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A non-invasive method for sexing first and second instar larvae of termites using external morphology

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Abstract

Division of labor is fundamental to the ecological success of social insects. In termites, both sexes engage in social tasks, and the sexual division of labor is common in many taxa. Each caste consisting of both sexes is supplied from the population of newly hatched larvae. To understand their social system, it is necessary to investigate the influence of larval sex composition on their developmental fate. However, no method currently exists for sexing young larvae non-invasively, which is essential for experimental manipulation of the sex composition in a society. Here, we report on sex-specific characteristics of the first and second instar larvae of the subterranean termite *Reticulitermes speratus*. Male larvae possess bristles near the center of the posterior margin of the eighth abdominal sternite that are absent in females. The bristles are detectable under a stereomicroscope without damaging the young instar larvae. The validity of morphological sexing was confirmed by a known sex-specific genetic marker. The sex-specific bristles were also useful to identify the sex of first and second instar larvae of the damp-wood termite *Zootermopsis nevadensis*, suggesting that morphological sexing is possible for a wide range of termite species. The morphological sexing presented here has broad applicability in studies addressing sex differences in development, caste differentiation, and behavior. These approaches will contribute to understanding why both sexes co-exist in a termite society and allocate tasks, and which tasks are completely compensable by the other sex, thereby deepening our understanding of social systems where both sexes engage in tasks.

Keywords: morphological sexing, sexual dimorphism, termites, social insects

Introduction

Division of labor is fundamental to the ecological success of social insects (Wilson 1971; Oster and Wilson 1978; Beshers and Fewell 2001; Hölldobler and Wilson 2009). In termites, tasks within the colony are performed by morphologically distinct castes (Oster and Wilson 1978; Robinson 1992). Sexual division of labor is common in many termite taxa (Noirot 1985; Roisin 2000, 2001; but also see Yashiro et al. 2018), even within a caste, where males and females engage in different tasks (Yanagihara et al. 2018). Each caste consisting of both sexes is supplied from the population of newly hatched larvae (Oster and Wilson 1978; Noirot and Pasteels 1987; Noirot 1989; Simpson et al. 2011), and the developmental fate of the larvae is largely determined by socioenvironmental factors (Haverty and Howard 1981; Roisin 2000), although transgenerational epigenetic factors also affect larval developmental fates (Matsuura et al. 2018; Matsuura 2020). To understand the regulatory mechanism and dynamics of the division of labor in societies with both sexes, we need to be able to manipulate the sex composition of the newly hatched larvae and investigate its influence on developmental fates in subsequent instars.

To date, no non-invasive method exists to separate the sexes of early instar larvae. Previous non-invasive methods of termite sexing using external morphology cannot be used for first and second instar larva. Sexing by external morphology is based on sexual dimorphism in the configuration of abdominal sternites. Females possess a posteriorly enlarged seventh sternite with a convex border and a notched or divided eighth sternite, whereas males possess a seventh sternite with a straight border and a smooth eighth sternite. This discrimination method can be used for alates, soldiers, workers (Imms 1920; Weesner 1969; Zimet and Stuart 1982), and third or older instar larva of Kalotermitidae (Luykx 1986; Neoh and Lee 2011), and Rhinotermitidae (Hayashi et al. 2007). Staining of the reproductive organs with hematoxylin (Noirot 1955) and sex-specific genetic markers (Hayashi et al. 2017) can be used for sexing colony members of any instar; however, these invasive methods are not suitable for developmental and behavioral experiments, which require intact individuals, and the known genetic markers are only applicable for *Reticulitermes speratus*.

In addition to the configuration of abdominal sternites, there is a sex difference in bristles on the eighth abdominal sternite, although no attention has been paid to it in terms of morphological sexing. In the alates, workers and soldiers, males possess bristles near the center of the posterior margin of the eighth sternite, while these are absent in females (Thompson and Snyder 1920;

Belyaeva and Dovgobrod 2006). The sex difference in the bristles may also exist in first and second instar larvae and be useful for non-invasive sexing.

