

Soldiers are differentiated from male larval stages in incipient colonies of *Nasutitermes takasagoensis* (Isoptera: Termitidae)

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Abstract In phylogenetically ancestral taxa of termites (the so-called lower termites), at least one soldier emerges and is maintained longitudinally in each incipient colony. However, in apical taxa (the so-called higher termites), the developmental pathway and regulation of soldiers in incipient colonies currently remain unknown. We therefore examined soldier and worker development in incipient colonies of higher termites (*Nasutitermes takasagoensis* Shiraki). Developmental stages and castes were successfully discriminated by head width in incipient colonies 4 months after colony foundation. Furthermore, differences were observed in the number of bristles on antennae between first- and second-instar larvae. In *N. takasagoensis*, there was more than one soldier in each incipient colony 4 months after its foundation. Presoldiers in the incipient colonies were differentiated from an earlier instar (male second-instar larvae), whereas, in mature colonies, they were differentiated from male third instars (= minor workers). The developmental period of the former (7 days) was markedly shorter than that of the latter (14 days). All female second-instar larvae molted into workers. The developmental processes shown here are useful for obtaining a clearer understanding of the mechanisms of soldier/worker differentiation in higher termites.

Keywords Termite · Soldier differentiation · Incipient colony · Sex ratio

Introduction

The soldier caste exists in colonies of some eusocial animals (Tian and Zhou 2014). All extant termite species possess soldiers [secondarily lost in some species (Bourguignon et al. 2016; Carrijo et al. 2015; Miller 1984; Scheffrahn 2013)]. Therefore, soldiers are considered to have evolved only once in the termite lineage (Roisin 2000). The soldier ratio differs among species and is generally maintained at a low level (Haverty 1977; Howard and Haverty 1981).

Soldier development is affected not only by environmental factors (e.g., nutrition, social interactions, and season) (Liu et al. 2005; Maekawa et al. 2012; Watanabe et al. 2014), but also by the age of a colony (Chouvenc and Su 2014; Noirot 1985). Due to differentiation from an earlier instar and/or a shortened larval instar, soldiers in incipient colonies of phylogenetically ancestral taxa (six out of seven extant families, so-called lower termites) are generally markedly smaller than those in mature colonies (Maekawa et al. 2012; Noirot 1985). Furthermore, one soldier basically emerges and is maintained for a long period of time in each incipient colony of lower termites (Castle 1934; Light and Weesner 1955). This soldier essentially serves as a signal for the larvae to inhibit new soldier differentiation and to develop into workers (Chouvenc et al. 2015; Watanabe et al. 2014). In an incipient colony of *Zootermopsis nevadensis* Hagen, the first-emerged third-instar larva always differentiates into a soldier via a presoldier (the intermediate stage of a soldier), while the other third instars differentiate into fourth instars, which function as workers (Maekawa et al. 2012). Using this information,

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developmental and physiological analyses have recently been performed in order to elucidate the intrinsic mechanisms responsible for soldier differentiation under natural conditions (Masuoka et al. 2015; Yaguchi et al. 2015, 2016). In contrast, in apical taxa (family Termitidae, the so-called higher termites), the developmental regulatory mechanism of the first soldier, for example, the detailed process of molting and/or regulatory factors for soldier differentiation, remains largely unknown. The only information currently available is that multiple soldiers emerge in each incipient colony of some species (Garcia and Becker 1975; Light and Weesner 1955). However, differences of soldier developmental pathways between incipient and mature colonies, for example, in *Z. nevadensis*, the third-instar molts into a presoldier in an incipient colony but the 5–7th instars usually molt into presoldiers in a mature colony (Maekawa et al. 2012), have not yet been clarified.

In the present study, we examined soldier/worker development in incipient colonies of *Nasutitermes takasagoensis* Shiraki (Termitidae: Nasutitermitinae). In mature colonies of *N. takasagoensis*, sterile developmental stages are divided into the following seven stages: first-instar larvae, male/female second-instar larvae, minor workers (male), male/female medium workers, major workers (female), presoldiers, and soldiers (Hojo et al. 2004; Toga et al. 2009). There are many species with a strongly biased sex ratio for caste proportions in higher termites (Bourguignon et al. 2012). Soldiers of *N. takasagoensis* are all male, and differentiate from male minor workers via a presoldier stage in mature colonies (Hojo et al. 2004). Incipient colonies were established under laboratory conditions, and the colonies were sampled 4 months after their foundation. We then clearly discriminated the developmental stages and sexes based on morphological observations according to previously described methods (Hojo et al. 2004; Miura et al. 1998). We investigated the numbers of individuals and caste compositions in each incipient colony. By using incipient colonies prior to presoldier differentiation, we traced the development of each colony member and measured developmental periods during soldier/worker differentiation in incipient colonies.

