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1 **Expansion of presoldier cuticle contributes to head elongation during**  
2 **soldier differentiation in termites**

3

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15

16 **Abstract**

17 In termites, the soldier caste possesses morphological features suitable for colony  
18 defence, despite some exceptions. Soldiers are differentiated via two moultings through  
19 a presoldier stage with dramatic morphogenesis. While a number of morphological  
20 modifications are known to occur during the presoldier moult, growth and  
21 morphogenesis seem to continue even after the moult. The present study, using the  
22 damp-wood termite *Hodotermopsis sjostedti*, carried out morphological and histological  
23 investigations on the developmental processes during the presoldier stage that is  
24 artificially induced by the application of a juvenile hormone analogue. Measurements of  
25 five body parameters indicated that head length significantly increased during the 14-  
26 day period after the presoldier moult, while it did not increase subsequent to the  
27 stationary moult (pseudergate moult as control). Histological observations also showed  
28 that the cuticular development played a role in the presoldier head elongation,  
29 suggesting that the soft and flexible presoldier cuticle contributed to the soldier  
30 morphogenesis in termites.

31

32 **Keywords:** Termite, Soldier differentiation, Presoldier, Cuticle, Intermoult

### 33 **Introduction**

34           In social-insect colonies, castes possess specific morphological characteristics  
35 that are specialized into their colony tasks (Miura 2005). The caste fates are determined  
36 during postembryonic development depending on social interactions and other  
37 environmental conditions (Nijhout and Wheeler 1982; Noirot 1991). In contrast to  
38 social hymenopterans, termites (superfamily Termitoidea in order Blattodea, or  
39 previously order Isoptera) exhibit hemimetabolous caste developmental patterns in  
40 which sterile castes are basically immature (Bordereau 1985; Noirot and Pasteels 1987;  
41 Miura 2005; Lo et al. 2007). Although caste developmental pathways are diverse among  
42 termite species, the soldier caste is thought to have evolved once in the ancestor of all  
43 termite species (Roisin 2000). Soldiers differentiate from workers, or pseudergates  
44 (later-stage larvae playing roles of workers and still having the potential to differentiate  
45 into reproductives) in relatively basal termite species, through two moulting events via a  
46 presoldier stage (Noirot 1969; Roisin 2000; Korb and Hartfelder 2008). Through soldier  
47 differentiation, the overall morphology, especially the anterior body parts, is modified to  
48 form weapons such as enlarged mandibles or frontal projections (Weesner 1969;  
49 Deligne et al. 1981; Prestwich 1984a; Koshikawa et al. 2002, 2003; Miura 2005),  
50 although other castes possess defensive organs in some species (e.g. Šobotník et al.  
51 2010, 2012).

52           While the overall shape of soldiers appears at the presoldier moult (Hare  
53 1937), the soldier morphology continues developing after the moult (Miller 1969).  
54 Although the majority of previous studies on soldier differentiation have focused on the  
55 hormonal mechanisms and the developmental processes leading the presoldier moult  
56 (Miura and Scharf 2011), knowledge on the subsequent development is limited except  
57 for developmental descriptions during presoldier stage (Prestwich 1984b; Bordereau et  
58 al. 1997; Hanus et al. 2006; Šobotník et al. 2004). Presoldiers are covered with whitish  
59 unsclerotized cuticles which are considered to allow the morphological modifications  
60 during the stage, although detailed morphological and histological observations after the  
61 presoldier moult have not yet been performed.

62           In this study, to test the hypothesis that presoldier cuticle enables the  
63 intermoult growth after the presoldier moult, developmental processes after the moult  
64 was observed in the damp-wood termite, *Hodotermopsis sjostedti* (Holmgren 1911). In  
65 this species, soldiers with larger head and mandibles differentiate from pseudergates  
66 through the dramatic morphogenesis via presoldiers (Miura et al. 2000, 2004;  
67 Koshikawa et al. 2002, 2003, 2005; **Fig.1a**). Subsequent to the presoldier moult  
68 (abbreviated as PsM) that was artificially induced by the application of a juvenile  
69 hormone analog (Miura et al. 1999; Cornette et al. 2008a; Ishikawa et al. 2008; Hattori  
70 et al. 2013), observations on body-part growth and cuticular structures were carried out  
71 and compared with a stationary moult (abbreviated as StM, moult from pseudergate to

72 pseudergate). The results indicated that the presoldier head continued to elongate even

73 during the presoldier stage, probably due to elastic nature of the presoldier cuticle.

