

Supplementary Material for
Age-based soldier polyethism: old termite soldiers take more risks
than young soldiers

Saki Yanagihara, Wataru Suehiro, Yuki Mitaka, Kenji Matsuura*

Laboratory of Insect Ecology, Graduate School of Agriculture, Kyoto University

Kitashirakawa Oiwakecho, Kyoto 606-8502, JAPAN

*correspondence to: kenjijpn@kais.kyoto-u.ac.jp

This file includes:

Text S1: Supplementary Notes

Text S2: Materials and Methods

Text S3: Supplementary Discussion

Figure S1 to S3

Table S1

Other Supplementary Materials for this manuscript includes the following:

Movie S1, S2

Dataset S1

Text S1: Supplementary Notes

Entrance guards and royal guards

Reticulitermes termites are classified as multiple-piece nesters, whereby nests of a single colony are interconnected by belowground tunnels and aboveground shelter tubes [1, 2]. Colonies have hidden royal chambers underground or deep inside wood (figure S1). In *R. speratus*, soldiers in the foraging area (entrance guards) typically perform head-plug (phragmotic) defence. The nests are divided into chambers connected by small openings that allow only one termite at a time to pass through. This arrangement allows chamber-by-chamber nest defence. When predatory ants attack the nest, soldiers retreat to the small openings between chambers and plug up the openings with their heads to prevent the ants from invading intact parts of the nest [3]. Soldiers comprise c.a. 4% of termites in foraging area of *R. speratus* nests [3], which is much smaller than in other Rhinotermitids such as *Coptotermes* termites, whose colonies consist of up to 40% soldiers [4]. This difference in soldier ratio can be explained by their distinct modes of defence: *Coptotermes* soldiers rush out and assault intruders, whereas *Reticulitermes* soldiers act phragmotically as ‘living plugs’.

Unlike the entrance-guard soldiers in the foraging area, the soldiers guarding royal chambers perform active defence by rushing out and establish the final defence line (movie S1, figure S2). In contrast to the foraging area, soldier ratio often exceeds 50% around the royal chamber. Therefore, entrance guards and royal guards are different in terms of defence position, defence tactics and density.

