University of Reading

Test of the negative feedback hypothesis of colony size sensing in social insects

Article

Accepted Version

Kikuchi, T., Hayashi, Y. ORCID: https://orcid.org/0000-0002-9207-6322, Fujito, Y., Fujiwara-Tsujii, N., Kawabata, K., Sugawara, K., Yamaoka, R. and Tsuji, K. (2024) Test of the negative feedback hypothesis of colony size sensing in social insects. Biology Letters, 20 (6). ISSN 1744-957X doi: 10.1098/rsbl.2024.0102 Available at https://centaur.reading.ac.uk/117037/

It is advisable to refer to the publisher's version if you intend to cite from the work. See Guidance on citing.

To link to this article DOI: http://dx.doi.org/10.1098/rsbl.2024.0102

Publisher: The Royal Society

All outputs in CentAUR are protected by Intellectual Property Rights law, including copyright law. Copyright and IPR is retained by the creators or other copyright holders. Terms and conditions for use of this material are defined in the End User Agreement.

www.reading.ac.uk/centaur

University of Reading

CentAUR

Central Archive at the University of Reading

Reading's research outputs online

BIOLOGY LETTERS

Test of the negative feedback hypothesis of colony-size sensing in social insects

Journal:	*Biology Letters*
Manuscript ID	RSBL-2024-0102.R1
Article Type:	Research
Date Submitted by the Author:	n/a
Complete List of Authors:	Kikuchi, Tomonori; Chiba University, Marine Biosystems Research Center Hayashi, Yoshikatsu; University of Reading, School of Systems Engineering Fujito, Yuka; Shimadzu Corporation, Division of Analytical and Measuring Instruments Fujiwara-Tsujii, Nao; NARO, Institute for Plant Protection Kawabata, Shun-ichi; Toyama University, Department of Bilogy Sugawara, K.; Tohoku Gakuin University, Department of Information Science Yamaoka, Ryohei; Kyoto Institute of Technology, Department of Applied Biology, Graduate school of Science and Technology Tsuji, Kazuki; University of the Ryukyus, Department of Subtropical Agro-Environmental Sciences; Kagoshima University, The United Graduate School of Agricultural Sciences
Categories:	Animal Behaviour
Subject:	Evolution < BIOLOGY, Ecology < BIOLOGY, Behaviour < BIOLOGY
Keywords:	self-organisation, contact, fertility, worker reproduction, queen pheromone

SCHOLARONE™
Manuscripts

Author-supplied statements

Relevant information will appear here if provided.

Ethics

Does your article include research that required ethical approval or permits?:
This article does not present research with ethical considerations

Statement (if applicable):
CUST_IF_YES_ETHICS :No data available.

Data

It is a condition of publication that data, code and materials supporting your paper are made publicly available. Does your paper present new data?:
Yes

Statement (if applicable):
https://datadryad.org/stash/share/yKuOrNWgkp5moayIR-Z1L1ZwtnwKDWRdClL_tGh9Ljg

Conflict of interest

I/We declare we have no competing interests

Statement (if applicable):
CUST_STATE_CONFLICT :No data available.

Use of AI

Please provide a statement of any use of AI technology in the preparation of the paper.

No, we have not used AI-assisted technologies in creating this article
CUST_IF_YES_DECLARATION_OF_AI_USE :No data available.

For Review Only

Test of the negative feedback hypothesis of colony-size sensing in social insects

T. Kikuchi[1], Y. Hayashi[2], Y. Fujito[3], N. Fujiwara-Tsujii[4], S. Kawabata[5], K. Sugawara[6], R. Yamaoka[7], K. Tsuji*[8,9]

Affiliations

[1] Marine Biosystems Research Center, Chiba University, Tokawa 1, Choshi City, Chiba 288-0014, Japan

[2] School of Biological Sciences, University of Reading, Reading, Berkshire RG6 6AH, United Kingdom

[3] Division of Analytical and Measuring Instruments, Shimadzu Corporation, 1 Kuwabaracho Nishinokyo Nakagyo-ku, Kyoto 604-8511, Japan