74

75 **Materials and methods**

76

77 **Insects**

78 Colonies of *Hodotermopsis sjostedti* Holmgren (family Archotermopsidae) inhabiting  
79 rotten wood were collected on Yakushima Island, Kagoshima Prefecture, Japan during  
80 May 2011 and May 2012 (Matsumoto and Hirono 1985). Stock colonies were  
81 maintained at approximately 25 °C under constant darkness and were occasionally fed  
82 with moistened pinewood.

83

84 **Induction of presoldier moult**

85 Pseudergaetes were identified based on the head width (seventh instar: approximately  
86 2.7mm) according to a previous study (Miura et al. 2000). The application of JHA was  
87 carried out based on Ogino et al. (1993). Briefly, 10 pseudergates were put in a petri  
88 dish (ø 70 mm) lined with filter paper containing 10 µg of pyriproxyfen (Sigma-  
89 Aldrich, St. Louis, USA), and moistened with distilled water. Eight replications (total of  
90 80 pseudergates) were prepared from 3 stock colonies. Petri dishes were monitored  
91 daily and maintained at 25 °C under constant darkness. The presoldier moult was  
92 typically induced around 14 days after JHA application.

93

94 *Comparison of the developmental processes that occurred after the presoldier and*  
95 *stationary moults*

96 The present study compared the developmental processes (size-growth process) that  
97 occurred subsequent to the presoldier moult with those that occurred subsequent to the  
98 StM. To obtain pseudergates prior to the stationary moult, individuals that exhibited  
99 whitish abdomens that resulted from gut purge were collected from stock colonies  
100 (Koshikawa et al. 2005). Collected individuals (40 for PsM, 55 for StM) were marked  
101 with oil paint to trace growth patterns of the intermoult stage individually. For termite  
102 sampling, time points at days 0, 1, 3, 7, and 14 after presoldier moult and after  
103 stationary moult were defined as PsM0, PsM1, PsM3, PsM7, and PsM14, and StM0,  
104 StM1, StM3, StM7, and StM14, respectively (**Fig. 1b**).

105

106 *Measurements of body parameters*

107 In order to determine whether intermoult growth occurred during the presoldier stage,  
108 five body parameters of presoldiers were measured and compared with those of  
109 pseudergates after a stationary moult. The following body parameters were measured at  
110 days 0, 1, 3, and 7 after both the presoldier and the stationary moults using an image  
111 analysis system with a CCD camera, 296 HIM-1N (HOGA, Kyoto, Japan) (**Fig. 2**);  
112 head length (from the base of the clypeus to the posterior margin of the head), head  
113 width (maximum head width), left-mandibular length (length from the basal articulation  
114 to the tip of left mandible), pronotum width (maximum pronotum width), and fore-tibial

115 length (length of the foreleg section between the femur and the tarsus). The  
116 measurements were carried out on live termites, and the same individuals were followed  
117 throughout the experiment period. Data obtained from these measurements (presoldier,  
118 n=19, pseudergate, n=10) was analysed using a general linear mixed model (GLMM)  
119 constructed using parameter size as a response variable, individual termites as random  
120 effects, and time (days) as a fixed effect. The influence of time on parameter size was  
121 examined using a likelihood ratio (LR) test. When the results of LR testing were  
122 significant, a post-hoc Tukey's multiple comparison was performed. Since termites  
123 exhibited continuous increases in head length during the 7-day period after the  
124 presoldier moult, measurements of body parameters at days 7 and 14 after the stationary  
125 moult were also analysed using a Welch's t test (n=11). All statistical analyses were  
126 performed using R3.2.0.