To develop a non-invasive sexing method for early instar larvae, we used the subterranean termite, *R. speratus* (Rhinotermitidae). We investigated sex-specific characteristics, particularly the bristles, in the first and second instar larvae under a stereomicroscope. The accuracy of morphological sexing by the characteristics was confirmed by RADtag_467410, a known sex-specific genetic marker (Hayashi et al. 2017). Next, the influence of the morphological sexing on larval survival was evaluated. To verify the cross-species applicability of this sexing method, we also investigated the sex-specific characteristics of the damp-wood termite, *Zootermopsis nevadensis* (Archotermopsidae).

Methods

Identification of sex-specific characteristics

Two mature colonies of *R. speratus* were collected from January to February 2020 in Kyoto, Japan, and the first, second, and third instar larvae were extracted. One hundred larvae for each instar from each colony were randomly selected and used for observation. The sex of the third instar worker larvae was distinguished by the morphology of the seventh and eighth sternites following Weesner (1969) and Zimet and Stuart (1982). Next, we searched for other sex-specific characteristics in the abdominal sternites. Larvae were individually placed in a clear plastic cardholder and observed under a stereomicroscope (SZX7, Olympus, Japan) with objective lens (DFPL1.5x-4), ocular lens (WHSZ10x-H or WHSZ30x-H), and a fiber illuminator (PICL-NEX, Nippon PI, Japan) at a magnification of 12 to 252x. An overview of the device setup is shown in Fig. 1. Similarly, we searched for the presence of these characteristics in the first and second instar larvae under a stereomicroscope.

Confirmation of sex-specificity using sex-specific genetic markers

Ten larvae with and without the characteristics for each instar were randomly selected, and their sex was identified using the RADtag_467410 sex-specific genetic marker (Hayashi et al. 2017). Two colonies were used for replication. Genomic DNA was isolated from the whole bodies of individuals by 2 h digestion with 200 μg/mL Proteinase K (Roche, Germany) in 30 μL of SNET buffer (20 mM Tris–HCl (pH 8.0), 5 mM EDTA, and 400 mM NaCl, 0.3% SDS), followed by precipitation with isopropanol and ethanol. A polymerase chain reaction (PCR) was performed in a

10-μL reaction mixture containing primers at 0.5 μM, dNTPs at 0.2 mM, 1 × Ex *Taq* Buffer, and 0.025 U Ex *Taq* (Takara Bio, Japan), as well as 15 ng of gDNA. The RADtag_467410 primer pair was used for sexing. PCR amplification was performed using the following PCR program: (a) predenaturation at 94 °C for 2 min, (b) 30 cycles of 10 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C, and (c) a final extension at 72 °C for 3 min. We used the primers for *internal transcribed spacer 2*, *ITS2* (Hayashi et al. 2017) as the internal control. Distilled water was used instead of DNA for the negative control, and the gDNA of fifth instar male workers was used as the positive control. The PCR products were electrophoresed in 1% agarose gel and visualized with Midori Green (Nippon Genetics, Japan). To visualize the sex-specific characteristics, both sexes of first, second, and third instar larvae were killed at −80 °C, and micrographs were taken under a scanning electron microscope (SEM:VE-8800, Keyence, Japan).

Effect of morphological sexing on larval survival

The survival rates of sex-identified and control larvae were compared. The first, second, and third instar larvae were extracted, and their sex was identified by the morphological sexing method. One hundred larvae were randomly selected for each sex of each instar. Each group of larvae was placed in a Petri dish (ca. 60 mm) with a moist, unwoven cloth with 100 workers. One hundred control larvae were directly placed in the dish after extraction from the colony without sexing. The termites were kept at 25 ± 1 °C in constant darkness, and the number of surviving larvae was recorded daily for 5 days. Two colonies were used for replication.

Applicability of morphological sexing for specimens

The sex-specific characteristics were investigated in the ethanol-preserved specimens of R. speratus. The first and second instar larvae were observed under both a stereomicroscope and SEM. Two larvae for each sex of each instar in two colonies were used for replication.

