers, but stimulate opposite-sex workers to  
61 differentiate into ergatoids. To test this, we evaluated ergatoid formation in response to the  
62 presence or absence of male or female reproductives. As soldier caste is present in all termite  
63 species (Tian & Zhou 2014), and previous studies suggest that soldiers potentially promote

64 differentiation of reproductives (Watanabe et al. 2014), we also examined the effect of soldiers  
65 on ergatoid formation.

66

## 67 **Methods**

### 68 **Study System**

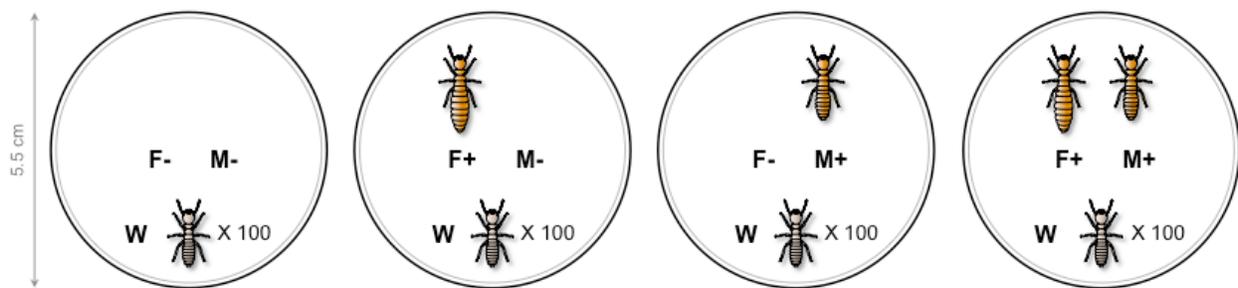
69 Colonies of *R. flavipes* were collected from the Arboretum (Lexington, Kentucky, USA), and the  
70 Red River Gorge area, Daniel Boone National Forest (Slade, Kentucky, USA). Colonies  
71 consisted of workers, soldiers and nymphs at the time of collection. Freshly collected termites  
72 were kept in Petri dishes and fed on moistened unbleached paper towel for one to two weeks.  
73 Neotenic-headed colonies were obtained by transferring field collected termites to closed plastic  
74 boxes (45.7 × 30.6 × 15.2 cm) filled with moistened wood mulch and pinewood blocks and  
75 maintained for 6 months, when eggs and larvae appeared indicating the presence of fertile  
76 neotenic reproductives. Primary-headed colonies were established by pairing female and male  
77 sibling alates collected in Lexington, Kentucky, and they had been maintained in laboratory for 5  
78 years by the time experiments started. All stock colonies and experimental termites were  
79 maintained at  $27 \pm 1^\circ\text{C}$  in complete darkness.

80

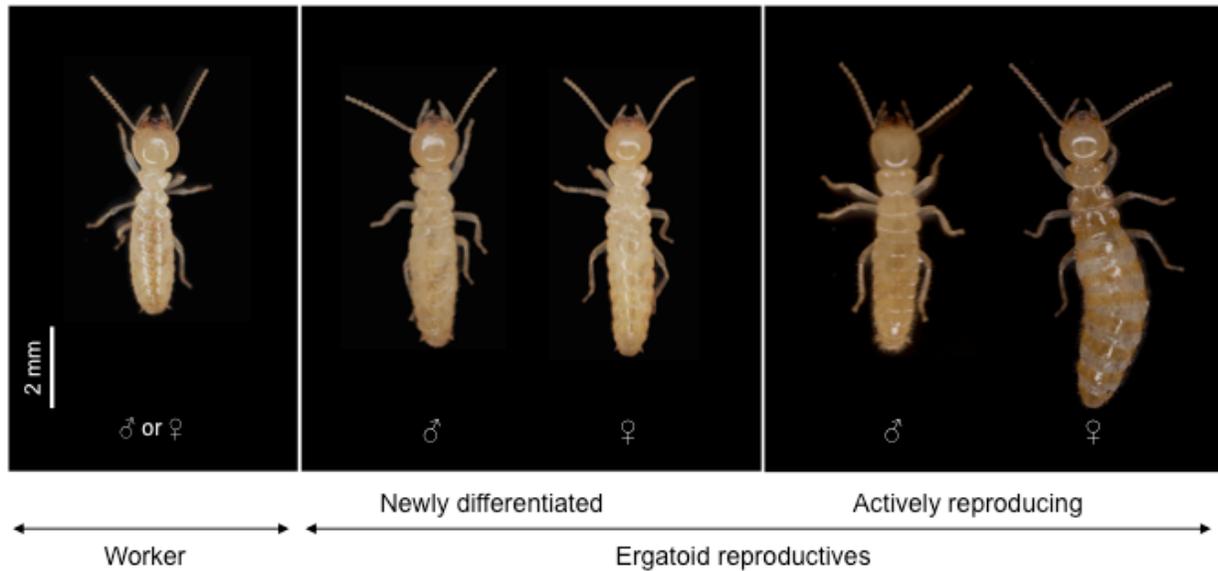
### 81 **Bioassay to Test Sex-specific Regulation**