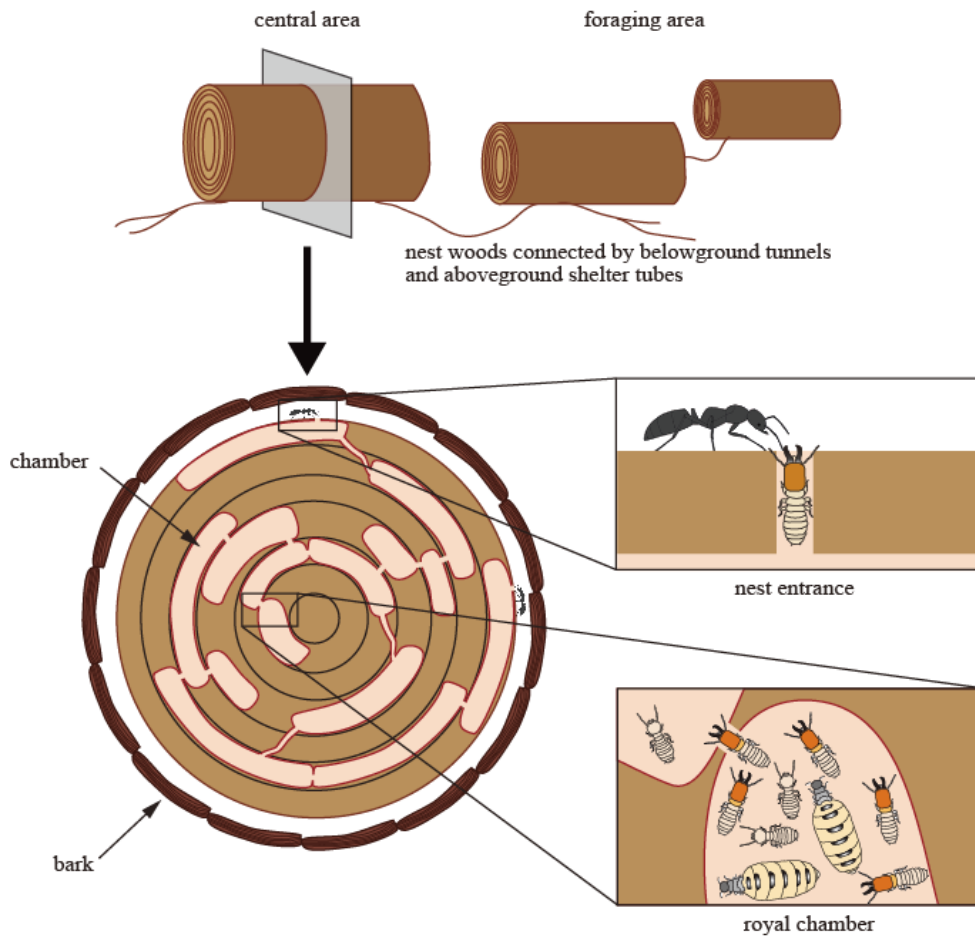


Figure S1. Schematic diagram of a nest structure of *Reticulitermes* termites. Royal chamber is typically located deep inside the wood of central area. The nests have multi-layered structures consisting of a number of chambers connected vertically and horizontally by small openings that allow only one termite at a time to pass through. Predatory ants need to break the multi-layered defence to access the royal chamber. Therefore, soldiers defending at the front line (outer layer) encounter predators more often than those defending the royal chamber.

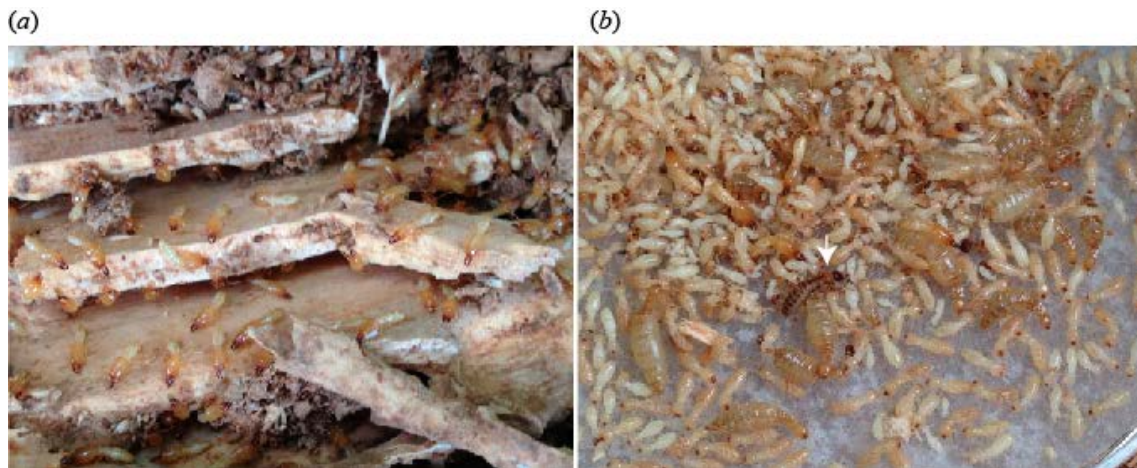


Figure S2. Royal guard soldiers observed in a field colony (a) Royal guard soldiers defending the royal chamber of a large field colony (colony code: MS150719E) Misawa, Aomori prefecture, Japan. (b) A primary king (arrowed) and multiple secondary queens extracted from the royal chamber.

Reproductive system of *Reticulitermes speratus* and soldier sterility

The reproductive system of *R. speratus* is characterized by asexual queen succession (AQS), which is a mode of reproduction whereby workers, soldiers and alates (dispersing reproductives) are produced sexually while neotenic queens (non-dispersing queens) arise through thelytokous parthenogenesis and eventually replace the old queens [5-7]. By using this system, founding queens are able to boost reproduction without reducing the transmission rate of their genes to future generations. This system also seems advantageous to the colony to promote its growth rate without losing genetic diversity in workers. In AQS species, reproduction is strictly limited to primary kings, primary queens and parthenogenetically-produced neotenic queens. In our recent study, we performed an extensive survey of reproductives in field colonies [8]. Among the 162 kings collected from 114 mature colonies in the field, 104 were primary, 58 were nymphoid, while no

ergatoid king was found in the field. Among the 6,824 queens, 6,812 (99.82%) were nymphoid and 6 were primary (alate-derived), while only 6 (0.088%) were ergatoid. We have never found reproductive soldiers in *R. speratus* and our genotyping data also showed no such inbreeding caused by soldier reproduction. Furthermore, there is no report of soldier reproduction in any species of the family Rhinotermitidae.