Cross-species applicability

Morphological sexing by the sex-specific characteristics was also conducted in *Zootermopsis nevadensis*. Two colonies were collected in April 2019 in Hyogo, Japan. The first, second, and third instar larvae were extracted from the colonies, and the sex was identified by the sex-specific characteristics. In *Z. nevadensis*, morphological sexing by the configuration of the seventh and eighth sternites (Weesner 1969; Zimet and Stuart 1982) was applicable for third instar and older

instar larvae. To validate the sex of first and second instar larvae, each larva was individually placed in a Petri dish (ca. 90 mm) with sawdust bait with 10 workers at 25 ± 1 °C in constant darkness and was left to develop into a third instar. Two larvae for each sex of each instar in two colonies were used for replication. To visualize the sex-specific characteristics, both sexes of first, second, and third instar larvae were killed at -80 °C, and micrographs were taken under SEM.

Statistical analysis

Survival of the larvae in sex-identified males, females, and the control groups was analyzed using Kaplan–Meier survival analysis. A complete pairwise comparison using the log-rank test with sequential Bonferroni correction was applied to test for differences among survival distributions across the three groups. The data were analyzed separately for each instar. Survival was measured as the number of days from the larva's placement on the dish until death. Larvae that were alive on the fifth day were censored. A two-sided Bonferroni-corrected significance level of $P < 0.0167$ was set. The statistical analyses were performed using R software v.3.3.3 (R Core Team 2018).

Results

Sex-specific characteristics and validity of morphological sexing in *R. speratus*

In the third instar larvae, the male possessed extra bristles near the center of the posterior margin of the eighth abdominal sternite that were absent in females (Fig. 2e, f). In the first and second instar, the two types of larvae could be distinguished by the presence of the bristles under a stereomicroscope (Fig. 2a–d). In the first instar, the extra bristles were bilaterally symmetric, and distance between them was similar to that between the styli. In the second instar, the bristle was located near the median line. Larvae possessing bristles also showed male-specific PCR amplification, whereas these were not detected in the larvae without bristles (Fig. 3) in all samples analyzed ($n = 10$ for each sex of each instar in two colonies). There was no variation in the number and location of sex-specific bristles in the first, second, and third instar in all samples analyzed ($n = 100$ for each instar in two colonies), although the number of the other bristles varied among larvae.

Effect of morphological sexing on larval survival in *R. speratus*

There were no differences in survival rates between sex-identified males and the control larvae (first instar: $\chi^2 = 0$, $df = 1$, $P = 1$, second instar: $\chi^2 = 0.1$, $df = 1$, $P = 0.8$), or between sex-identified

females and the control larvae (first instar: $\chi^2 = 0$, $df = 1$, $P = 1$, second instar: $\chi^2 = 0$, $df = 1$, $P = 1$). There was also no sexual difference in the survival rate (first instar: $\chi^2 = 0$, df = 1, $P = 1$, second instar: $\chi^2 = 0.1$, $df = 1$, $P = 0.8$). The 5-day survival rates in sex-identified males, females, and the control groups were 93.0%, 93.0%, and 93.0% in the first instar and 96.0%, 95.5%, and 95.5% in the second instar, respectively.

Applicability of the morphological sexing for specimens in *R. speratus*

The sex-specific bristles were detectable under a stereomicroscope and an SEM in 99.5% of the ethanol-preserved specimens of the first and second instar larvae.

Cross-species applicability

As in *R. speratus*, third instar males of *Z. nevadensis* possessed extra bristles on the eighth abdominal sternite (Fig. 4). The first and second instar larvae possessing the bristles developed into third instar males, whereas the larvae without the bristles developed into third instar females. The morphological sexing in *Z. nevadensis* by the sex-specific bristles under the stereomicroscope was correct in all the samples analyzed, irrespective of instar and colony ($n = 2$ for each sex of instar in two colonies).

Discussion

We identified sex-specific bristles on the eighth abdominal sternite in first, second, and third instar larvae in *R. speratus*. There was no variation in the bristles, and PCR amplification of the sex-specific genetic marker indicated that sexing by the bristles under a stereomicroscope was 100% accurate in the first to third instars. The morphological sexing did not increase the mortality rate of the larvae. The results indicate that the bristles provide a precise method for sexing under a stereomicroscope without damaging the larvae. The morphological sexing was also applicable to nondestructive determination of the sex of ethanol-preserved specimens. Conventionally, the histological method (Noirot 1955) has been used for sexing dead larvae. Compared to the histological method, the morphological sexing method only requires a stereomicroscope or an SEM, saving economic resources and time. The morphological sexing presented here has broad applicability in studies investigating the sex of termites.