82 Fertile ergatoids of different sexes were used to test their influences on worker-reproductive  
83 transition. These ergatoids were obtained from an orphaning assay, in which groups of 100  
84 workers were kept in Petri dishes (6.0 cm in diameter, 1.5 cm in height) lined with moistened  
85 unbleached paper towel for 60 days. Ergatoids that were actively reproducing (i.e., with eggs  
86 present in dishes) were used in the subsequent assay.

87           The same set-up was used to test how workers differentiate in response to ergatoids (Fig.  
88 1). Groups of 100 workers were kept with: 1) no reproductives (F-M-); 2) one female ergatoid  
89 (F+M-); 3) one male ergatoid (F-M+); or 4) one pair of ergatoids (F+M+). The ergatoids and  
90 workers were from the same colony in each group. Fourteen replications using three colonies  
91 were conducted, with one colony originally primary-headed and two colonies originally  
92 neotenic-headed. Worker differentiation was observed daily for 60 consecutive days, and newly  
93 formed ergatoids were removed and their sex was determined. Ergatoids were recognized by  
94 slightly heavier cuticle pigmentation, elongated abdomen, and wider thorax than workers (Fig.  
95 2). Female ergatoids were distinguished from males by their enlarged 7th abdominal sternite. We  
96 also removed eggs, newly formed pre-soldiers and any inter-caste (individuals with the degree of  
97 mandible development intermediate between workers and pre-soldiers) every day to prevent their  
98 potential influence on worker development.  
99



100  
101 **Figure 1. Experimental set-up.** Each group of 100 workers were placed in a Petri dish lined with moistened  
102 unbleached paper. Workers were kept with no reproductives (F-M-), one female ergatoid (F+M-), one male ergatoid  
103 (F-M+), or one pair of ergatoids (F+M+). Newly formed ergatoids, pre-soldiers, and eggs laid by reproductives were  
104 removed every day for 60 consecutive days. FR: female ergatoid reproductive; MR: male ergatoid reproductive; W:  
105 worker. Ergatoid reproductives used in the assay were actively reproducing, and female ergatoids were physogastric.



106

107 **Figure 2. Photographs of a worker, young ergatoids, and mature ergatoids.** The young ergatoids were about 10  
108 days post differentiation. The mature ergatoids were six months post differentiation and actively reproducing.

109

### 110 **Bioassays to Test Soldier Effect**

111 Two orphaning assays were conducted to test influence of soldier caste on ergatoid  
112 differentiation. The first assay (“single-soldier orphaning assay”) simulated natural orphaning  
113 condition where freshly collected workers were separated into groups of 100 individuals, and  
114 each group was placed with one soldier (Soldier+) or no soldier (Soldier-). Termites were  
115 maintained in Petri dishes (6.0 cm in diameter, 1.5 cm in height) lined with moistened  
116 unbleached paper towel as food source. Termites were allowed to undergo changes in caste  
117 differentiation in the dishes without disturbance, and caste composition and mortality of each  
118 group was documented at the end of 60 days. A total of 42 and 45 replicates from four colonies  
119 were conducted for soldier+ and soldier- treatment, respectively.

120 We further performed the second assay (“multiple-soldier orphaning assay”), which was  
121 conducted with an increased soldier stimulus and observed daily for 60 consecutive days. Groups

122 of 100 workers were isolated from neotenic-headed colonies, and they were companioned with  
123 either four soldiers (Soldier++) or no soldier (Soldier-). Newly differentiated individuals  
124 (ergatoids and pre-soldiers) were removed to prevent their potential influence on worker  
125 development, and an equal number of workers to the removed individuals were added to the  
126 group to keep group size consistent. Termites were maintained in Petri dishes (3.5 cm in  
127 diameter, 1.5 cm in height) provided with moistened unbleached paper towel. A total of 10  
128 replicates from two colonies were conducted for both soldier++ and soldier- treatments.