Materials and methods

Incipient colony foundation

Colonies ($n = 2$) inside the arboreal nests of *N. takasagoensis* were collected from Ishigakijima (Yaeyama Islands), Okinawa Prefecture, Japan in February 2015. These colonies were maintained in separate plastic boxes with well-moistened cotton wool in constant darkness at room temperature (20–25 °C). After sex determination,

dealted adults from each colony were paired in snap vials (As one, Osaka, Japan) with oak sawdust (Mitani, Tokyo) ($n = 40$). The snap vials were kept at 25 °C under constant darkness. The established incipient colonies were sampled 4 months after their foundation. Specimens were fixed using formaldehyde:ethanol:acetic acid (FAA) (6:16:1) for at least 24 h and then used in the following analyses (sex determination, scanning electron microscopy, and morphometric analyses). We used newly established incipient colonies to trace individual development (see below).

Sex discrimination

Sex determination was performed on larvae and workers as described by Miura et al. (1998); females possess rudiments of (1) an oviduct at the posterior margin on sternite 7, (2) a spermatheca on sternite 8, and (3) colleterial glands on sternite 9, while males possess the rudiments of seminal vesicles on sternite 9. The guts and fat bodies of samples preserved in 70% ethanol were removed, and the remaining body parts were stained with 1% hematoxylin for 15 min. Samples were destained with 0.5% HCl in 80% ethanol until the stains of rudiments were observed. Photographs were taken using a Digital Sight DS-U1 (Nikon, Tokyo) attached to a SZX12 binocular microscope (Olympus, Tokyo).

Scanning electron microscopy

We randomly selected two colonies after sex discrimination. We discriminated each larval instar ($n = 5$) by the head width (see the following section). Larvae fixed in FAA were dehydrated in increasing concentrations of ethanol (70–100%). After preservation in 3-methylbutyl acetate for 10 min, specimens were dried in a HCP-2 critical point drier (Hitachi, Tokyo) and coated with gold in an E-1030 ion sputter (Hitachi). Scanning electron micrographs were taken with an S-3000 N microscope (Hitachi).

Morphometric analyses

Since head width was previously used as one of the criteria for caste discrimination in *N. takasagoensis* (Hojo et al. 2004), those of larvae (first instar, $n = 6$; male second instar, $n = 4$; female second instar, $n = 3$) and workers (male workers, $n = 6$; female workers, $n = 5$) were measured using a Digital Sight DS-U1 (Nikon) attached to a SZX12 binocular microscope (Olympus) after sex determination as described above. Statistical analyses [one-way ANOVA and post hoc Tukey honest significant difference (HSD)] were performed using a four-step Excel statistical analysis (OMS, Saitama, Japan).

Tracing individual development

We selected incipient colonies including parents with eggs ($n = 13$ colonies). The duration of hatching was observed in five out of the 13 colonies. After some eggs in each colony had hatched, all colony members including parents, first-instar larvae and remaining eggs were transferred to one of the 24-well plates (Greiner Bio-One, Frickenhausen, Germany) with well-moistened cotton. These colonies were observed every 24 h until the first instar molted into second-instar larvae. The head of each second instar was marked using a different colored permanent marker. These marked second-instar larvae were examined every 24 h before they molted into presoldiers or workers.

