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

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

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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 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 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 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 workers (never engaged in dominance interactions). Alpha workers occasionally lay male eggs even if a queen is present [18,20]. We then put the queen and three low-rank workers into a plastic Petri dish (12 cm in diameter) for 1 h to acclimatise the queen. Then we introduced an additional worker (either the alpha or a randomly selected low-rank worker) into the Petri dish and video-recorded the behavioural interaction between the focal worker and the queen for 15 min. Immediately after this trial the focal worker was dissected to examine her ovarian condition. To minimise the impact of handling, the queen and the three low-rank workers were returned to the original nest, and 1 or 2 days were allowed to pass before conducting another trial. Trial order was randomised. We defined an encounter as any physical contact between queen and worker. We counted the number of encounters that caused the queen to respond, either by antennation or by orientation (turning her body to orient her head toward the worker). We compared the ratio of response to non-response encounters between alpha and low-rank workers by using a generalised linear mixed model (GLMM) with a Poisson distribution. We set worker rank (alpha or low-rank) as the fixed factor, colony as a random factor, the total number of encounters as an offset, and the number of encounters that led to a queen response (antennation or orientation) as the response variable. Similarly, we compared focal workers' ovarian condition (the number of oocytes in ovaries) between alpha and low-rank workers by Wilcoxon singed rank test regarding colony as the block.

Bioassay on CHCs' queen pheromone activity

Using another four colonies we tested if the queen CHCs have a suppressive effect on worker reproduction. We used the dominance behaviour as a proxy of a worker's tendency to perform self-reproduction [13-15, 18]. First, more than 2 days before the experiment, we standardized all colonies to have the same size of 80 workers and a queen. Then each colony was randomly divided into two orphan groups (each with 40 workers), each of which was placed in a plastic container (6 × 4.7 × 2.5 cm). During this procedure, we killed the queen and quickly extracted the CHCs in the following way. To retrieve the crude extract, the queen was immersed in 1.5 ml of *n*-hexane. Then the solvent was evaporated and re-dissolved in 70 µl of *n*-hexane. This total crude hexane extract was separated into HC fraction (HC fr.) and non-HC fraction (non-HC fr.) on a silica-gel column (0.04–0.063 mm mesh silica-gel) by stepwise solution with 3 ml of n-hexane and dichloromethane. This n-hexane fluid extract was applied to 1/8 of a piece of a glass-fibre filter paper (2.1 mm in diameter, 1 mm width) by a microsyringe (0.5 individual equivalent amount). At 30 min after the orphaning, we gently used tweezers 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' 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.

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.

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, $N = 4$).

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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**

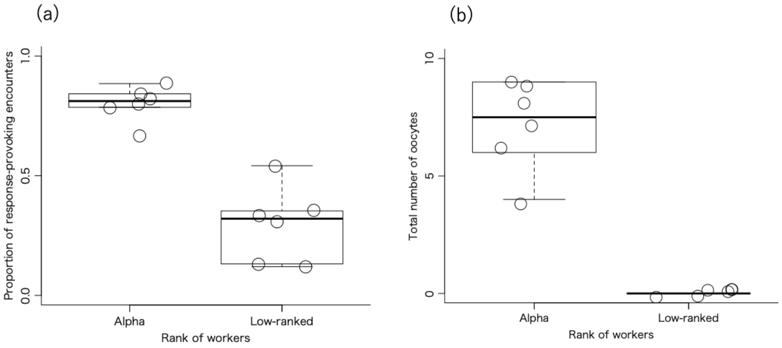

Figure 2. (a) Proportion of encounters that led to a queen response (either by antennation or by orientation) to alpha (N = 6) and low-rank workers (N = 6). (b) Number of oocytes in the ovaries of each worker. Circles represent individual colony values. For an explanation of the components of the box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

338x190mm (108 x 108 DPI)

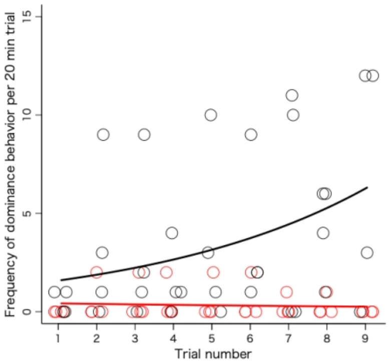

Figure 3. Frequency of dominance behaviour of workers under repeated exposure to queen's cuticular hydrocarbon extract (red circles, N = 4) or to n-hexane (black circles, N = 4).

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