129

### 130 **Data Analyses**

131 Data were analysed using Statistix 10 (Analytical Software, Tallahassee, FL, USA). In the assay  
132 that tested sex-specific regulation, Wilcoxon rank-sum test was performed on the cumulative  
133 numbers ergatoids between each treatment and the control on every assay day. In both single-  
134 and multiple-soldier orphaning assays, unpaired *t*-test was performed on numbers of  
135 differentiated individuals and mortality. To obtain values that fit the assumptions of parametric  
136 test, data were transformed through square root ( $x + 0.5$ ) on the combined numbers of pre-  
137 soldiers and soldiers in the single-soldier orphaning assay, and numbers of female and male  
138 ergatoids in the multiple-soldier orphaning assay. Because the pattern of regulation was  
139 consistent in all colonies, results were pooled for statistical analyses.

140

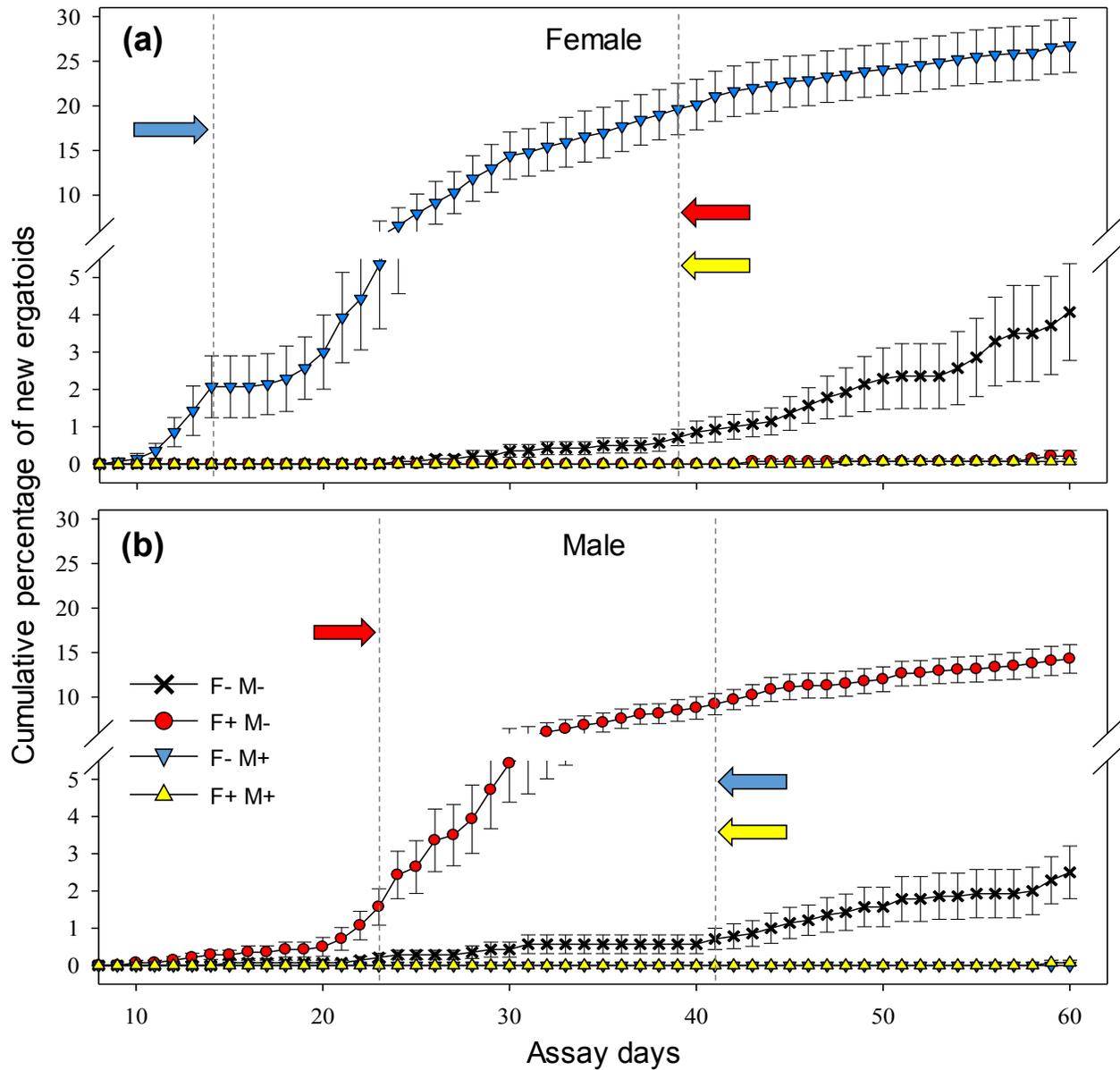
### 141 **Results**

#### 142 **Ergatoid Formation in Response to Reproductives of Different sexes**

143 Under orphaning condition (F-M-),  $4.1 \pm 1.3\%$  and  $2.5 \pm 0.7\%$  of workers differentiated into  
144 female and male ergatoids, respectively, in 60 days (mean  $\pm$  SEM, Fig. 3). The presence of a