Results and discussion

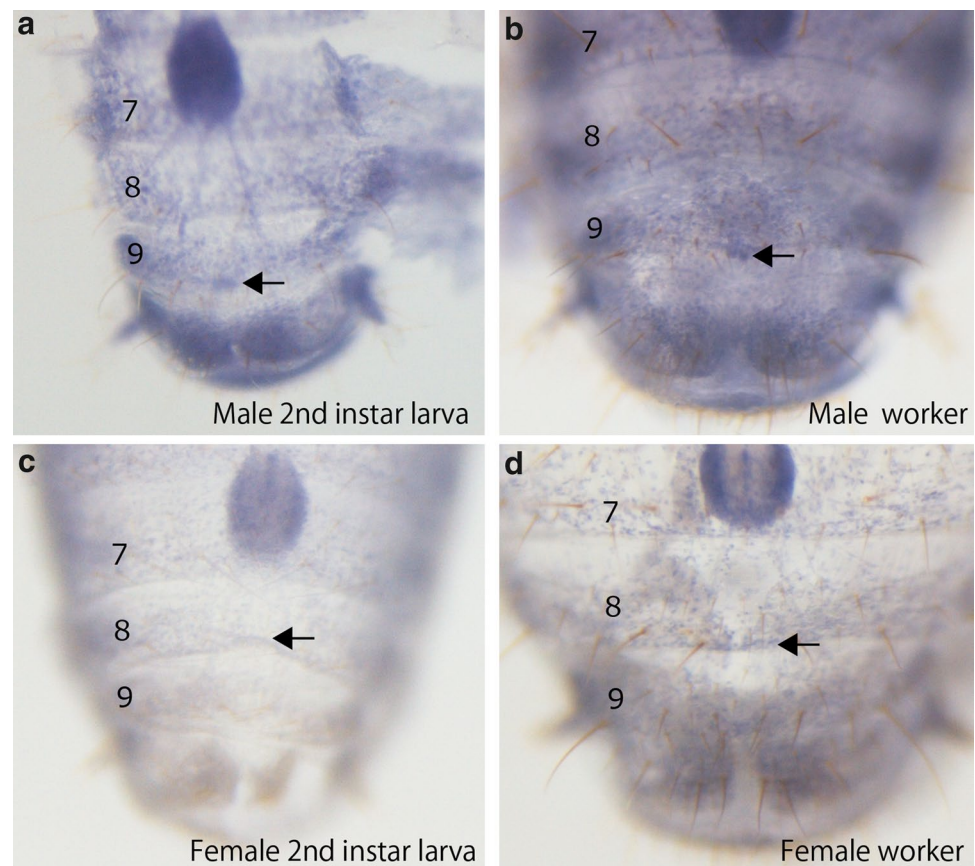
Caste composition in the incipient colony

We attempted to discriminate the developmental stage and sex of all individuals in the incipient colonies 4 months after colony foundation. A specific signal due to hematoxylin stain was observed on sternite 8 or 9 (Fig. 1). In *Hospitalitermes medioflavus* Holmgren (Nasutitermitinae),

rudiments of a spermatheca on sternite 8 were visible in females, and seminal vesicles were visible on sternite 9 in males (Miura et al. 1998). Thus, we concluded that an individual showing a signal from sternite 8 was female, while those that did not were male. In the present study, we were unable to select the sex of first-instar larvae because they were too small to be dissected.

After sex determination, we measured the head widths of these individuals. The head widths of males were significantly smaller than those of females within the same instar (Fig. 2). Significant differences were observed in head widths among all developmental stages (one-way ANOVA and post hoc Tukey HSD, $p < 0.05$). Therefore, we found that it is possible to discriminate the developmental stages of individuals in incipient colonies by head width. Although previous studies indicated that there are four types of male/female workers in mature colonies (Hojo et al. 2004; Toga et al. 2009), we only detected minor workers (male workers; Fig. 2) and medium workers (female workers; Fig. 2) in the colonies examined. In order to clearly discriminate between the first- and second-instar larvae, morphological differences in antennae were investigated by scanning electron microscopy. Both male and female second-instar larvae possessed long bristles on antennal segments 3 and 4 ($n = 5$), whereas none of the first-instar larvae examined

Fig. 1 Sex determination of larvae and workers of *Nasutitermes takasagoensis*. Numbers (7, 8, and 9) indicate abdominal segments. Each arrow shows the rudiments of seminal vesicles on sternite 9 (male; **a**, **b**) or a spermatheca on sternite 8 (female; **c**, **d**)