127

### 128 Scanning electron microscopy

129 For detailed morphological examinations, termite heads after the presodier moults (n =  
130 3, respectively for PsM0, 7 and 14) and after the stationary moult (n = 3, respectively  
131 for PsM0, 7 and 14) were observed by a scanning electron microscope JSM-5510LV  
132 (JEOL Ltd., Tokyo, Japan), according to our previous studies (e.g. Koshikawa et al.  
133 2003). The sizes of cuticle patches on the obtained SEM images were measured a  
134 maximum distance along anterior-posterior direction (PsM0: n = 26, PsM7: n = 21,

135 PsM14: n = 22, StM0: n = 20, StM7: n = 25, StM14: n = 19). Image analysis was  
136 conducted by ImageJ (ver. 1.49). Statistical analysis was performed for the obtained  
137 data to compare among days of post-moult (day 0, 7 and 14) for each caste (presoldier  
138 and pseudergate), using one-way ANOVA followed by Tukey's multiple comparisons  
139 test.

140

#### 141 **Histological observations**

142 Histological examinations were conducted using dissected termite heads after the  
143 presoldier moults and those after the stationary moult (5 individuals for each category).  
144 Paraffin sections were prepared and stained as performed in our previous studies (e.g.  
145 Corenette et al. 2008b) and were placed on slides and observed using a BZ-9000 HS  
146 All-in-one fluorescence microscope (KEYENCE, Osaka, Japan). Thickness of the head  
147 cuticle was defined as the distance between the surface of outer cuticle and the bottom  
148 of the inner cuticle adjacent to the epidermal cells, and was measured using a BZ-II  
149 Analyzer (KEYENCE, Osaka, Japan). To examine differences in cuticular thickening  
150 among castes and regions of the head, mean values of cuticular thickness were  
151 compared across the two castes (presoldier and pseudergate) for each day post-moult  
152 (days 0, 7, and 14) and each head region (frons, postmentum, and occipital) using a  
153 Welch's t test (n = 5).

154 **Results**

155 **Measurement of body parameters**

156 The measurements of five body parameters after the two types of moult showed clear  
157 differences (**Fig. 2**). In post-PsM termites, head length, left-mandibular length, and fore-  
158 tibial length significantly increased during the first 7 days post-moult (**Table 1**). The  
159 sizes at PsM7 against the initial sizes (at day 0) for all measured body parts except for  
160 head width increased. Furthermore, when body parameters were measured at day 14  
161 after the presoldier moult (PsM14), only head length had increased (**Table 2**). In post-  
162 StM termites, fore-tibial length exhibited a dramatic increase during the first 7 days  
163 post-moult, while head length did not exhibit any growth (**Table 1**). Interestingly, fore-  
164 tibial length in both PsM and StM termites exhibited dramatic growth at 1 day post-  
165 moult (**Fig. 2f**), likely due to rapid expansion of the new cuticle.

166

167 **Morphological comparison using a scanning electron microscope**

168 Since it was determined that head length increased during the presoldier stage, detailed  
169 structures of the head cuticle were examined using a scanning electron microscope  
170 (SEM) in order to identify areas of the head that contributed to head elongation during  
171 the presoldier stage. Surface structures of the head cuticle of presoldiers after the moult  
172 were compared with those of pseudergates subsequent to a stationary moult.

173 Immediately after the presoldier moult (PsM0), a distinctive wrinkle structure was  
174 observed on the frons, an area defined as the cuticular plate between the ecdysial line  
175 and the clypeus (**Fig. 3a, b**). At day 7 (PsM7), the wrinkles had expanded and the  
176 cuticle itself was flattened (**Fig. 3b**). Furthermore, the scale-like cuticle patches that  
177 corresponded to the underlying epidermal cells became larger during the period between  
178 PsM0 and PsM7 (**Fig. 3c**); PsM0:  $7.12 \pm 0.89 \mu\text{m}$  (n=26), PsM7:  $9.11 \pm 1.14 \mu\text{m}$   
179 (n=21), PsM14:  $9.63 \pm 1.10 \mu\text{m}$  (n=22) (mean  $\pm$  s.d., one-way ANOVA,  $F = 39.52$ ,  $p <$   
180  $0.001$ ). Statistical analyses showed that the significant growth was detected between  
181 PsM0 and Psm7 (Tukey's test,  $p < 0.001$ ), and between PsM0 and PsM14 ( $p < 0.001$ )  
182 although it was not detected between PsM7 and PsM14 ( $p > 0.05$ ). Similar wrinkle  
183 structures were not observed on the head cuticle of pseudergates subsequent to a  
184 stationary moult (**Fig. 3b**). In post-StM pseudergates, the head surface was smooth and  
185 the size of cuticle patches did not increase (**Fig. 3c**); StM0:  $8.32 \pm 1.19 \mu\text{m}$  (n=20), StM  
186 7:  $8.38 \pm 0.79 \mu\text{m}$  (n=25), StM 14:  $8.49 \pm 1.75 \mu\text{m}$  (n=19) (mean  $\pm$  s.d., one-way  
187 ANOVA,  $F = 0.096$ ,  $p > 0.05$ ).