145 single female (F+M-) or a pair of ergatoids (F+M+) significantly inhibited the formation of  
146 additional female ergatoids ( $0.2 \pm 0.2\%$  and  $0.1 \pm 0.1\%$ , respectively); however, the presence of  
147 a single male ergatoid (F-M+) significantly stimulated the formation of female ergatoid ( $26.8 \pm$   
148  $3.0\%$ ) (Fig. 3(a);  $P < 0.01$ , Wilcoxon rank-sum test, one-sided,  $n = 14$ ). Similarly, significantly  
149 fewer male ergatoids differentiated in the presence of a single male (F-M+) or a pair of ergatoids  
150 (F+M+) ( $0\%$  and  $0.1 \pm 0.1\%$ , respectively), while significantly more of them formed when a  
151 single female ergatoid was present (F+M-) ( $14.3 \pm 1.6\%$ ) (Fig. 3(b);  $P < 0.01$ , Wilcoxon rank-  
152 sum test, one-sided,  $n = 14$ ).

153           Within 60 days, female and male ergatoids differentiated in 10 and 9 replicates,  
154 respectively, out of 14 total replicates under the orphaning condition (F-M-). The formation of  
155 ergatoids in all 14 replicates was stimulated by a single ergatoid of the opposite sex. Under this  
156 stimulation, developmental time for the first ergatoid was significantly reduced (female:  $19 \pm 3.3$   
157 days,  $n = 14$  in F-M+, compared with  $38 \pm 3.7$  days,  $n = 10$  in F-M-; male:  $21 \pm 1.8$  days,  $n = 14$   
158 in F+M-, compared with  $35 \pm 5.4$  days,  $n = 9$  in F-M-; Wilcoxon rank-sum test, one-sided,  $P <$   
159  $0.01$ ). There was no significant difference on mortality within 60-day assay period among  
160 treatments (Fig. 4; ANOVA,  $F_{3,52} = 1.5$ ,  $P > 0.05$ ;  $n = 14$ ).



161

162 **Figure 3. Ergatoid formation in response to fertile ergatoids of different sexes.** Cumulative percentage of newly

163 differentiated female (a) and male (b) ergatoids is shown (mean  $\pm$  SEM). Stimulation (forward arrows) and

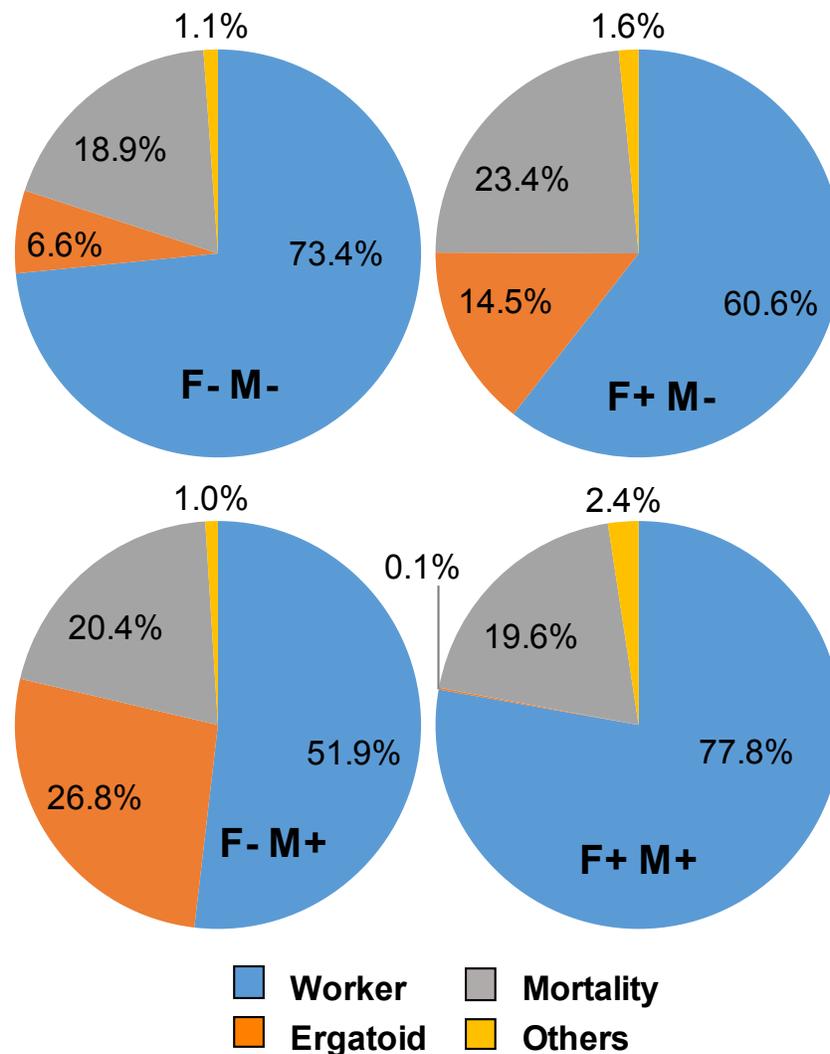
164 inhibition (reverse arrows) refer to significantly more and fewer ergatoids formed, respectively, relative to control