The sex-specific bristles were found in the central area of eighth sternite which is known to have sexual difference in development in termites. In the late instars including sterile worker and soldier

castes, the central area of eighth sternite in females develops into a part of a membranous genitalia where spermathecal opening locates on its center and no bristles around it, whereas the sternite develops into sclerotized plate with bristles on its posterior margin in males (Thompson and Snyder 1920; Belyaeva and Dovgobrod 2006). Since the morphology of internal reproductive organs is detectable even in first instar (Miura et al. 1998; Roisin and Lenz 1999; Parmentier and Roisin 2003), the sex-specific bristles in first to third instar larvae appear to be associated with the sexual difference of reproductive organs.

The sex-specific bristles were identified in *R. speratus* and *Z. nevadensis* which belong to two phylogenetically distinct families of Isoptera, Rhinotermitidae, and Archotermopsidae, respectively (Bourguignon et al. 2015), suggesting that the morphological sexing by the bristles is applicable to larvae in a wide range of termite species. As described above, the bristles on the eighth sternite are likely to be associated with the reproductive organs. Previous histological studies in a variety of taxa including species with permanently sterile workers have shown that the internal reproductive organs of early instar larvae are morphologically different between sexes (Miura et al. 1998; Roisin and Lenz 1999; Parmentier and Roisin 2003). Therefore, the method presented here is likely to be applicable to the other termite taxa, although it is the issue to be addressed in future studies.

Morphological sexing by the sex-specific bristles is applicable to non-invasive identification of the sex of first and second instar larvae. Using our method and previous non-invasive methods (Imms 1920; Weesner 1969; Zimet and Stuart 1982) together, we can identify the sex of any instar and caste before observation and experimental manipulation. Tracking of larval growth, developmental fate, and survivorship over time in both sexes will reveal the underlying processes that produce the sex composition in each caste and the age demography in termite colonies. Furthermore, manipulation of socioenvironmental factors will reveal the regulatory mechanism of their developmental fate, which contributes to maintaining the sex composition in a colony. Our sexing method is also applicable to investigating sex differences in behavior, immunity, and stress resistance at an early developmental stage. These approaches will contribute to understanding why both male and female termites co-exist in a society and allocate tasks, and which tasks are completely compensable by the other sex and which tasks are not. In summary, non-invasive sexing has broad applicability in studies addressing the role and contribution of each sex in each caste, opening new avenues for understanding the social system where both sexes engage in tasks.

Data availability

The dataset is available in Mendeley data (https://data.mendeley.com/datasets/prpdfghdz8/draft?a=c2137322-a305-43e0-a5db-e8a5a3018a90).

Code availability

Not applicable

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Contributions

MT and KM designed experiments. All authors contributed to collect termites. MT, TI, TI, and ET performed experiments. MT, TI, TI, and KM wrote the manuscript, and all authors are accountable for the content and approved the final version of the manuscript.

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Ethics declarations

Conflict of interest

The authors declare no competing interests.

Authors' contributions:

M. T. and K. M. designed experiments. All authors contributed to collect termites. M. T., T. In., T. Is. and E. T. performed experiments. M. T., T. In., T. Is. and K. M. wrote the manuscript, and all authors are accountable for the content and approved the final version of the manuscript.