[4] Institute for Plant Protection, National Agriculture and Food Research Organization, Tsukuba 305-8666, Ibaraki, Japan

[5] Department of Biology, Toyama University, Toyama 930-8555, Japan

[6] Department of Information Science, Faculty of Liberal Arts, Tohoku-gakuin University, 2-1-1, Tenjinzawa, Izumi, Sendai, Miyagi 981-3193, Japan

[7] Kyoto Institute of Technology (Emeritus), Kyoto 606-8287, Japan

[8] Department of Subtropical Agro-Environmental Sciences, University of the Ryukyus, Nishihara, Okinawa 903-0123, Japan

[9] The United Graduate School of Agricultural Sciences, Kagoshima University, Kagoshima 890-0065 Japan

* Corresponding author: Kazuki Tsuji, Department of Subtropical Agro-Environmental Sciences, University of the Ryukyus, Nishihara, Okinawa 903-0123, Japan
E-mail: tsujik@agr.u-ryukyus.ac.jp
Phone number: +81-098-895-8797

Abstract

Social insects can sense colony size—even without visual information in a dark environment. How they achieve this is yet largely unknown. We empirically tested a hypothesis on the proximate mechanism using ant colonies. In *Diacamma* colonies the monogynous queen is known to increase the effort devoted to queen pheromone–transmission behaviour (patrolling) as the colony grows, as if she perceives colony size. The negative feedback hypothesis assumes that through repeated physical contacts with

For Review Only

workers the queen monitors the physiological state (fertility) of workers and increases her patrolling effort when she encounters more fertile workers. Supporting this hypothesis, we found that queens increased patrol effort in response to a higher ratio of fertile workers under the experimental condition of constant colony size. Furthermore, chemical analyses and bioassays suggested that cuticular hydrocarbons have queen pheromone activity and can mediate the observed queen–worker communication of fertility state. Such a self-organising mechanism of sensing colony size may also operate in other social insects living in small colonies.

Keywords: self-organisation, contact, fertility, worker reproduction, queen pheromone, eusociality

Background

Colony size is related to social complexity, and is important for understanding the evolution of sociality in social insects [1,2]. Phylogenetic analysis indicates that in eusocial insects, large complex colonies are derived from small simple colonies [2,3]. From an ontogenetic perspective, various individual and group traits change as the colony grows. For example, in eusocial taxa of Hymenoptera and Isoptera, reproductive castes are generally produced when colony size (worker number) exceeds a certain level [4]. Also in some eusocial insects, individual body size, foraging strategy, aggressiveness, response to stress, sex ratio, and policing behaviour also change with colony size [5–9]. Many of these trait changes are considered adaptive and are therefore likely to be controlled in a colony size–dependent manner [4,10,11]. However, the proximate mechanisms by which colony size controls these traits are not yet well understood.

Colony size–dependent behaviour is observed in queens of the ant *Diacamma* cf. *indicum* from Japan. (In this article we use the functional definition of queen caste [12], though queens of *Diacamma* are called gamergates, i.e. mated workers, in other contexts [13–15] since all females are morphologically identical) In this monogynous species, queens mate once (sometimes twice) and worker reproduction is inhibited via queen pheromone and policing behaviours [13–15]. Queen pheromone has only a short-term effect, and the frequency of aggressive dominance interactions among workers competing for oviposition rights significantly increases by 3 hours after queen removal [13,14]. Queen pheromone is transmitted to workers by direct physical contact [15]. The queen distributes her pheromone by regularly walking around the nest and touching workers with her antennae and other body parts (patrol behaviour) [13,16]. Queens

For Review Only

devote more effort to this pheromone-transmission behaviour in large colonies than in small colonies by patrolling more frequently [13,16]. However, it is not known how the queen perceives colony size and is triggered to switch her behaviour.