165 (F-M-), and dash line next to the tip of arrow indicates the day when the significant difference started (Wilcoxon

166 rank-sum test, one-sided,  $P < 0.01$ ;  $n = 14$  for all treatments). Symbols and arrows of the same colour correspond to

167 the same treatment.

168



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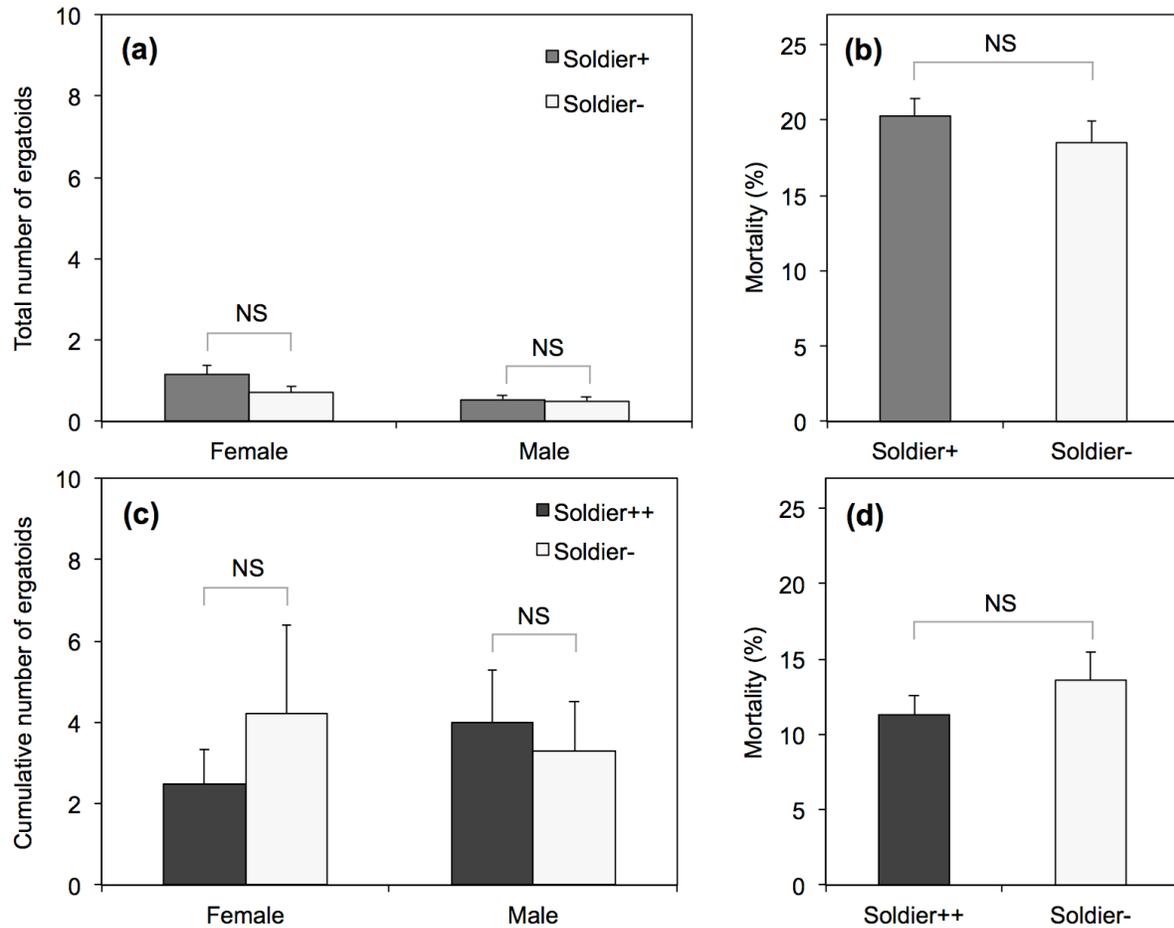
170 **Figure 4. Developmental endpoint of workers in 60 days.** “Others” includes pre-soldiers and inter-castes, and the  
171 latter refers to individuals with the degree of mandible development between workers and pre-soldiers after  
172 moulting. There was no significant effect of treatment on mortality (ANOVA,  $F_{3,52} = 1.5$ ,  $P > 0.05$ ;  $n = 14$  pooled  
173 from three colonies).