Figures

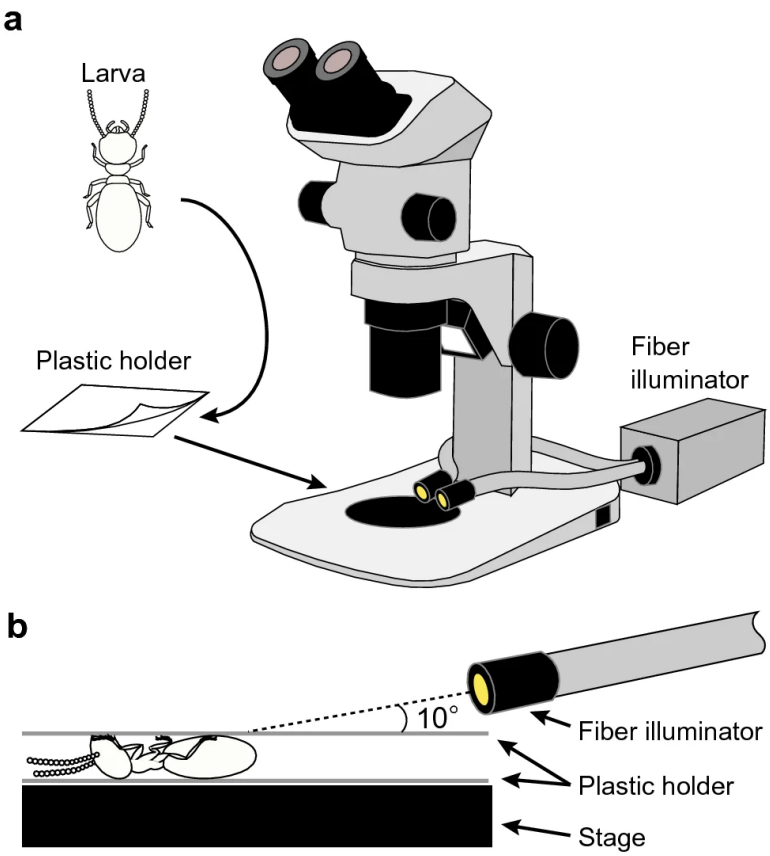

Figure 1 Schematic showing **a** the device used to observe the characteristics of the abdominal sternites and **b** a side view of the stage and lighting angle. A larva is placed in the clear plastic cardholder and observed under a stereomicroscope. The sample is illuminated from the posterior side by a fiber illuminator. The lighting angle was set to 10° from the horizontal plane to make the characteristics visible