Adejumo and co-workers [16] created a computer model to test whether this phenomenon can be self-organising, controlled by a feedback mechanism through repeated interactions between the queen and workers (see also [17]). Their model was based on the following four assumptions. First, when a worker has lost contact with the queen for a long time, the worker's physiological state becomes more prone to self-reproduction (i.e., more fertile). This has been empirically established [13,14,18]. Second, if the queen encounters fertile workers during her patrols, she devotes more effort to pheromone-transmission behaviour. This behaviour generates negative feedback, because encounters with fertile workers promote the queen's pheromone transmission behaviour, which suppresses worker fertility. Note that the queen is assumed to respond only to local information, that is, recent contact with fertile workers. Third, the queen can perceive the fertility of workers that she contacts. Last, the presence of queen pheromone. We assume cuticular hydrocarbons (CHCs) can play the role, since queens and workers have markedly different CHC profiles (Electronic Supplementary Material 1). However, the second to last assumptions underlying the feedback hypothesis have not been empirically tested. Here, we tested those assumptions in *D.* cf. *indicum*. This is a rare rigorous empirical test of an explicit colony size–related feedback mechanism in social insects.

Methods

Study materials

We used colonies of *D.* cf. *indicum* collected on Okinawa Island, Okinawa Prefecture, Japan, during 2001–2022. Each colony was kept in a plastic container (26.5 cm length × 18.5 cm width × 5 cm height) with a plaster floor (1.5 cm thick). In the middle of the floor a 13 cm × 9 cm depression (1 cm deep) covered with a glass plate acted as an artificial nest. Ants were kept in the laboratory at 25 ± 1°C with a light:dark cycle of 12 h:12 h and fed honey water and mealworms ad libitum three or four times a week. Field colonies contain 20–300 workers [19,20]. We used medium to large colonies containing more than 100 workers.

Effect of fertile workers on queen patrol behaviour (negative feedback test)

We investigated whether the proportion of fertile workers in a colony influenced the queen's patrol behaviour. First, we created nine queen-right colonies of equal size (100

For Review Only

workers) by removing workers at random. After an acclimation period of 1 month, we divided each colony into an orphan subcolony (40 workers) and a queen-right subcolony (60 workers) that were maintained for the subsequent 2 weeks. Then, after eliminating all brood (non-adults), the queen-right subcolony was transferred to a new observation nest of the above-mentioned design. Two days later we recorded the queen's behaviour for 12 h with a digital video camera. Then a portion of the workers were replaced with workers from the orphan subcolony at an exchange ratio of 0% (0 workers), 25% (15 workers), or 50% (30 workers). The majority of orphan workers become fertile within 2 weeks after queen removal [17]. One hour after this worker exchange, we resumed recording the queen's behaviour for 12 h. The queen and all workers were then combined into a single colony and maintained in the nest for at least 3 weeks to ensure the restoration of queen-right physiological conditions in both workers and the queen. After the restoration period, the same colonies underwent this procedure two more times until each queen had undergone all treatments (0%, 25%, and 50%); treatment order was randomly assigned. For analysis, we regarded each queen as a block, because there was large individual variation in queen patrol behaviour for unknown reasons. The queen's activity mostly consists of simple repeats of patrolling and resting, with occasional rare oviposition and feeding events [13]. Patrol behaviour was defined as either walking or antennating a worker while not walking. Resting behaviour was defined as either remaining motionless or self-grooming in the same location for longer than 5 sec.

We focused on four behavioural metrics: total patrol time per 12 h, patrol frequency (number of patrol bouts per 12 h), mean patrol duration and mean rest duration. We analysed the effect of treatments on each of the above behavioural metrics by using generalised linear mixed models (GLMMs) with likelihood ratio tests. We set the exchange ratio as the fixed factor, the colony (or queen) as a random factor, the behavioural metric (such as patrol frequency) before the worker exchange as an offset, and that after the worker exchange as the response variable. A negative binomial distribution was assumed for total patrol time per 12 h, and a gamma distribution was assumed for the remaining behavioural performances.