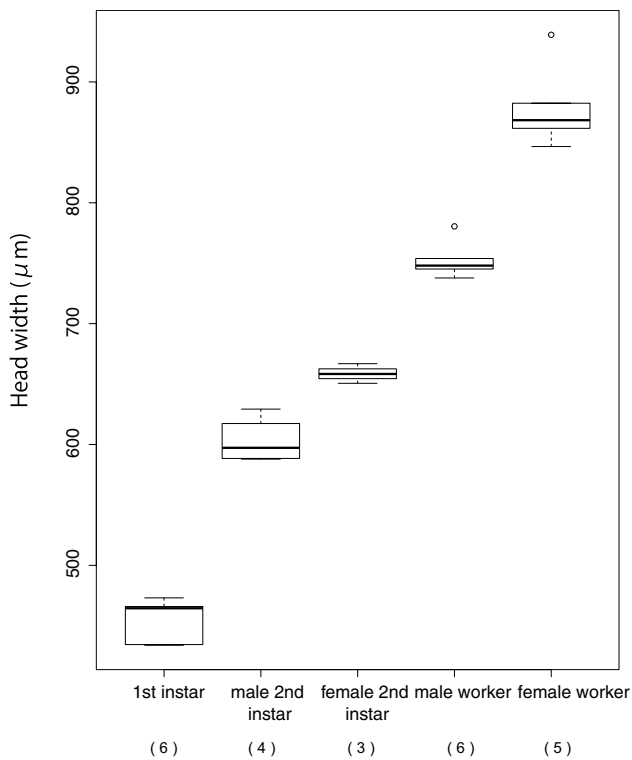


Fig. 2 Head widths of larvae and workers of *N. takasagoensis*. All individuals except for the first instar were measured after sex determination. It was not possible to sex first-instar larvae because they were too small to be dissected. Boxes and whiskers indicate the median, quartiles and range, respectively circles indicate outliers. Numbers of individuals examined in parentheses

had these on the same segments ($n = 5$) (Fig. 3). These bristles were also visible by binocular observations. Based on the results obtained above, we examined caste compositions in incipient colonies 4 months after their foundation (Table 1; $n = 7$ colonies). All colonies examined possessed more than one presoldier and soldier. This result is consistent with previous findings in *Nasutitermes nigriceps* Halderman (Garcia and Becker 1975).

Presoldier and soldier differentiation in the incipient colony

We traced larval development of a newly formed incipient colony in order to identify instars that differentiated into presoldiers and soldiers ($n = 13$ colonies). Hatching from oviposition required approximately 25 days ($n = 5$ colonies). Incipient colonies were observed every 24 h until second-instar larvae emerged. No presoldiers were differentiated from first-instar larvae. We traced the development of second-instar larvae marked with waterproof ink. We were unable to trace the development of first-instar larvae because of the high mortality associated with marking (100%, $n = 5$ individuals). Most male second-instar larvae