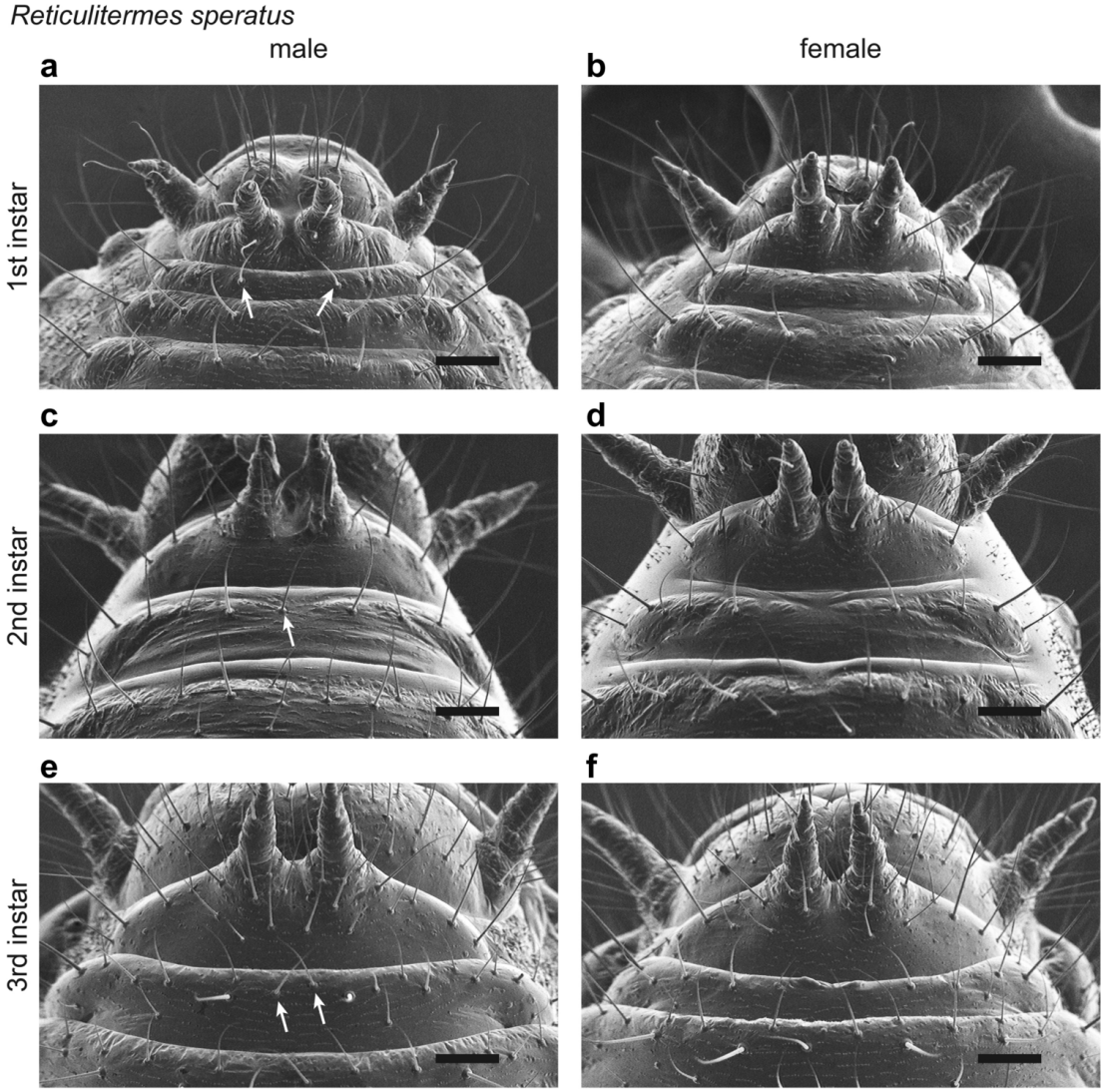

Figure 2 Scanning electron microscope images of the abdominal sternites in *Reticulitermes speratus*: **a** first instar male; **b** first instar female; **c** second instar male; **d** second instar female; **e** third instar male; **f** third instar female. White arrows indicate the sex-specific bristles on the eighth sternite. Scale bars indicate 50 μm.

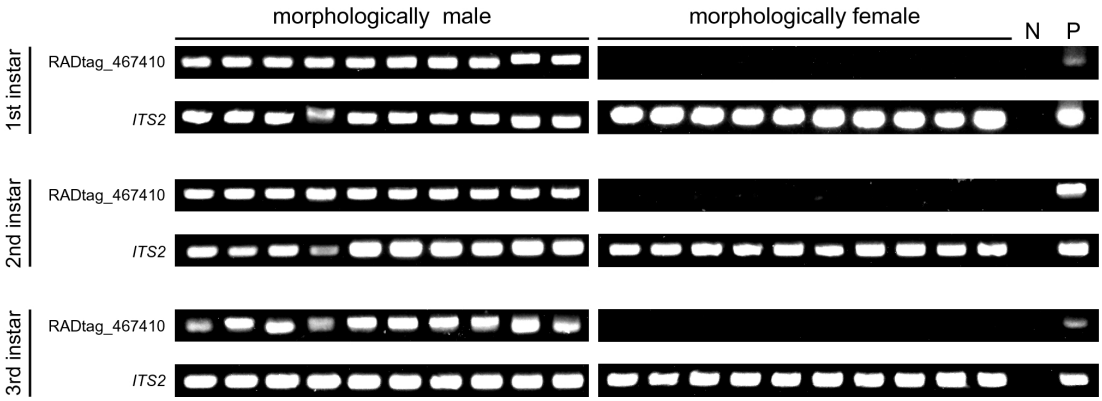

Figure 3 Electropherograms of the male-specific marker (RADtag_467410) and an internal control gene (*Internal transcribed spacer 2*, *ITS2*). Male-specific markers were amplified in the larvae evaluated as male by morphological sexing and not in the larvae evaluated as female. "N" and "P" indicate the lanes of the negative control (distilled water) and positive control (a fifth instar male worker), respectively. Although ten individuals for each sex of each instar were analyzed in two colonies, electropherograms of one representative colony are shown, because the colonies yielded similar electropherograms.