Queen's ability to discriminate fertile workers

To examine whether queens can discriminate fertile from non-fertile workers, we video-recorded colonies ($N = 6$) in which all adults were in individually marked in advance with enamel paint; recordings lasted 4–5 h. We identified the most dominant alpha worker (showed aggression to others but had never received aggression) and low-rank

For Review Only

145 workers (never engaged in dominance interactions). Alpha workers occasionally lay
146 male eggs even if a queen is present [18,20]. We then put the queen and three low-rank
147 workers into a plastic Petri dish (12 cm in diameter) for 1 h to acclimatise the queen.
148 Then we introduced an additional worker (either the alpha or a randomly selected low-
149 rank worker) into the Petri dish and video-recorded the behavioural interaction between
150 the focal worker and the queen for 15 min. Immediately after this trial the focal worker
151 was dissected to examine her ovarian condition. To minimise the impact of handling,
152 the queen and the three low-rank workers were returned to the original nest, and 1 or 2
153 days were allowed to pass before conducting another trial. Trial order was randomised.
154 We defined an encounter as any physical contact between queen and worker. We
155 counted the number of encounters that caused the queen to respond, either by
156 antennation or by orientation (turning her body to orient her head toward the worker).
157 We compared the ratio of response to non-response encounters between alpha and low-
158 rank workers by using a generalised linear mixed model (GLMM) with a Poisson
159 distribution. We set worker rank (alpha or low-rank) as the fixed factor, colony as a
160 random factor, the total number of encounters as an offset, and the number of
161 encounters that led to a queen response (antennation or orientation) as the response
162 variable. Similarly, we compared focal workers' ovarian condition (the number of
163 oocytes in ovaries) between alpha and low-rank workers by Wilcoxon singed rank test
164 regarding colony as the block.
165
166 *Bioassay on CHCs' queen pheromone activity*
167 Using another four colonies we tested if the queen CHCs have a suppressive effect on
168 worker reproduction. We used the dominance behaviour as a proxy of a worker's
169 tendency to perform self-reproduction [13-15, 18]. First, more than 2 days before the
170 experiment, we standardized all colonies to have the same size of 80 workers and a
171 queen. Then each colony was randomly divided into two orphan groups (each with 40
172 workers), each of which was placed in a plastic container (6 × 4.7 × 2.5 cm). During this
173 procedure, we killed the queen and quickly extracted the CHCs in the following way.
174 To retrieve the crude extract, the queen was immersed in 1.5 ml of *n*-hexane. Then the
175 solvent was evaporated and re-dissolved in 70 µl of *n*-hexane. This total crude hexane
176 extract was separated into HC fraction (HC fr.) and non-HC fraction (non-HC fr.) on a
177 silica-gel column (0.04–0.063 mm mesh silica-gel) by stepwise solution with 3 ml of n-
178 hexane and dichloromethane. This n-hexane fluid extract was applied to 1/8 of a piece
179 of a glass-fibre filter paper (2.1 mm in diameter, 1 mm width) by a microsyringe (0.5
180 individual equivalent amount). At 30 min after the orphaning, we gently used tweezers

For Review Only

to place the filter on one of the two orphan groups and exposed workers to it for 5 min. It is known that *D.* cf. *indicum* workers are attracted and exposed to the queen's HC fr. soaked in the filter paper (Electronic Supplementary Material 2). We then gently removed the filter from the nest container, allowed 5 min to lapse so the colony could calm down, and counted the incidence of worker–worker dominance behaviour for 20 min. This procedure (one cycle = 30 min) was repeated nine times. As the control treatment, the other orphan groups were exposed to n-hexane in the same way. Finally, we analyzed the effect of queen HC fr. on dominance behaviour by using a GLMM with likelihood ratio tests, in which the fixed factors were treatment (queen HC fr. or *n*-hexane), time after orphaning, and treatment × time after orphaning, the random factor was colony, and the response variable was assumed to show a gamma distribution.

All statistical tests were performed using R statistical software version 4.2.2 (20).

Results

Test of the negative feedback loop

The key assumption of the negative feedback hypothesis is that queens invest more effort in patrol behaviour after contact with fertile workers. This assumption was empirically supported; when a portion of workers were swapped for orphan workers while keeping the colony size constant (60 workers), queens significantly increased the total patrol time per 12 h (GLMM with likelihood ratio tests, $\chi^2 = 13.91$, $p = 0.0009$). More precisely, this was achieved by more frequent switches in queen behaviour; when workers were replaced by orphan workers, queens significantly increased their patrol frequency ($\chi^2 = 14.10$, $p < 0.0001$, figure 1*a*) and decreased both the average duration of resting ($\chi^2 = 17.06$, $p = 0.0002$, figure 1*b*) and that of patrolling ($\chi^2 = 5.621$, $p = 0.060$). For the effect size of each treatment (each exchange ratio) see also Table 1.