Text S2: Materials and Methods

Termite preparation

We collected five colonies of *R. speratus* in secondary forests in Kyoto and Shiga, Japan, from January to April 2017. Each colony was placed outdoors until use in the experiment. Soldier differentiation is a seasonal event in *Reticulitermes speratus*, with new soldiers differentiating from June to September [9]. Therefore, soldiers collected before June can be classified as ‘old soldiers’, i.e. soldiers older than one year. To obtain old soldiers, we extracted all soldiers from each colony in late April and separated them by sex based on their external abdominal characters [10]. Male and female old soldiers were marked with oil paint and then housed with nestmate workers in Petri dishes with mixed sawdust bait [11]. To obtain new soldiers, we housed the remaining workers (ca. 1500 workers) of each colony in Petri dishes with mixed sawdust bait. New soldiers differentiated in the soldier-less group from June to July. We maintained new soldiers for one month for cuticle sclerotisation before use in the experiment. There was no significant difference in body size between old and young soldiers (likelihood ratio test, head width: $\chi_1^2 = 1.050$, $p = 0.306$, pronotum width: $\chi_1^2 = 1.856$, $p = 0.173$).

Nest defence experiment

To observe soldier defence behaviour, we prepared experimental nests (figure 1a) as previously described [3]. Experimental nests were made of cardboard ($25 \times 50 \times 1.5$ mm), with a cylindrical nest chamber (15 mm in diameter) with an entrance (1.5 mm wide), covered with a 20×70 -mm microscope slide (figure 1a). The experimental nest was placed in the centre of a 90-mm petri dish, and then two soldiers and five workers were placed in the nest chamber. Termites were maintained for an hour to acclimatise them to the experimental nest. Then we recorded the position of each soldier. Thereafter, a single predatory ant, i.e. the termite-hunting needle ant *Brachyponera chinensis*, was placed in the petri dish. Subsequent termite defence and ant predation behaviour was recorded for 30 minutes. We designated a soldier in the defensive position if it was located at the nest entrance (figure 1b). Using this experimental setup, we compared the defensive rates (no. of soldiers defending at the nest opening per no. of trials) between old and young soldiers of each sex, and between male and female soldiers of each age class. We performed five to twenty replicates (depending on soldier availability from each colony) for each treatment, for each colony. This experiment was repeated using the five colonies.

Table S1. Number of replications for each treatment

soldier combination	before ant introduction (without ant)	after ant introduction (with ant)
old ♂ vs young ♂	68	68 (68)*
old ♀ vs young ♀	41	41 (41)
old ♂ vs old ♀	81	81 (81)
young ♂ vs young ♀	48	48 (41)

*The number of trials in which predatory ants approached the nest entrance within 30 minutes was indicated in the parenthesis (See Dataset S1 for details of the number of replication per colony).

Test of age-dependent mortality

We compared the survival rates of old and young soldiers under poor nutritional condition to investigate the higher age-dependent mortality. A group of 10 old soldiers, 10 young soldiers and 100 workers was made from each colony. The termites were placed in a 90mm petri dish lined with moist quartz filter paper (090270N-SPGFD, AS ONE Co.). The petri dishes were kept in a plastic container and maintained at 25°C. Soldier survival was recorded at every 24 hours.

Distribution of young and old soldiers in the nest

To investigate the distribution of old and young soldiers in the nest, we prepared an artificial nest imitating the multiple-piece nest structure, i.e., multiple nests interconnected by belowground tunnels and aboveground shelter tubes [1, 2]. The artificial nest consists of two large plastic cases (140×220×35mm) coupled with each other via an acrylic tube (figure 2a). In each large case, five small plastic cases (30×30×20mm) with 5mm opening on each side were arranged in a row at 10mm interval, which simulated the arrangement of nest chambers in a log (figure 2a). Each of the small plastic case was filled with mixed sawdust bait to half the depth. We placed a group of termite consisting of two neotenic queens, an old male soldier, an old female soldier, a young male soldier, a young female soldier, 10 larva and 100 workers in each of the five cells in one of the large plastic case. The artificial nest was kept at 25°C in the dark for 30 days, and then we counted the number of individuals by castes and by sexes in each cell. We replicated this experiment twice using two colonies collected in secondary forests in Kyoto, Japan in June, 2017.