188

### 189 **Histological observations**

190 To determine whether or not the wrinkle structure on the presoldier head cuticle  
191 expanded as the head elongated, histological observations of 3 different regions of the  
192 head (the frons, postmentum, and occipital) were carried out along the median line (**Fig.**

193 **4a)**. At PsM0, the frons cuticle exhibited an undulated structure (**Fig. 4b**), while  
194 cuticular surfaces of the postmentum and occipital regions were smooth (**Fig. 4c, d**). At  
195 PsM7, the frons cuticle became flattened (**Fig. 4b**), and cuticles in all three regions  
196 thickened (**Fig. 4b, c, d**). At PsM14, the frons cuticle was thinner than it was at PsM7  
197 and was almost completely flat (**Fig. 4b**). Although the cuticle in the postmentum region  
198 was thinner at PsM14, the occipital cuticle had thickened (**Fig. 4c, d**). Similar  
199 observation of pseudergates after the stationary moult revealed that no undulated  
200 structures were present on any of the head regions at StM0, and that the cuticles in each  
201 head region gradually increased in thickness between StM7 and StM14 (**Fig. 4b-d**).

202           Differences in cuticular thickening between head regions of post-moult  
203 presoldiers and pseudergates were also compared visually (**Fig. 5**). Results of these  
204 comparisons indicated that patterns of cuticular thickening at the frons differed between  
205 PsM and StM termites (Welch's t-test, day 0:  $t = 1.84$ ,  $p > 0.05$ , day 7:  $t = 4.81$ ,  $p <$   
206  $0.05$ , day 14:  $t = -0.53$ ,  $p > 0.05$ ; **Fig. 5a**). The frons cuticle was significantly thicker at  
207 PsM7 than it was at StM7. At PsM14, the thickness of the frons cuticle in presoldiers  
208 had decreased and was almost equal to that of post-StM pseudergates (**Fig. 5a**). In the  
209 postmentum region, the cuticle of post-PsM termites was significantly thicker than that  
210 of post-StM ones at day 0 and at day 7 (day 0:  $t = 3.54$ ,  $p < 0.05$ , day 7:  $t = 6.01$ ,  $p <$   
211  $0.05$ ; **Fig. 5b**); however, at day 14, cuticular thickness of post-StM termites was greater  
212 than post-PsM (day 14:  $t = -2.76$ ,  $p < 0.05$ ; **Fig.5b**). The occipital cuticle of post-PsM

213 termites was thicker than post-StM at day 7, and seemed to increase in thickness at a  
214 constant rate until PsM14 (day 0:  $t = 1.26$ ,  $p > 0.05$ , day 7:  $t = 12.65$ ,  $p < 0.05$ , day 14:  $t$   
215  $= 1.51$ ,  $p > 0.05$ ; **Fig.5c**).

## 216 **Discussion**

217       Some previous studies in the focal and other termite species have shown the  
218 drastic morphogenesis at the presoldier moult (e.g. Koshikawa et al. 2002, 2003; Xing  
219 et al. 2013). Under the old cuticle, newly synthesized cuticle was prepared with wrinkle  
220 structure that expands at the time of exuviation. The present study demonstrated, in  
221 contrast, that head length exhibited continuous growth even after the PsM (**Fig. 2**).  
222 Although the mandibles were slightly elongated after the presoldier moult (**Fig. 2c**), the  
223 intermoult growth did not contribute much to the mandibular morphogenesis as it did in  
224 the head elongation. Moreover, Fore-tibial length increased rapidly post-moult (within 1  
225 day), likely due to the preparation of folded cuticles that occurred prior to the both type  
226 of moults (**Fig. 2f**). These results suggest that, depending on body parts, the growth  
227 differently contributes to the soldier morphogenesis, probably due to temporal  
228 differences in cell proliferation or sclerotization of the cuticle.