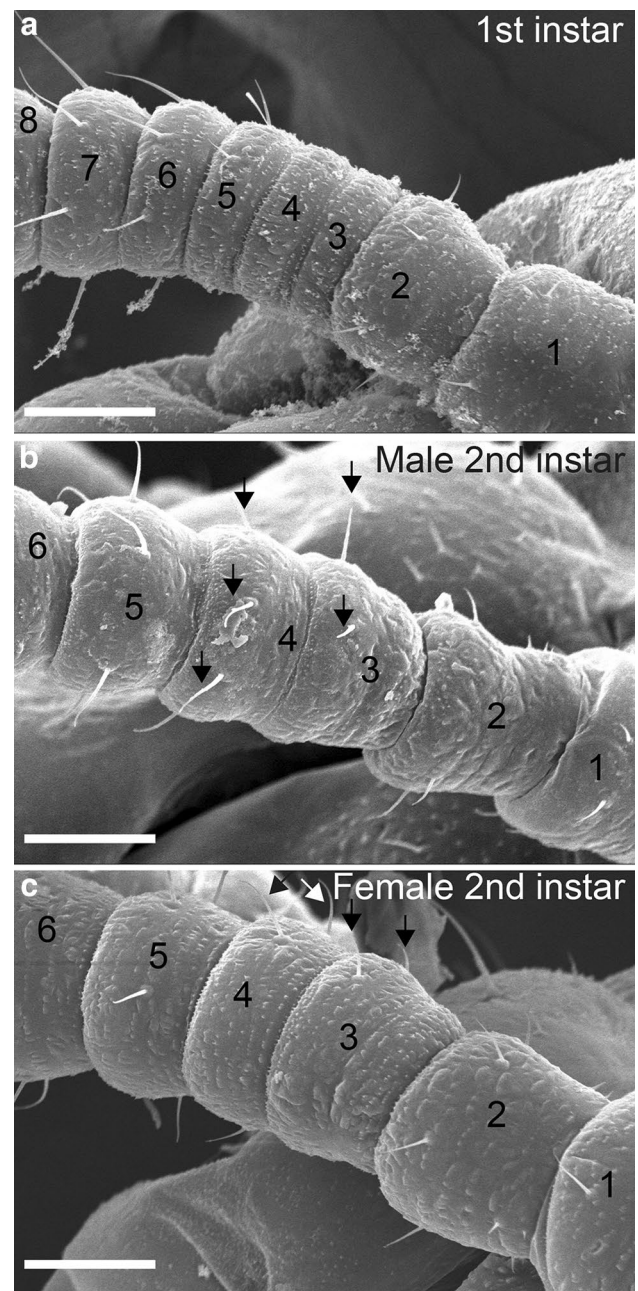


Fig. 3 Scanning electron microscopic photographs of antennae of first-instar larvae (a), male second-instar larvae (b), and female second-instar larvae (c). Numbers (1–8) indicate antennal segments. Each arrow shows specific bristles on antennal segments 3 and 4 of second-instar larvae. Scale bars indicate 50 μm

differentiated into presoldiers regardless of the molting order (Table 2). In mature colonies, soldiers are normally differentiated from minor workers, but not from male second-instar larvae (Hojo et al. 2004). Two individuals, which molted into second-instar larvae thirdly, molted into the third instar (i.e., minor worker) and functioned as workers (Table 2). All female second-instar larvae molted into

Table 1 Numbers of all members in incipient colonies four months after colony foundation

Colony no.	Egg	1st instar	Male 2nd instar	Female 2nd instar	Male minor	Female medium	Presoldier	Soldier
1	5	2	1	3	3	22	0	10
2	18	5	0	1	6	13	1	7
3	8	0	1	3	3	18	0	8
4	7	1	1	0	4	18	1	7
5	9	3	1	3	6	14	0	5
6	13	5	3	0	3	13	0	7
7	19	5	0	4	0	0	2	0

Table 2 Numbers of individuals molted from male 2nd instar larvae

The order in which 1st instar larvae molt into 2nd instar larvae	To a presoldier	To a minor worker
1	13	0
2	4	0
3	2	2
4	13	0

Table 3 Numbers of individuals molted from female 2nd instar larvae

The order in which 1st instar larvae molt into 2nd instar larvae	To a presoldier	To a medium worker
1	0	7
2	0	3
3	0	3
4	0	2

Table 4 Developmental period from the 2nd instar to the presoldier or worker

Sex, stage	<i>n</i>	Duration (day, average \pm S.D.)
Male, 2nd instar to a presoldier	23	7.42 \pm 1.62
Male, 2nd instar to a minor worker	2	14.50 \pm 0.50
Female, 2nd instar to a medium worker	15	12.26 \pm 1.91

third instars (i.e., medium workers) (Table 3). The interval between the emergence of male second instars and their molt into presoldiers was markedly shorter than that into workers (Table 4). This difference in developmental period between worker and presoldier molts is consistent with