174

### 175 **Ergatoid Formation in Response to Soldier Caste**

176 In both assays, ergatoids were differentiated from workers in response to orphaning condition,  
177 and there were no significant effects of soldiers on ergatoid formation (Fig. 5). In the single-  
178 soldier orphaning assay, soldier caste did not significantly influence the number of female or

179 male ergatoids in 60 days (Fig. 5(a); female:  $t_{85} = 1.64$ ,  $P > 0.05$ ; male:  $t_{85} = 1.64$ ,  $P > 0.05$ ;  
180 unpaired  $t$ -test, two-sided,  $n = 42$  for Soldier+;  $n = 45$  for Soldier-). At the end of the assay,  
181 mortality between Soldier+ and Soldier- groups were not significantly different (Fig. 5(b);  $t_{42} =$   
182  $0.67$ ,  $P > 0.05$ ; unpaired  $t$ -test, two-sided,  $n = 22$  randomly selected from both soldier+ and  
183 soldier- groups). The presence of one soldier significantly suppressed the differentiation of pre-  
184 soldiers and soldiers, and the increased total number of pre-soldiers and soldiers are  $0.43 \pm 0.10$   
185 in Soldier+ groups and  $0.91 \pm 0.14$  in Soldier- groups ( $t_{85} = 2.71$ ,  $P < 0.01$ ; unpaired  $t$ -test, two-  
186 sided; data were transformed (square root ( $x + 0.5$ )));  $n = 42$  for Soldier+,  $n = 45$  for Soldier-).  
187 Similarly, in the multiple-soldier orphaning assay, the soldier caste did not significantly  
188 influence the accumulative number of female or male ergatoids in 60 days (Fig. 5(c); female:  $t_{18}$   
189  $= 0.57$ ,  $P > 0.05$ ; male:  $t_{18} = 0.44$ ,  $P > 0.05$ ; unpaired  $t$ -test, two-sided; data were transformed  
190 (square root ( $x + 0.5$ )));  $n = 10$  for both Soldier++ and Soldier- groups). Mortality was not  
191 significantly influenced by the presence of 4 soldiers (Fig. 5(d);  $t_{18} = 1.02$ ,  $P > 0.05$ ; unpaired  $t$ -  
192 test, two-sided,  $n = 10$  for both Soldier++ and Soldier- groups).  
193



194

195 **Figure 5. Soldier impacts on ergatoid formation.** In a single-soldier orphaning assay, total numbers of female and

196 male ergatoids presented (mean ± SEM) (a) and mortality (%; mean ± SEM) (b) between soldier present and absent

197 groups in 60 days are shown. In a multiple-soldier orphaning assay, cumulative numbers of female and male

198 ergatoids differentiated (mean ± SEM) (c) and mortality (%; mean ± SEM) (d) between soldier present and absent

199 groups in 60 days are shown. In the single-soldier assay, newly differentiated ergatoids were left in groups;

200 Soldier+: each group consisted of 100 workers and one soldier at the start of assay; Soldier-: each group consisted of

201 100 workers only; NS: no significant difference (unpaired *t*-test, two-sided,  $P > 0.05$ ;  $n = 42$  for Soldier+,  $n = 45$  for

202 Soldier-). In the multiple-soldier assay, newly differentiated ergatoids were removed and replaced with workers

203 every day; Soldier++: each group consisted of 100 workers and four soldiers at the start of assay; Soldier-: each

204 group consisted of 100 workers only; NS: no significant difference (unpaired *t*-test, two-sided,  $P > 0.05$ ;  $n = 10$  for

205 both Soldier++ and Soldier-).

