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

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Abstract

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

Keywords: Termite, Soldier differentiation, Presoldier, Cuticle, Intermoult
Introduction

In social-insect colonies, castes possess specific morphological characteristics that are specialized into their colony tasks (Miura 2005). The caste fates are determined during postembryonic development depending on social interactions and other environmental conditions (Nijhout and Wheeler 1982; Noirot 1991). In contrast to social hymenopterans, termites (superfamily Termitoidea in order Blattodea, or previously order Isoptera) exhibit hemimetabolous caste developmental patterns in which sterile castes are basically immature (Bordereau 1985; Noirot and Pasteels 1987; Miura 2005; Lo et al. 2007). Although caste developmental pathways are diverse among termite species, the soldier caste is thought to have evolved once in the ancestor of all termite species (Roisin 2000). Soldiers differentiate from workers, or pseudergates (later-stage larvae playing roles of workers and still having the potential to differentiate into reproductives) in relatively basal termite species, through two moulting events via a presoldier stage (Noirot 1969; Roisin 2000; Korb and Hartfelder 2008). Through soldier differentiation, the overall morphology, especially the anterior body parts, is modified to form weapons such as enlarged mandibles or frontal projections (Weesner 1969; Deligne et al. 1981; Prestwich 1984a; Koshikawa et al. 2002, 2003; Miura 2005), although other castes possess defensive organs in some species (e.g. Šobotník et al. 2010, 2012).
While the overall shape of soldiers appears at the presoldier moult (Hare 1937), the soldier morphology continues developing after the moult (Miller 1969). Although the majority of previous studies on soldier differentiation have focused on the hormonal mechanisms and the developmental processes leading the presoldier moult (Miura and Scharf 2011), knowledge on the subsequent development is limited except for developmental descriptions during presoldier stage (Prestwich 1984b; Bordereau et al. 1997; Hanus et al. 2006; Šobotník et al. 2004). Presoldiers are covered with whitish unsclerotized cuticles which are considered to allow the morphological modifications during the stage, although detailed morphological and histological observations after the presoldier moult have not yet been performed.

In this study, to test the hypothesis that presoldier cuticle enables the intermoult growth after the presoldier moult, developmental processes after the moult was observed in the damp-wood termite, *Hodotermopsis sjostedti* (Holmgren 1911). In this species, soldiers with larger head and mandibles differentiate from pseudergates through the dramatic morphogenesis via presoldiers (Miura et al. 2000, 2004; Koshikawa et al. 2002, 2003, 2005; Fig.1a). Subsequent to the presoldier moult (abbreviated as PsM) that was artificially induced by the application of a juvenile hormone analog (Miura et al. 1999; Cornette et al. 2008a; Ishikawa et al. 2008; Hattori et al. 2013), observations on body-part growth and cuticular structures were carried out and compared with a stationary moult (abbreviated as StM, moult from pseudergate to
pseudergate). The results indicated that the presoldier head continued to elongate even 
during the presoldier stage, probably due to elastic nature of the presoldier cuticle.
Materials and methods

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

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

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

Measurements of body parameters

In order to determine whether intermoult growth occurred during the presoldier stage, five body parameters of presoldiers were measured and compared with those of pseudergates after a stationary moult. The following body parameters were measured at days 0, 1, 3, and 7 after both the presoldier and the stationary moults using an image analysis system with a CCD camera, 296 HIM-1N (HOGA, Kyoto, Japan) (Fig. 2); head length (from the base of the clypeus to the posterior margin of the head), head width (maximum head width), left-mandibular length (length from the basal articulation to the tip of left mandible), pronotum width (maximum pronotum width), and fore-tibial
length (length of the foreleg section between the femur and the tarsus). The measurements were carried out on live termites, and the same individuals were followed throughout the experiment period. Data obtained from these measurements (presoldier, n=19, pseudergate, n=10) was analysed using a general linear mixed model (GLMM) constructed using parameter size as a response variable, individual termites as random effects, and time (days) as a fixed effect. The influence of time on parameter size was examined using a likelihood ratio (LR) test. When the results of LR testing were significant, a post-hoc Tukey's multiple comparison was performed. Since termites exhibited continuous increases in head length during the 7-day period after the presoldier moult, measurements of body parameters at days 7 and 14 after the stationary moult were also analysed using a Welch’s t test (n=11). All statistical analyses were performed using R3.2.0.