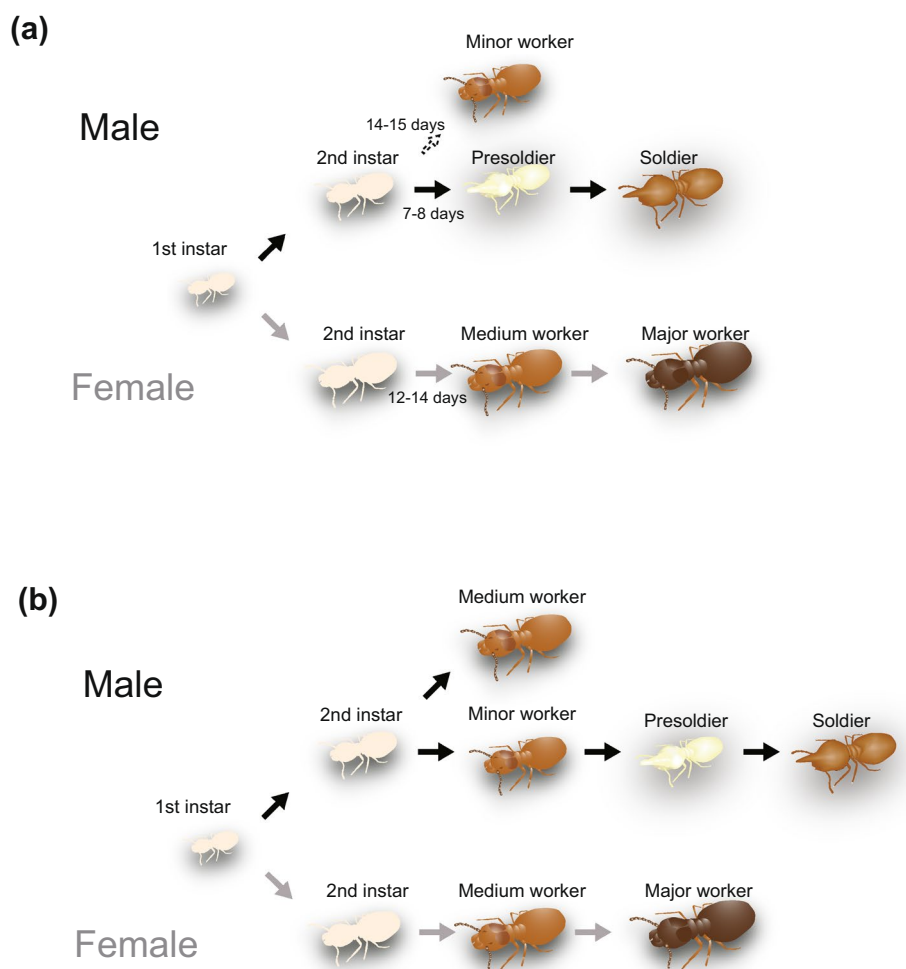
findings obtained for incipient colonies of *Z. nevadensis* [for which these molts took about 22 and 7 days, respectively (Maekawa et al. 2012)]. Presoldier development has been suggested to require more cell proliferation for specific morphogenesis than worker development (Toga et al. 2013). Since presoldier and soldier differentiation require high juvenile hormone (JH) titers in workers, JH synthesis and signaling are considered to be involved in the promotion of cell proliferation and shortening of molting periods. JH biosynthetic genes were previously reported to be up-regulated in soldier differentiation in incipient colonies of *Z. nevadensis* (Yaguchi et al. 2015).

Regulation of soldier differentiation in incipient colonies of *N. takasagoensis*

In mature colonies of *N. takasagoensis*, soldiers were differentiated from male minor workers (Hojo et al. 2004; Toga et al. 2009), but in incipient colonies, differentiated from male second-instar larvae (present study). In *Z. nevadensis*, parental proctodeal trophallaxis was shown to be involved in soldier differentiation in incipient colonies (Maekawa et al. 2012). In higher termites including *N. takasagoensis*, proctodeal trophallaxis generally never occurs, whereas stomodeal trophallaxis is frequently observed (Noirot 1985). Further analyses are needed in order to establish whether stomodeal trophallaxis regulates earlier male-specific soldier differentiation in incipient colonies in *N. takasagoensis*.

In *N. takasagoensis*, The JH titers of male minor workers were higher than those of female workers (Toga et al. 2016). Furthermore, the application of a JH analog may induce soldier differentiation from female workers in *N. takasagoensis* (Toga et al. 2016). The results of the present study suggest that sex differences in JH titers and sensitivities existed even in larval stages, and played a role in sex-specific soldier differentiation. The caste differentiation pathway clarified in the

Fig. 4a, b Soldier and worker developmental pathways in *N. takasagoensis*. **a** In incipient colonies, most male second-instar larvae differentiate into presoldiers within 7–8 days, whereas some individuals rarely molt into minor workers within 14–15 days. All female second-instar larvae differentiate into medium workers within 12–14 days. **b** In mature colonies, presoldiers are differentiated from male minor workers (=third instars) (Hojo et al. 2004; Toga et al. 2009)



present study (Fig. 4) will be useful for obtaining a clearer understanding of the developmental mechanisms involved in sex-specific soldier differentiation.

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