206

## 207 **Discussion**

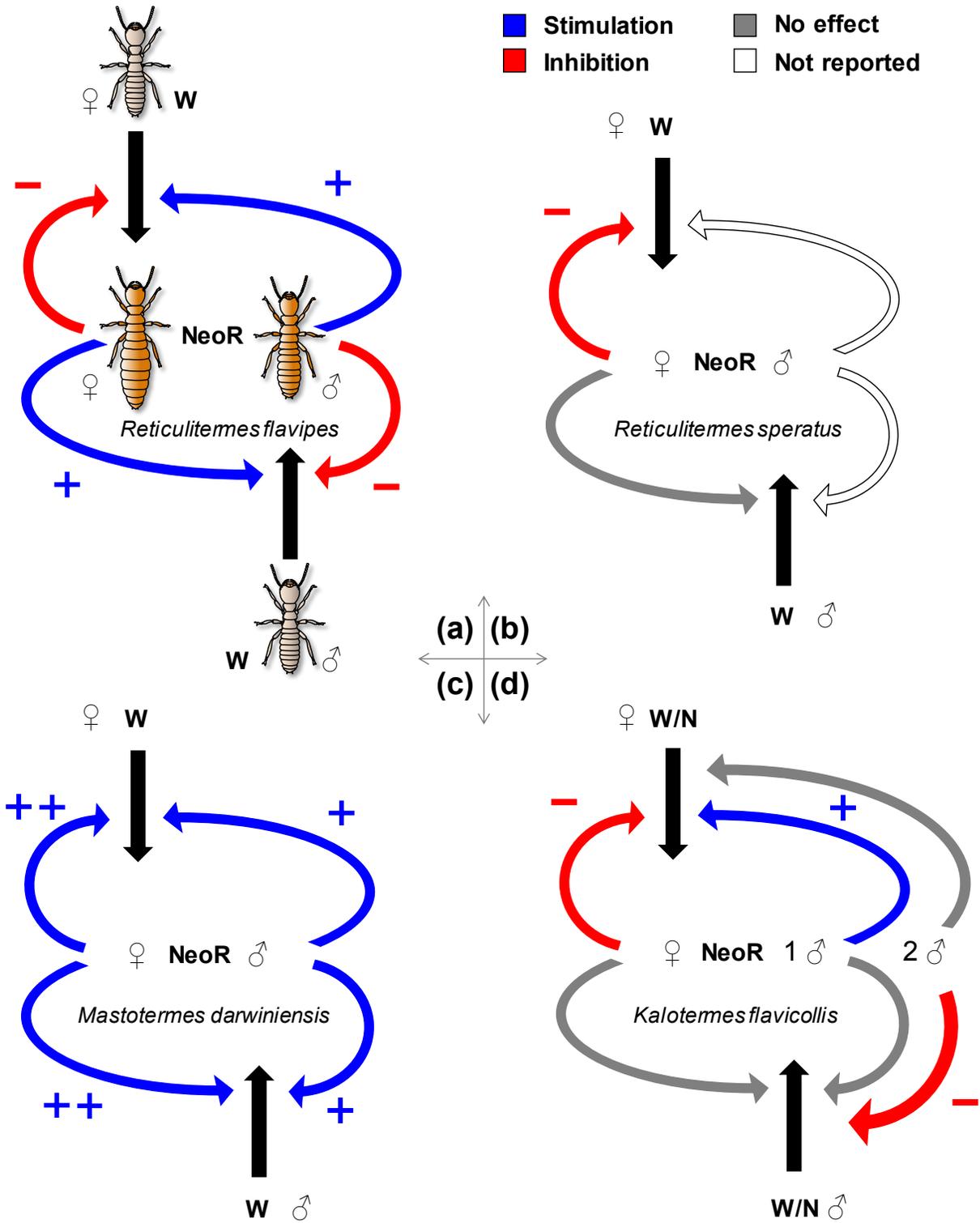
208 These results support our hypothesis that regulation of worker-reproductive transition by fertile  
209 reproductives is sex-specific. More importantly, our empirical evidence demonstrated that the  
210 dual regulation (inhibition and stimulation) is employed by both sexes. Ergatoid differentiation  
211 occurs after more than 30 days in response to orphaning, but can be significantly accelerated by  
212 the presence of a potential mate. Such stimulation by the opposite sex benefits the colony by  
213 enabling it to resume reproduction soon after the loss of a queen or a king. Inhibition of  
214 development by the same sex, on the other hand, prevents unnecessary investments in  
215 reproduction, which, in turn, would be a loss in the labour force. This sex-specific regulation  
216 suggests that the development of reproductives is strictly dependent on the reproductive needs of  
217 the colony.