229       Histological observations on the head parts indicated that, after the presoldier  
230 moult, the thickness of endocuticle on frons and postmenum once increased but later  
231 decreased (**Figs. 4, 5**). Furrow-like structures on the frons cuticle were somewhat  
232 stretched at day 7 (PsM7), but had become completely flat by day 14 (PsM14). These  
233 results suggested that the presoldier cuticle was thickened by the synthesis of cuticular  
234 components by day 7, and that the frons cuticle expanded until at least day 14 (**Fig. 6**).

235 This scheme of cuticular expansion corresponds with the hypothesis that the presoldier  
236 cuticle is soft and elastic.

237 Cuticle proteins in insects are diverse, not only among insect species, but also  
238 among body parts and developmental stages even within a single species (Andersen et  
239 al. 1995). Actually, insect cuticles exhibit various physical properties with variety of  
240 strength and elasticity (Hackman 1984; Hopkins and Kramer 1992). Results of this  
241 study suggested that the physical properties of the presoldier cuticle differed from other  
242 developmental stages, so that the constituent substances of the presoldier cuticle  
243 differed from other castes and stages. One of our previous studies on the gene  
244 expression during soldier differentiation identified a presoldier-specific cuticle protein  
245 termed as CP4 (Koshikawa et al. 2005), suggested that CP4 may be responsible for the  
246 presoldier-specific cuticular properties. Since gene expressions of cuticle proteins were  
247 under the control of hormonal actions (Charles 2010), together with the fact that the  
248 presoldier moult was induced by the JH action (Ogino et al. 1993, Cornette et al.  
249 2008b), the gene expression of CP4 may be regulated by the action of JH as well.  
250 Actually, knockdown of the putative JH receptor gene (*Met*) reduced the head  
251 elongation in the closely related species (Masuoka et al. 2015).

252 The presoldier is a unique developmental stage seen only in termites, and many  
253 enigmas regarding its developmental and physiological mechanisms as well as its  
254 evolutionary origins remain unanswered. In all termite species (except those that have

255 secondarily lost the soldier caste), the presoldier stage exists as a preparatory stage  
256 required for the soldier morphogenesis (Hare 1937). Results of the present study firstly  
257 suggested that intermolt development during the presoldier stage that utilized the  
258 elastic presoldier cuticle played an important role in soldier-specific morphogenesis,  
259 and possibly in the evolutionary diversification of different soldier morphologies as  
260 well. Interestingly, this study showed that the anatomical position of furrowed cuticle  
261 formed at frons part of the head, where the frontal projection is formed in some higher  
262 termite species (Deligne et al. 1981). The developmental process for head elongation  
263 might be a pre-adaptive requirement for the evolution of soldier morphologies (e.g.  
264 Miura and Matsumoto 2000; Šobotník et al. 2004; Toga et al. 2009).

265 Future analyses of the regulatory mechanisms affecting presoldiers, including  
266 hormonal actions and cuticle proteins, are necessary in order to develop a greater  
267 understanding of the mechanisms that affect the presoldier stage in termites as well as  
268 its evolutionary origins.

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415

416 **Tables**

417

418 **Table 1.**

419 Intermoult growth during the initial 7 day period after the presoldier moult (PsM) and  
420 the stationary moult (StM).