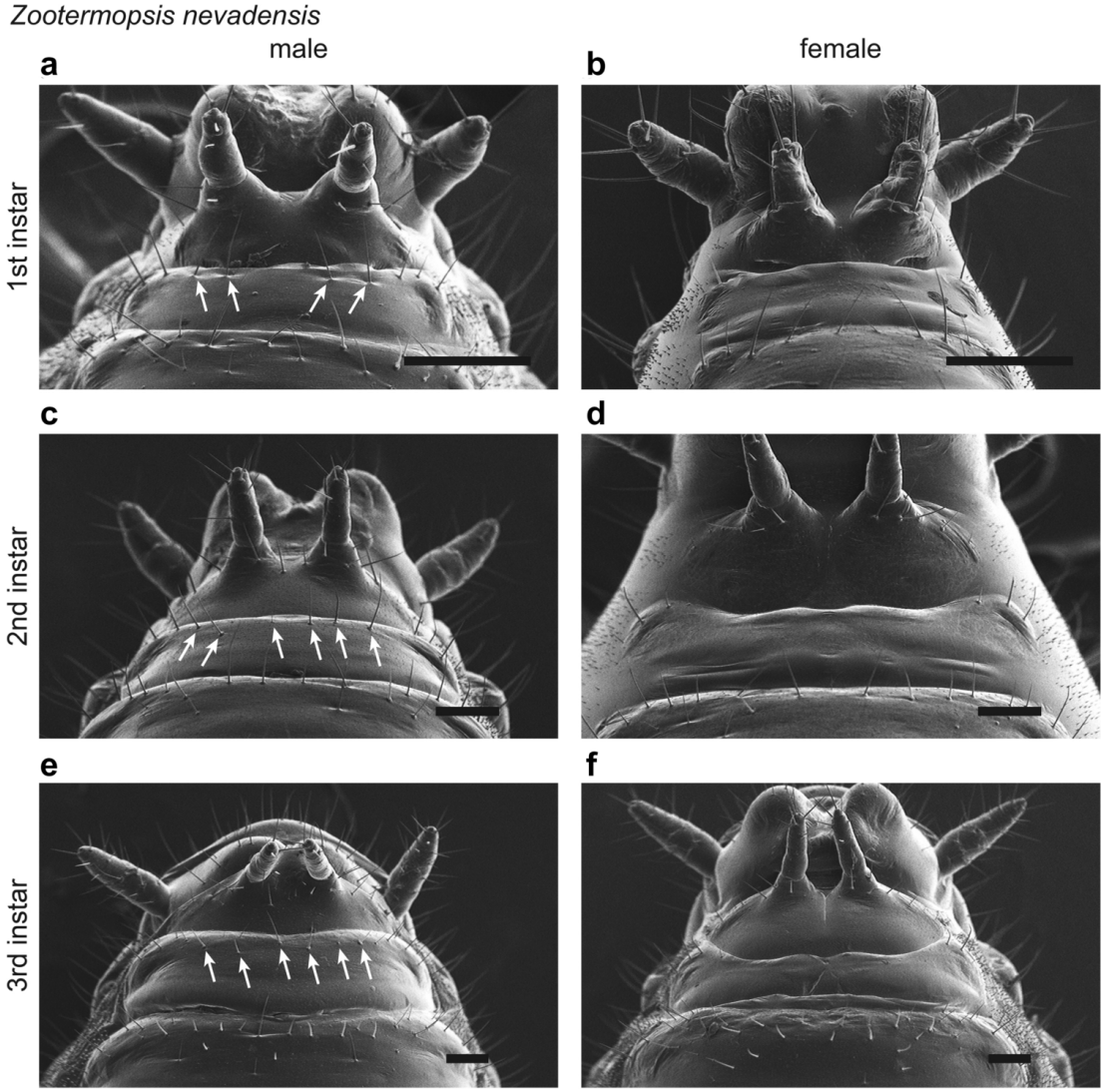

Figure 4 Scanning electron microscope images of the abdominal sternites in *Zootermopsis nevadensis*: **a** first instar male; **b** first instar female; **c** second instar male; **d** second instar female; **e** third instar male; **f** third instar female. White arrows indicate the sex-specific bristles on the eighth sternite. Scale bars indicate 100 μm.