We defined the distance ranking as follows: distance ranking 1: in the royal

chamber itself, 2: in the nest chambers of the royal chamber, 3: in the chamber which is one chamber apart from the royal chamber, 4: in the chamber which is two chamber apart from the royal chamber, 5: any other place in the central area, 6: in the satellite area.

Statistical analysis

All analyses were performed using R v3.3.2 (R Core Team, Vienna, Austria, <https://www.R-project.org/>). Comparisons of defense rates in each treatment were performed with a generalized linear mixed model (GLMM) with binomial distribution followed by likelihood ratio tests. In the model, individual and colony were included as random effects to account for repeated measures, and age and sex as fixed effects in the test of age- and sex-dependent defense, respectively. Comparison of survivability of old/young soldiers under starved condition were performed with Kaplan–Meier survival analysis followed by Wald tests. Comparisons of the each soldiers' distribution in the article nest were performed with an ordered logistic regression followed by likelihood ratio tests. In the model, age, sex and colony as fixed effect.

Text S3: Supplementary Discussion

Life expectancy, task performance and allocation of risky tasks

The division of labour is caused by multiple factors including proximate internal and external factors [12-14]. A number of studies reported worker age polyethism in eusocial Hymenoptera, i.e., ants [15], honeybees [16], stingless bees [17] and social wasps [18], and also in some termite species [19, 20]. However, it has remained unclear whether life expectancy is an ultimate factor in allocating risky tasks among colony members because

workers undertake a variety of tasks in their life and different tasks requires different morphological and physiological adaptations. For example, in ant species with polymorphic worker caste, major workers are larger and often engage in defence [21, 22] but live longer than minor workers [23]. This pattern seems inconsistent with the hypothesis of shorter-lived workers engaging in more risky tasks. However, this could be explained by the better defensive performance of major workers than minor workers due to morphological adaptations. Similarly, it has been reported that larger and thus older workers perform most tasks in a lower termite *Reticulitermes fukienensis* [19]. Old workers of a neotropical higher termite *Neocapritermes taracua* perform suicidal defence by bursting [20]. These age polyethism in termite workers would be reasonably explained by task performance of different size classes (and thus age classes). It is impossible to separate the effects of age and size in worker polyethism in termites because workers go through multiple moults increasing in size with age. Therefore, to understand the effect of age in allocating risky tasks among workers we need to investigate whether workers from the same physical caste show age dependent division of labour.

Recent studies of Moroń et al. [24, 25] demonstrated that workers with an experimentally reduced life span foraged under risk more often than control workers in the ant *Myrmica scabrinodis*. In these studies, the life expectancy of workers were shortened by poisoning with carbon dioxide or by injury through removal of their propodeal spines. Such an artificial damage may influence ant behaviour not only by shortening the life expectancy but also by its neurophysiological impact.

In contrast to earlier studies of worker age polyethism in eusocial Hymenoptera and termites, we separated the effects of size and age by using termite soldiers, a physical caste specialized to nest defence. We demonstrated that old soldiers go to the front line

of nest defence more often than young soldiers and that young soldiers are more biased toward choosing central nest defence as royal guards. Nevertheless, we found no significant difference in task performance (defence efficiency) between young and old soldiers at least in this study system. When only young soldiers were placed in the experimental nest, young soldiers themselves located at the nest entrance and performed fighting against the ants (41 of 41 trials in which the ants approached at the entrance) as well as old soldiers. Our study suggests that spatial distribution of risk is crucial for the allocation of soldiers with different life expectancies.

Sexual difference in defensive behaviour

In termites, unlike the eusocial Hymenoptera, both males and females are engaged in social labour. We found that female soldiers performed defensive behaviour on the front line more often than male soldiers. This may imply sexual division of labour in the termite soldier caste, although the adaptive significance of this sex difference requires further study. Sexual size dimorphism, in that female soldiers are larger than male soldiers, may explain the better phragmotic defence of females [26]. A recent study of immune-related gene expression in *R. speratus* revealed soldier-specific immune genes, some of which exhibit sexually dimorphic expression by which male soldiers show significantly higher expression levels of immune genes, including peptidoglycan recognition protein, cathepsin, lysozyme-like protein, and serine protease [27]. Our data in conjunction with male-biased immune defence suggest that female soldiers may engage more in anti-predatory defence, and male soldiers perform more anti-pathogenic defence.