218 Neotenic reproduction is common in termites; however, regulation of neotenic  
219 differentiation varies among species (Grassé & Noirot 1960; Lüscher 1964; Matsuura et al.,  
220 2010; Miyaguni, Sugio & Tsuji 2013; Watson, Metcalf & Sewell 1975). In *R. speratus*, female  
221 reproductives inhibit the differentiation of female neotenic, but do not influence the formation  
222 of male neotenic (Matsuura et al. 2010). Compared with *R. flavipes*, female ergatoid formation  
223 is faster in *R. speratus* in response to orphaning, and the formation of nymphoids are faster than  
224 ergatoids in *R. speratus* (Matsuura et al. 2010; Miyata, Furuichi & Kitade 2004). Orphaning  
225 assays have also been conducted in other congeneric species including *R. grassei* (Pichon et al.  
226 2007) and *R. urbis* (Ghesini & Marini 2009), which confirmed the inhibitory effect of  
227 reproductive pairs, but the regulation by each sex remains unclear. Stimulation by neotenic  
228 reproductives has been suggested previously in primitive termite species. In *K. flavicollis*, the  
229 formation of female neotenic was stimulated by the extracts of male reproductives (Lüscher

230 1964). In *M. darwiniensis*, formation of neotenics was promoted in the presence rather than the  
231 absence of other neotenics. Although sex-specificity was not confirmed, female neotenics  
232 exhibited stronger stimulatory effects than males and the pair (Watson & Abbey 1985; Watson,  
233 Metcalf & Sewell 1975).

234 In comparison, *R. flavipes* neotenics exhibit sex-specific inhibition and stimulation in  
235 both sexes (Fig. 6a). Such a regulatory pattern consists of all possible directions of social  
236 regulation, therefore presents a model for understanding the pheromonal and developmental  
237 mechanisms underlying neotenic reproduction. Given that previous studies on the differentiation  
238 of neotenic reproductives were incomplete or inconclusive (Fig. 6b-6d), this study also provides  
239 an opportunity for us to re-examine the sex-specificity hypothesis across termite taxa. Foraging  
240 populations of *R. flavipes* contain about 2% or less soldiers (Haverty & Howard 1981; Howard &  
241 Haverty 1980), while higher proportions (close to 4%) were observed in nest areas where  
242 neotenics are present (Howard & Haverty 1980). Soldiers were considered to induce the  
243 differentiation of reproductives (Tian & Zhou 2014; Watanabe et al. 2014), however, our results  
244 indicated that soldier caste does not play a significant role in regulating ergatoid formation in *R.*  
245 *flavipes*. It is worth noting that if ergatoids were not removed from the groups (single-soldier  
246 orphaning assay), the total number of ergatoids was lower than the cumulative number of  
247 ergatoids if they were constantly removed (multiple-soldier orphaning assay) within the same  
248 period of time. Although the two assays were conducted separately and not entirely comparable,  
249 this result could be explained by newly formed ergatoids suppressing formation of additional  
250 ergatoids through pheromones or policing behaviour.

251



252

253 **Figure 6. Comparison of feedback regulation of neotenic differentiation in four termites.** (a) Sex-specific  
254 inhibition and stimulation are demonstrated for both females and males in *R. flavipes* (this study). (b) In *R. speratus*,  
255 female neotenic inhibit differentiation of females, but does not influence males; the effects of male neotenic were  
256 not reported. (c) In *M. darwiniensis*, both female and male neotenic stimulate neotenic differentiation, but not in a  
257 sex-specific manner; females exhibit stronger stimulation. (d) In *K. flavicollis*, female neotenic inhibit  
258 differentiation of females, but the effect of males depends on number. One male neotenic shows opposite-sex  
259 stimulation, while two males exhibit same-sex inhibition. W: worker; N: nymph; NeoR: neotenic reproductive.

260

261 Our study represents a first step in understanding sex-specific worker-reproductive  
262 differentiation in response to social cues. The results from this study add a new dimension to the  
263 prevailing view that reproductives inhibit worker-reproductive transition in termites (Noirot  
264 1990). Much remains to be investigated about the regulatory mechanisms of caste differentiation,  
265 including the identification of inhibitory and stimulatory pheromones from reproductives. The  
266 active substances or blends must be sex-specific. The search of reproductive pheromones in  
267 termites should include volatile compounds (Matsuura et al. 2010), cuticular hydrocarbons  
268 (Liebig, Eliyahu & Brent 2009) as often observed in Hymenoptera (Van Oystaeyen et al. 2014),  
269 and proteinaceous secretions (Hanus et al. 2010). The sex-specificity and the dual effect of  
270 reproductive cues reflect unique adaptation and regulation of caste differentiation in  
271 hemimetabolous termites.

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284 preparation of the manuscript.

285

286 **Author Contributions**

287 Q.S., K.F.H. and X.Z. designed the experiments, Q.S. and J.D.H conducted the experiments,  
288 Q.S. and K.F.H analysed the data, Q.S. drafted the manuscript, and K.F.H and X.Z. revised the  
289 manuscript. All authors approved the final manuscript.

290

291

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