Queens can recognise fertile workers

Queens responded (by antennation or by orientation) more frequently to alpha workers than to low-rank workers ($\chi^2 = 34.1$, $p < 0.001$; figure 2*a*). Dissections showed alpha workers had more developed ovaries than low-rank workers ($z = 2.29$, $p = 0.022$; figure 2*b*).

CHCs show queen pheromone activity

The queen CHC extract suppressed dominance behaviour of orphan workers. The frequency of dominance behaviour among workers steadily increased with time after the queen removal in the control treatment (exposed to n-hexane), whereas workers'

For Review Only

dominance behaviour remained at a low level when orphan workers were exposed to the HC fraction of queen extract (GLMM with likelihood ratio tests, time: $\chi^2 = 19.41$, $P < 0.001$; treatment: $\chi^2 = 52.52$, $P < 0.001$; time × treatment: time × treatment: $\chi^2 = 4.067$, $P = 0.0437$; figure 3). For details see also Electronic Supplementary Material 3.

Discussion

The negative feedback hypothesis for the colony size-dependent patrol behaviour of *Diacamma* queens [16,17] assumes that a queen can sense the reproductive state of workers through physical contact. It also assumes that the queen changes her patrol behaviour to reflect this information; thus, her colony size–dependent behaviour emerges without direct knowledge of colony size. Our experiments empirically support this hypothesis. This queen behaviour is adaptive because it effectively prevents worker reproduction until it is constrained by the high cost of patrol in very large colonies [16].

There are other hypotheses regarding the mechanisms of ant colony size sensing, including an increase in the frequency of worker–worker encounters [22–24] or an increase in the concentration of carbon dioxide [25] or other chemicals [26] in the nest. Those hypothetical mechanisms differ from the negative feedback hypothesis in that the former implicitly assume an increase in individual density per nest space with colony growth. However, the relevant factor in the negative feedback hypothesis is not worker density or frequency of queen–worker encounters, but the ratio of fertile to infertile workers that the queen encounters. In our experiment, colony size was kept constant and only the ratio of fertile workers was manipulated. Furthermore, in *Diacamma* the density of individuals per nest area appears to be controlled by the ants themselves, with the number of individuals per floor area in the centre of the nest (near the queen) remaining almost constant regardless of colony size [16]. This observation makes sense for ants that nest in soil, because they are highly likely to dig to expand nest space as colony size increases [27] to prevent overcrowding, which would hinder colony efficiency. In addition, under crowded conditions ants are known to change behaviour and keep the worker–worker encounter rate low [28].

A series of our chemical analyses and bioassays suggested that cuticular hydrocarbons (CHCs) underpin the communication between workers and queens. CHC profiles were associated with fertility and differed between fertile and sterile workers as well as between queens and workers (Electronic Supplementary Materials). Most importantly, queen CHCs suppressed orphan workers' dominance behaviour (figure 3) that reflects competition over egg-laying opportunities. A wide range of social insects use CHCs as the queen pheromone [29–32]. This also appears to be the case in *D.* cf.

For Review Only

indicum from Japan, although the specific CHC component that shows queen pheromone activity has yet to be specified.

Insect CHCs are a mixture of various hydrocarbon molecules, the primary function of which is considered dehydration prevention. The queen pheromone activity of some long-chain linear and methyl-branched saturated CHCs is conserved across a broad array of social taxa [31]. The phylogenetic conservation of CHCs as queen pheromones may support the honest fertility signature hypothesis [31–33] rather than the chemical weapon hypothesis (but see [34,35]). Provided that the honest fertility signature hypothesis is also valid in *Diacamma*, a single CHC may allow workers to sense the queen and for the queen to sense fertile workers. In this scenario, the individual bearing a large amount of the pheromone on its cuticle would be identified as the queen, whereas an individual bearing a small amount of the pheromone would be identified as an ovary-developing worker. Future studies should test this hypothesis.