**Scanning electron microscopy**

For detailed morphological examinations, termite heads after the presoldier moults (n = 3, respectively for PsM0, 7 and 14) and after the stationary moult (n = 3, respectively for PsM0, 7 and 14) were observed by a scanning electron microscope JSM-5510LV (JEOL Ltd., Tokyo, Japan), according to our previous studies (e.g. Koshikawa et al. 2003). The sizes of cuticle patches on the obtained SEM images were measured a maximum distance along anterior-posterior direction (PsM0: n = 26, PsM7: n = 21,
PsM14: n = 22, StM0: n = 20, StM7: n = 25, StM14: n = 19. Image analysis was conducted by ImageJ (ver. 1.49). Statistical analysis was performed for the obtained data to compare among days of post-moult (day 0, 7 and 14) for each caste (presoldier and pseudergate), using one-way ANOVA followed by Tukey’s multiple comparisons test.

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

Measurement of body parameters

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

Morphological comparison using a scanning electron microscope

Since it was determined that head length increased during the presoldier stage, detailed structures of the head cuticle were examined using a scanning electron microscope (SEM) in order to identify areas of the head that contributed to head elongation during the presoldier stage. Surface structures of the head cuticle of presoldiers after the moult were compared with those of pseudergates subsequent to a stationary moult.
Immediately after the presoldier moult (PsM0), a distinctive wrinkle structure was observed on the frons, an area defined as the cuticular plate between the ecdysial line and the clypeus (Fig. 3a, b). At day 7 (PsM7), the wrinkles had expanded and the cuticle itself was flattened (Fig. 3b). Furthermore, the scale-like cuticle patches that corresponded to the underlying epidermal cells became larger during the period between PsM0 and PsM7 (Fig. 3c); PsM0: 7.12 ± 0.89 µm (n=26), PsM7: 9.11 ± 1.14 µm (n=21), PsM14: 9.63 ± 1.10 µm (n=22) (mean ± s.d., one-way ANOVA, F = 39.52, p < 0.001). Statistical analyses showed that the significant growth was detected between PsM0 and PsM7 (Tukey’s test, p < 0.001), and between PsM0 and PsM14 (p < 0.001) although it was not detected between PsM7 and PsM14 (p > 0.05). Similar wrinkle structures were not observed on the head cuticle of pseudergates subsequent to a stationary moult (Fig. 3b). In post-StM pseudergates, the head surface was smooth and the size of cuticle patches did not increase (Fig. 3c); StM0: 8.32 ± 1.19 µm (n=20), StM7: 8.38 ± 0.79 µm (n=25), StM 14: 8.49 ± 1.75 µm (n=19) (mean ± s.d., one-way ANOVA, F = 0.096, p > 0.05).

Histological observations

To determine whether or not the wrinkle structure on the presoldier head cuticle expanded as the head elongated, histological observations of 3 different regions of the head (the frons, postmentum, and occipital) were carried out along the median line (Fig.
At PsM0, the frons cuticle exhibited an undulated structure (Fig. 4b), while cuticular surfaces of the postmentum and occipital regions were smooth (Fig. 4c, d). At PsM7, the frons cuticle became flattened (Fig. 4b), and cuticles in all three regions thickened (Fig. 4b, c, d). At PsM14, the frons cuticle was thinner than it was at PsM7 and was almost completely flat (Fig. 4b). Although the cuticle in the postmentum region was thinner at PsM14, the occipital cuticle had thickened (Fig. 4c, d). Similar observation of pseudergates after the stationary moult revealed that no undulated structures were present on any of the head regions at StM0, and that the cuticles in each head region gradually increased in thickness between StM7 and StM14 (Fig. 4b-d).

Differences in cuticular thickening between head regions of post-moult presoldiers and pseudergates were also compared visually (Fig. 5). Results of these comparisons indicated that patterns of cuticular thickening at the frons differed between PsM and StM termites (Welch’s t-test, day 0: $t = 1.84, p > 0.05$, day 7: $t = 4.81, p < 0.05$, day 14: $t = -0.53, p > 0.05$; Fig. 5a). The frons cuticle was significantly thicker at PsM7 than it was at StM7. At PsM14, the thickness of the frons cuticle in presoldiers had decreased and was almost equal to that of post-StM pseudergates (Fig. 5a). In the postmentum region, the cuticle of post-PsM termites was significantly thicker than that of post-StM ones at day 0 and at day 7 (day 0: $t = 3.54, p < 0.05$, day 7: $t = 6.01, p < 0.05$; Fig. 5b); however, at day 14, cuticular thickness of post-StM termites was greater than post-PsM (day 14: $t = -2.76, p < 0.05$; Fig. 5b). The occipital cuticle of post-PsM
termites was thicker than post-StM at day 7, and seemed to increase in thickness at a
constant rate until PsM14 (day 0: $t = 1.26, p > 0.05$, day 7: $t = 12.65, p < 0.05$, day 14: $t = 1.51, p > 0.05$; Fig.5c).
Some previous studies in the focal and other termite species have shown the drastic morphogenesis at the presoldier moult (e.g. Koshikawa et al. 2002, 2003; Xing et al. 2013). Under the old cuticle, newly synthesized cuticle was prepared with wrinkle structure that expands at the time of exuviation. The present study demonstrated, in contrast, that head length exhibited continuous growth even after the PsM (Fig. 2). Although the mandibles were slightly elongated after the presoldier moult (Fig. 2c), the intermoult growth did not contribute much to the mandibular morphogenesis as it did in the head elongation. Moreover, Fore-tibial length increased rapidly post-moult (within 1 day), likely due to the preparation of folded cuticles that occurred prior to the both type of moults (Fig. 2f). These results suggest that, depending on body parts, the growth differently contributes to the soldier morphogenesis, probably due to temporal differences in cell proliferation or sclerotization of the cuticle.