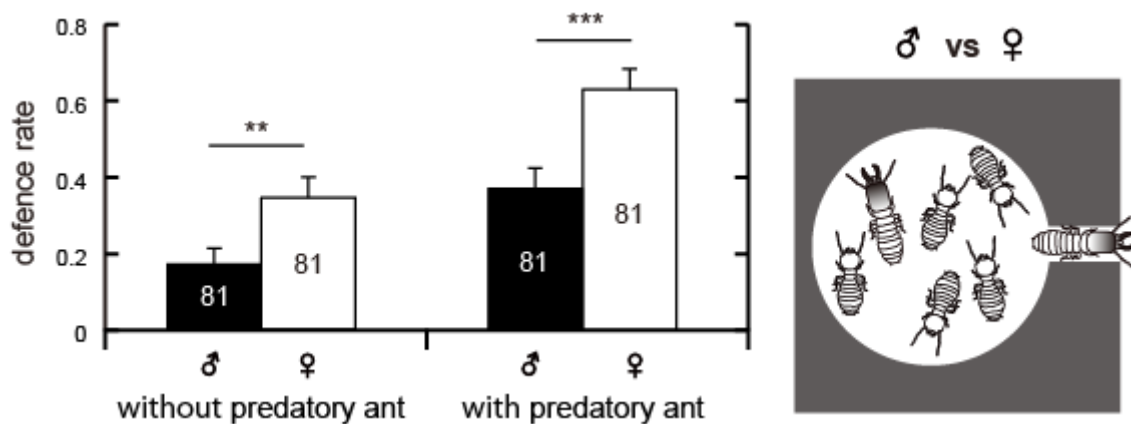


Figure S3. Comparison of defence behavior between male and female soldiers. Comparison of defence rates of old soldiers between male and female with (right) and without (left) predatory ants (** $p < 0.01$, *** $p < 0.001$, generalised linear mixed model [GLMM]). Values denote the means \pm standard error. n is indicated on each bar.

References

- [1] Mizumoto, N. & Matsuura, K. 2013 Colony-specific architecture of shelter tubes by termites. *Insectes Soc.* **60**, 525-530. (doi:10.1007/s00040-013-0319-1).
- [2] Abe, T. 1987 Evolution of life types in termites. In *Evolution and Coadaptation in Biotic Communities*. (eds. S. Kawano, J.H. Connell & T. Hidaka), pp. 125-148. Tokyo, University of Tokyo Press.
- [3] Matsuura, K. 2002 Colony-level stabilization of soldier head width for head-plug defense in the termite *Reticulitermes speratus* (Isoptera : Rhinotermitidae). *Behav. Ecol. Sociobiol.* **51**, 172-179. (doi:10.1007/s00265-001-0426-2).
- [4] Waller, D.A. & La Fage, J.P. 1987 Nutritional ecology of termites. In *Nutritional ecology of insects, mites, spiders, and related invertebrates* (eds. F. Slansky & J.G. Rodriguez), pp. 487-532, John Wiley & Sons, New York.
- [5] Matsuura, K., Vargo, E.L., Kawatsu, K., Labadie, P.E., Nakano, H., Yashiro, T. & Tsuji, K. 2009 Queen succession through asexual reproduction in termites. *Science* **323**, 1687-1687. (doi:10.1126/science.1169702).
- [6] Matsuura, K. 2017 Evolution of the asexual queen succession system and its underlying mechanisms in termites. *J. Exp. Biol.* **220**, 63-72. (doi:10.1242/jeb.142547).
- [7] Yashiro, T. & Matsuura, K. 2014 Termite queens close the sperm gates of eggs to switch from sexual to asexual reproduction. *Proc. Natl. Acad. Sci. USA* **111**, 17212-17217. (doi:10.1073/pnas.1412481111).
- [8] Matsuura, K., Mizumoto, N., Kobayashi, K., Nozaki, N., Fujita, T., Yashiro, T., Fuchikawa, T., Mitaka, Y. & Vargo, E.L. in press A genomic imprinting model of termite caste determination: not genetic but epigenetic inheritance influences offspring caste fate *Am. Nat.*
- [9] Matsuura, K. 2002 Sociobiology of the termite *Reticulitermes speratus*, Doctoral thesis,

Kyoto University, Kyoto.