We believe that the mechanism of colony-size sensing via queen–worker contact that we suggest here may be generally operative in other social insects, at least in those with small colony sizes. Indeed, the importance of combination of physical interaction and pheromone for suppression of reproduction has been suggested in a polistine wasp [36]. More studies, including studies in other taxa, are needed.

Table caption

Table 1. GLMM results of four behavioural metrics of the queens: total patrol time per 12 h, patrol frequency (number of patrol bouts per 12 h), mean patrol duration and mean rest duration. Different exchange ratios were regarded as different treatments (categorical variables and fixed effects) and their effects on each of the above behavioural metrics in comparison to the control (0% swapping) were analysed. One can also see some non-linear effects.

Figure legends

Figure 1. Queen responses when a proportion of workers were swapped with orphan workers ($N = 9$). (*a*) Frequency of queen patrol per 12 h after the worker swap divided by that before the swap. (*b*) Mean resting duration after the worker swap divided by that before the swap. Box boundaries reflect the inter-quartile range (IQR), the vertical range is the maximum value in Q3+1.5*IQR and the minimum value in Q1+1.5*IQR, and the horizontal bar is the median.

For Review Only

Figure 2. (*a*) Proportion of encounters that led to a queen response (either by antennation or by orientation) to alpha ($N = 6$) and low-rank workers ($N = 6$). (*b*) Number of oocytes in the ovaries of each worker. Circles represent individual colony values. For an explanation of the components of the box plots, see figure 1.

Figure 3. Frequency of dominance behaviour of workers under repeated exposure to queen's cuticular hydrocarbon extract (red circles, $N = 4$) or to n-hexane (black circles, $N = 4$).

Acknowledgments

We thank Toshiharu Akino for helping with the bioassay and Ryo Hosomi, Mieko Okai, and Nao Shigenari of Toyama University for collecting preliminary data.

Funding statements

This study was supported in part by Japan Society for the Promotion of Science KAKENHI (grant nos. 17657029, 18047017, 20033015, 23870003, 26249024, 15H02652, 16F16794, 17H01249, 22H02702, 23K18155).

References

1. Bonner JT. 2004 Perspective: the size complexity rule. *Evolution* **58**, 1883–1990.
2. Bourke AFG. 2011 Principles of Social Evolution. (Oxford University Press).
3. Wilson, EO. 1971 The Insect Societies (The Belknap Press of Harvard University Press).
4. Oster GF, Wilson EO. 1978 Caste and Ecology in the Social Insects (Princeton University Press).
5. Houston A, Schmid-Hempel P, Kacelnik A. 1988 Foraging strategy, worker mortality, and the growth of the colony in social insects. *Am. Nat.* **131**, 107–114.
6. Karsai I, Wenzel W. 1998 Productivity, individual-level and colony-level flexibility, and organization of work as consequences of colony size. *Proc. Natl. Acad. Sci. USA*. **95**, 8665–8669
7. Matsuura K, Kobayashi N. 2010 Termite queens adjust egg size according to colony development. *Behav. Ecol.* **21**, 1018–1023.
8. Shimoji H, Kikuchi T, Ohnishi H, Kikuta N, Tsuji K. 2018 Social enforcement depending on the stage of colony growth in an ant. *Proc. R. Soc. Lond. B.* **285**,

For Review Only

20172548.