Histological observations on the head parts indicated that, after the presoldier moult, the thickness of endocuticle on frons and postmenum once increased but later decreased (Figs. 4, 5). Furrow-like structures on the frons cuticle were somewhat stretched at day 7 (PsM7), but had become completely flat by day 14 (PsM14). These results suggested that the presoldier cuticle was thickened by the synthesis of cuticular components by day 7, and that the frons cuticle expanded until at least day 14 (Fig. 6).
This scheme of cuticular expansion corresponds with the hypothesis that the presoldier cuticle is soft and elastic.

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

The presoldier is a unique developmental stage seen only in termites, and many enigmas regarding its developmental and physiological mechanisms as well as its evolutionary origins remain unanswered. In all termite species (except those that have
secondarily lost the soldier caste), the presoldier stage exists as a preparatory stage required for the soldier morphogenesis (Hare 1937). Results of the present study firstly suggested that intermoult development during the presoldier stage that utilized the elastic presoldier cuticle played an important role in soldier-specific morphogenesis, and possibly in the evolutionary diversification of different soldier morphologies as well. Interestingly, this study showed that the anatomical position of furrowed cuticle formed at frons part of the head, where the frontal projection is formed in some higher termite species (Deligne et al. 1981). The developmental process for head elongation might be a pre-adaptive requirement for the evolution of soldier morphologies (e.g. Miura and Matsumoto 2000; Šobotník et al. 2004; Toga et al. 2009).

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

Table 1.
Intermoult growth during the initial 7 day period after the presoldier moult (PsM) and the stationary moult (StM).

Table 2.
Growth sizes and rates between 7 and 14 days after the presoldier moult (PsM7 and PsM14).
Figure legends

Fig. 1.

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

Fig. 2.

Comparison of intermoult growth between presoldiers (post-presoldier moult [PsM] growth) and pseudergates (post-stationary moult [StM] growth) at days 0, 1, 3 and 7.
post-moult (mean ± S.E.). (a) Five body parameters were measured during the intermoult period in presoldiers (n = 19) and pseudergates (n = 10). (b) Head length (from the base of the clypeus to the posterior margin of the head along the ecdysial line), (c) Left-mandibular length (distance from the condyle to the tip of the left mandible), (d) Head width (maximum head width), (e) Pronotum width (maximum pronotum width), and (f) Fore-tibial length (distance from the boundary of the femur and the tarsus on the foreleg). Different letters indicate statistical significances among samples from a given day (Tukey’s test, $p < 0.05$). Head length (b) exhibited dramatic growth subsequent to the PsM.

Fig. 3.

Morphological observation of the frons cuticle after the presoldier (PsM) and stationary moults (StM) carried out using a scanning electron microscope (SEM). (a) Diagram indicating the observed regions (frons) shown in (b) and (c). (b) Cuticular structures on the frons indicating that the folds (furrows) seen at day 0 had disappeared by day 14 in PsM termites, while relatively few folds were observable in StM termites. (c) Magnified images of the frons cuticle indicating that cuticle patches expanded during the first 14 days post-PsM in presoldiers. Numbers following PsM and StM in (b) and (c) indicate the number of days post-moult at which the images were obtained.
Fig. 4.

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

Fig. 5.

Post-moult cuticle thickness determined through histological observation. Cuticle thicknesses of the three different regions observed in Fig. 4 (a: frons, b: postmentum, c: occipital) measured at days 0, 7, and 14 after presoldier (PsM, n = 5) and stationary moults (StM, n = 5). Black and white columns indicate thicknesses associated with PsM and StM, respectively. Asterisks indicate statistically significant differences between the two castes (Welch’s t-test, $p < 0.05$).
Fig. 6.
Schematic drawing of frons-cuticular development subsequent to the presoldier moult (PsM) in *H. sjostedti*. Bars at top represent the intermoult periods of pseudergate and presoldier termites. Outer cuticle (epicuticle and exocuticle) and inner cuticle (endocuticle) are represented by black sections and striped sections, respectively. Cuticular thickening occurs during the first 7 days of the intermoult stage (post-PsM), while cuticular extension occurs over the first 14 days.