- [10] Roonwal, M.L. 1975 Sex ratios and sexual dimorphism in termites. *J. Sci. Ind. Res.* **34**, 402-416.
- [11] Matsuura, K. & Nishida, T. 2001 Comparison of colony foundation success between sexual pairs and female asexual units in the termite *Reticulitermes speratus* (Isoptera : Rhinotermitidae). *Popul. Ecol.* **43**, 119-124. (doi:10.1007/pl00012022).
- [12] Robinson, G.E. 1992 Regulation of division of labor in insect societies. *Annu. Rev. Entomol.* **37**, 637-665. (doi:10.1146/annurev.en.37.010192.003225).
- [13] Gordon, D.M. 1996 The organization of work in social insect colonies. *Nature* **380**, 121-124. (doi:10.1038/380121a0).
- [14] Giraldo, Y.M. & Traniello, J.F.A. 2014 Worker senescence and the sociobiology of aging in ants. *Behav. Ecol. Sociobiol.* **68**, 1901-1919. (doi:10.1007/s00265-014-1826-4).
- [15] Hölldobler, B. & Wilson, E.O. 1990 *The Ants*. Cambridge, MA, Harvard University Press.
- [16] Seeley, T.D. 1982 Adaptive significance of the age polyethism schedule in honeybee colonies. *Behav. Ecol. Sociobiol.* **11**, 287-293. (doi:10.1007/BF00299306).
- [17] Sommeijer, M. 1984 Distribution of labour among workers of *Melipona favosa* F.: age-polyethism and worker oviposition. *Insectes Soc.* **31**, 171-184. (doi:10.1007/BF02228754).
- [18] Jeanne, R.L., Williams, N.M. & Yandell, B.S. 1992 Age polyethism and defense in a tropical social wasp (Hymenoptera: Vespidae). *J. Insect Behav.* **5**, 211-227. (doi:10.1007/BF01049290).
- [19] Crosland, M.W.J., Lok, C.M., Wong, T.C., Shakarad, M. & Traniello, J.F.A. 1997 Division of labour in a lower termite: the majority of tasks are performed by older workers. *Anim. Behav.* **54**, 999-1012. (doi:10.1006/anbe.1997.0509).
- [20] Šobotník, J., Bourguignon, T., Hanus, R., Demianová, Z., Pytelková, J., Mareš, M., Foltynová, P., Preisler, J., Cvačka, J., Krasulová, J., et al. 2012 Explosive backpacks in old termite workers. *Science* **337**, 436-436. (doi:10.1126/science.1219129).
- [21] Wilson, E.O. 1976 The organization of colony defense in the ant *Pheidole dentata* Mayr (Hymenoptera: Formicidae). *Behav. Ecol. Sociobiol.* **1**, 63-81. (doi:10.1007/bf00299953).
- [22] Hasegawa, E. 1993 Nest defense and early production of the major workers in the dimorphic ant *Colobopsis nipponicus* (Wheeler)(Hymenoptera: Formicidae). *Behav. Ecol. Sociobiol.* **33**, 73-77. (doi:10.1007/BF00171658).
- [23] Shik, J.Z. 2010 The metabolic costs of building ant colonies from variably sized subunits. *Behav. Ecol. Sociobiol.* **64**, 1981-1990. (doi:10.1007/s00265-010-1009-x).
- [24] Moroń, D., Witek, M. & Woyciechowski, M. 2008 Division of labour among workers with different life expectancy in the ant *Myrmica scabrinodis*. *Anim. Behav.* **75**, 345-350. (doi:10.1016/j.anbehav.2007.06.005).
- [25] Moroń, D., Lenda, M., Skórka, P. & Woyciechowski, M. 2012 Short-Lived Ants Take Greater Risks during Food Collection. *Am. Nat.* **180**, 744-750. (doi:10.1086/668009).
- [26] Matsuura, K. 2006 A novel hypothesis for the origin of the sexual division of labor in termites: which sex should be soldiers? *Evol. Ecol.* **20**, 565-574. (doi:10.1007/s10682-006-9117-9).
- [27] Mitaka, Y., Kobayashi, K. & Matsuura, K. 2017 Caste-, sex-, and age-dependent expression of immune-related genes in a Japanese subterranean termite, *Reticulitermes speratus*. *PLoS ONE* **12**, e0175417. (doi:10.1371/journal.pone.0175417).

Movie S1: Royal guard soldiers defending the royal chamber of a large field colony (colony code: MS150719E) Misawa, Aomori prefecture, Japan.

Movie S2: A soldier blocking the entrance of the nest against a predatory ant *Brachyponera chinensis*.