9. Gal A, Kronauer D. 2022 The emergence of a collective sensory response threshold in ant colonies. *Proc. Natl. Acad. Sci. USA*. **119**, e2123076119
10. Ohtsuki H, Tsuji K. 2009 Adaptive reproduction schedule as a cause of worker policing in social Hymenoptera: a dynamic game analysis. *Am. Nat.* **173**, 747–758.
11. Hou C, Kaspari M, Vander Zanden HB, Gillooly JF. 2009 Energetic basis of colonial living in social insects. *Proc. Natl. Acad. Sci. USA*. **107**, 3634–3638.
12. Buschinger A, Heinze J. 1992 Polymorphism of female reproductives in ants. *Biology and evolution of social insects* (ed Billen J), pp.21-23, Leuven: Leuven University Press.
13. Kikuchi T, Nakagawa T, Tsuji K. 2008 Changes in relative importance of multiple social regulatory forces with colony size in the ant *Diacamma* sp. from Japan. *Anim. Behav*. **76**, 2069–2077.
14. Kikuchi T, Suwabe M, Tsuji K. 2010 Durability of information concerning the presence of a gamergate in *Diacamma* sp. from Japan. *Physiol. Entomol.* **35**, 93–97.
15. Tsuji K, Egashira K, Hölldobler B. 1999 Regulation of worker reproduction by direct physical contact in the ant *Diacamma* sp. from Japan. *Anim. Behav.* **58**, 337–343.
16. Adejumo S, Kikuchi T, Tsuji K, Maruyama-Onda K, Sugawara K, Hayashi Y. 2023 A real-time feedback system stabilises the regulation of worker reproduction under various colony sizes. *PLoS Computational Biology*, doi: 10.1371/journal.pcbi.1010840.
17. Sugawara K, Yaegashi K, Hayashi Y, Kikuchi T, Tsuji K. 2009 Modeling of patrol behavior of *Diacamma*'s gamergate. *Artif. Life. Robot.* **14**, 318–320.
18. Peeters C, Tsuji K. 1993 Reproductive conflict among ant workers in *Diacamma* sp. from Japan: dominance and oviposition in the absence of the gamergate. *Insect. Soc.* **40**, 119–136.
19. Fukumoto Y, Abe T, Taki A. 1989 A novel form of colony organization in the 'queenless' ant *Diacamma rugosum. Physiol. Ecol. Jpn.* **26**, 55–61.
20. Nakata K, Tsuji K.1996 The effect of colony size on conflict over male-production between gameragte and dominant workers in the ponerine ant *Diacamma* sp. *Ethol. Ecol. Evol.* **8**, 147–156.
21. R Core Team. 2022 R: A Language and Environment for Statistical Computing (version 4.2.2). R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/
22. Pratt SC. 2005 Quorum sensing by encounter rates in the ant *Temnothorax*

For Review Only

albipennis. Behav. Ecol. **16**, 488–496.
23. Musco C, Su H-H, Lynch NA. 2017 Ant-inspired density estimation via random walks. *Proc. Natl. Acad. Sci. USA*. **114**, 10534–10541.
24. Gordon DM. 2021 Movement, encounter rate, and collective behavior in ant colonies. *Ann. Entomol. Soc. Am*. **114**, 541–546.
25. Tschinkel WR. 1999 Sociometry and sociogenesis of colonies of the Florida harvester ant (Hymenoptera: Formicidae). *Ann. Entomol. Soc. Am.* **92**, 80–89.
26. Smith ML, Kingwell CJ, Boroczky K, Kessler A. 2020 Colony-level chemical profiles do not provide reliable information about colony size in the honey bee. *Ecol. Entomol.* **3**, 679–687.
27. Franks NR, Wilby A, Silverman B, Toft C. 1992 Self-organizing nest construction in ants: Sophisticated building by blind bulldozing. *Anim. Behav.* **44**, 357–375.
28. Gordon DM, Paul RE, Thorpe K. 1993 What is the function of encounter patterns in ant colonies? *Anim. Behav.* **45**, 1083–1100.
29. Holman L, Jorgensen CG, Nielsen J, d'Ettorre P. 2010 Identification of an ant queen pheromone regulating worker sterility. *Proc. R. Soc. Lond. B.* **27**, 3793–3800.
30. Van Oystaeyen A, Oliveria RC, Holman L, van Zweden JS, Romero C, Oi CA, d'Ettorre P, Khalesi M, Billen J, Wackers F, Millar JG, Weseleers T. 2014 Conserved class of queen pheromones stops social insect workers from reproducing. *Science* **343**, 287–290.
31. Smith AA, Liebig J. 2017 The evolution of cuticular fertility signals in eusocial insects. *Curr. Opin. Insect. Sci.* **22**, 79–84.
32. Holman L. 2018 Queen pheromones and reproductive division of labor: a meta-analysis. Behav. Ecol. **29**, 1199–1209.
33. Keller L, Nonacs P.1993 The role of queen pheromones in social insects: queen control or queen signal? *Anim. Behav.* **45**, 787–794.
34. Olejarz J, Veller C, Nowak MA. 2017 The evolution of queen control over worker reproduction in the social Hymenoptera. *Ecol. Evol.* **7**, 8427–8441.
35. Villalta I, Abril S, Cerda X, Boulay R. 2018 Queen control or queen signal in ants: What remains of the controversy 25 years after Keller and Nonacs' seminal paper? *J. Chem. Ecol.* **44**, SI 805–817.
36. Oi CA, Oliveira1 RC, van Zweden JS, Mateus S, Millar JG, Nascimento FS, Wenseleers T. 2019 Do primitively eusocial wasps use queen pheromones to regulate reproduction? A case study of the paper wasp *Polistes satan*. *Front. Ecol. Evol,* **7**, 7199.