421

422 **Table 2.**

423 Growth sizes and rates between 7 and 14 days after the presoldier moult (PsM7 and  
424 PsM14).

425

426

427

## 428 **Figure legends**

429

### 430 **Fig. 1.**

431 Diagrams of the caste differentiation pathway and the sampling scheme used in the  
432 present study. (a) Caste developmental pathway of the damp-wood termite,  
433 *Hodotermopsis sjostedti*. Each arrow indicates a moulting event. Soldier differentiation  
434 begins in the pseudergate stage and occurs over two moulting events via the presoldier  
435 stage (after Miura et al. 2000, 2004). Pseudergates typically undergo a moult into an  
436 identical pseudergate stage (stationary moult [StM]). (b) In the present study,  
437 developmental processes that occurred after the presoldier moult (PsM) were compared  
438 with that those that occurred after the StM. Comparisons were carried out at day 0  
439 (PsM0 or StM0), day 7 (PsM7 or StM7), and day 14 (PsM14 or StM14) post-moult.  
440 Additional morphometric analyses were performed at days 1 and 3 post-moult.

441

442

### 443 **Fig. 2.**

444 Comparison of intermoult growth between presoldiers (post-presoldier moult [PsM]  
445 growth) and pseudergates (post-stationary moult [StM] growth) at days 0, 1, 3 and 7

446 post-moult (mean  $\pm$  S.E.). (a) Five body parameters were measured during the  
447 intermoult period in presoldiers (n = 19) and pseudergates (n = 10). (b) Head length  
448 (from the base of the clypeus to the posterior margin of the head along the ecdysial  
449 line), (c) Left-mandibular length (distance from the condyle to the tip of the left  
450 mandible), (d) Head width (maximum head width), (e) Pronotum width (maximum  
451 pronotum width), and (f) Fore-tibial length (distance from the boundary of the femur  
452 and the tarsus on the foreleg). Different letters indicate statistical significances among  
453 samples from a given day (Tukey's test,  $p < 0.05$ ). Head length (b) exhibited dramatic  
454 growth subsequent to the PsM.

455

456 **Fig. 3.**

457 Morphological observation of the frons cuticle after the presoldier (PsM) and stationary  
458 moults (StM) carried out using a scanning electron microscope (SEM). (a) Diagram  
459 indicating the observed regions (frons) shown in (b) and (c). (b) Cuticular structures on  
460 the frons indicating that the folds (furrows) seen at day 0 had disappeared by day 14 in  
461 PsM termites, while relatively few folds were observable in StM termites. (c) Magnified  
462 images of the frons cuticle indicating that cuticle patches expanded during the first 14  
463 days post-PsM in presoldiers. Numbers following PsM and StM in (b) and (c) indicate  
464 the number of days post-moult at which the images were obtained.

465

466

467 **Fig. 4.**

468 Histological observations focusing on the cuticular and epithelial structures at three  
469 different regions on the termite head. (a) Median section indicating the three cuticular  
470 regions on the termite head. (b) Frons cuticle observed at days 0, 7, and 14 post-  
471 presoldier moult (PsM) and post-stationary moult (StM). (c) Postmentum cuticle  
472 observed at days 0, 7, and 14 post-PsM and post-StM. (d) Occipital cuticle observed at  
473 days 0, 7, and 14 post-PsM and post-StM. Numbers following PsM and StM in (b), (c),  
474 and (d) indicate the number of days post-moult at which the images were obtained.

475

476

477 **Fig. 5.**

478 Post-moult cuticle thickness determined through histological observation. Cuticle  
479 thicknesses of the three different regions observed in Fig. 4 (a: frons, b: postmentum, c:  
480 occipital) measured at days 0, 7, and 14 after presoldier (PsM,  $n = 5$ ) and stationary  
481 moults (StM,  $n = 5$ ). Black and white columns indicate thicknesses associated with  
482 PsM and StM, respectively. Asterisks indicate statistically significant differences  
483 between the two castes (Welch's t-test,  $p < 0.05$ ).

484

485

486 **Fig. 6.**

487 Schematic drawing of frons-cuticular development subsequent to the presoldier moult  
488 (PsM) in *H. sjostedti*. Bars at top represent the intermoult periods of pseudergate and  
489 presoldier termites. Outer cuticle (epicuticle and exocuticle) and inner cuticle  
490 (endocuticle) are represented by black sections and striped sections, respectively.  
491 Cuticular thickening occurs during the first 7 days of the intermoult stage (post-PsM),  
492 while cuticular extension occurs over the first 14 days.