For Review Only

Average duration of patrolling			
fixed effects	estimate ±SE	t value	P
Intercept	0.074±0.077	0.963	0.336
25% introduction	**-0.226±0.095**	-7.453	**0.016**
50% introduction	-0.049±0.095	-3.069	0.605

Average duration of resting			
fixed effects	estimate ±SE	t value	P
Intercept	-0.041±0.114	-0.364	0.716
25% introduction	-0.052±0.071	-0.736	0.462
50% introduction	**-0.341±0.071**	-4.760	**< 0.001**

Total patorol time			
fixed effects	estimate ±SE	t value	P
Intercept	0.077±0.116	0.668	0.504
25% introduction	-0.125±0.087	-1.439	0.150
50% introduction	**0.242±0.087**	2.780	**0.005**

Patrol frquency			
fixed effects	estimate ±SE	t value	P
Intercept	0.029±0.102	0.287	0.774
25% introduction	0.099±0.083	1.199	0.230
50% introduction	**0.353±0.082**	4.252	**< 0.001**

For Review Only

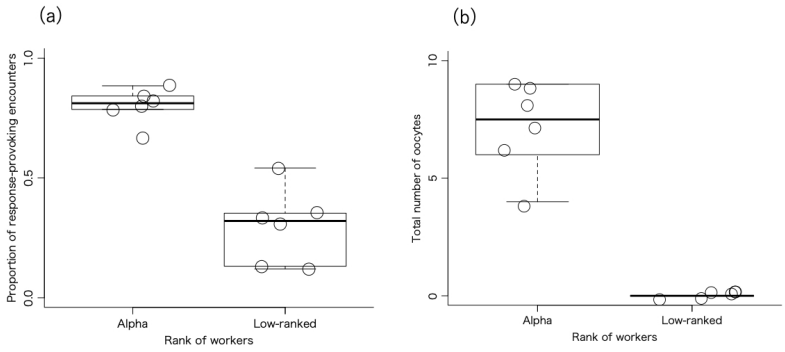

Figure 2. (a) Proportion of encounters that led to a queen response (either by antennation or by orientation) to alpha (N = 6) and low-rank workers (N = 6). (b) Number of oocytes in the ovaries of each worker. Circles represent individual colony values. For an explanation of the components of the box plots, see figure 1.

338x190mm (108 x 108 DPI)

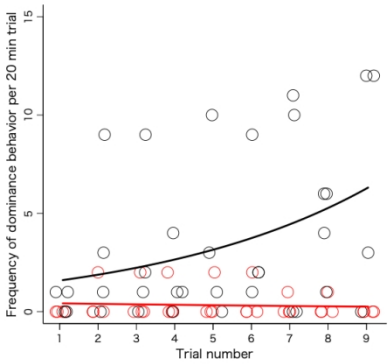

Figure 3. Frequency of dominance behaviour of workers under repeated exposure to queen's cuticular hydrocarbon extract (red circles, N = 4) or to n-hexane (black circles, N = 4).

338x190mm (108 x 108 DPI)