Colony co-founding in ants is an active process by queens

Serge Aron1,2* & Jean-Louis Deneubourg2

Cooperative breeding may be selected for in animals when, on average, it confers greater benefits than solitary breeding. In a number of eusocial insects (i.e., ants, bees, wasps, and termites), queens join together to co-create new nests, a phenomenon known as colony co-founding. It has been hypothesised that co-founding evolved because queens obtain several fitness benefits. However, in ants, previous work has suggested that co-founding is a random process that results from high queen density and low nest-site availability. We experimentally examined nest-founding behaviour in the black garden ant, Lasius niger. We gave newly mated queens the choice between two empty nesting chambers, and compared their distribution across the two chambers with that expected under random allocation. We found that queens formed associations of various sizes; in most instances, queens group together in a single chamber. Across all experiments, the frequency of larger groups of queens was significantly higher than expected given random assortment. These results indicate colony co-founding in ants may actually be an active process resulting from mutual attraction among queens. That said, under natural conditions, ecological constraints may limit encounters among newly mated queens.

Cooperative breeding is a social system in which organisms create communal nests, and it has evolved repeatedly in a range of taxa, including insects, fish, birds, and mammals1–7. In cooperative breeding, several adults engage in social behaviours that benefit both themselves individually and the group as a whole. This system may be selected for when ecological constraints (e.g., nest-site limitation, predation, parasitism, unpredictable resource availability) and competition greatly diminish the expected fitness payoff of solitary breeding. Cooperative breeding can result in greater nesting success because it enhances survival and reproduction, alloparental care, and/or collective nest defence8–13. Related individuals may nest together because they obtain fitness benefits, either directly or indirectly (i.e., via kin selection)14. Unrelated individuals may also nest together because they derive benefits arising from mutualism, reciprocity, and/or group selection15–19.

Ecological constraints on solitary breeding appear to be major drivers of collaborative colony founding in the four main groups of eusocial insects—ants, bees, wasps and termites20–25. In the majority of ant species, foundation of a colony is the deadliest phase of the life cycle because newly mated queens are exposed to predation, starvation, disease, competition, and adverse environmental conditions (e.g. desiccation). Colony founding events have a very high failure rate, as high as 99% in some species [26–29 and references therein]. Although new colonies are created by single queens (haplometrosis) in most ants, the process can involve multiple queens (pleometrosis) in several species20,26,30. Founding associations have been documented across a dozen genera from three different ant subfamilies20. Collaborative colony founding, hereafter referred to as colony co-founding, is usually carried out by unrelated queens; therefore, it is unlikely to have evolved as a result of indirect fitness benefits20,26,28,30.

In ants, colony co-founding enhances the productivity and success of incipient colonies because it increases queen survival during the early founding phase31–33; promotes faster brood development31–39; and boosts the size of the initial workforce, providing greater protection against intraspecific brood raiding, predation, and/or adverse abiotic conditions31,32,37,40–43. However, there is a cost associated with colony co-founding. In most species, the collaboration among queens is transient, and, after the first workers emerge, all but one of the queens are usually eliminated via queen fighting and/or culling by workers20,42. Co-founding a colony is therefore a risky endeavour: while the surviving queen will reap the full reproductive benefits of the colony, the defeated queens will have zero fitness. Thus, co-founding should be selected for when, on average, queens achieve higher fitness than they could have as solitary foundresses; conversely, it should be selected against when fitness benefits are significantly lower. Then, co-founding would result from random encounters when co-founding and solitary founding provide roughly equal benefits.

1Evolutionary Biology and Ecology, Université Libre de Bruxelles, Brussels, Belgium. 2Center for Nonlinear Phenomena and Complex Systems, Université Libre de Bruxelles, Brussels, Belgium. 25email: saron@ulb.ac.be
Although considerable attention has been paid to the benefits of colony co-founding in eusocial insects, the proximate factors underlying the phenomenon have remained largely unexplored. In particular, it is unclear whether co-founding results from a random process in which queens are simply tolerant of one another (i.e., there is neither attraction nor repulsion) or whether it results from attraction among queens. Studies have shown that group size increases with increasing queen density in some ant species\(^\text{43-45}\). However, whether or not such associations were random was unclear. A laboratory study of co-founding in the ant *Lasius pallitarsis* suggested that queen association resulted from random allocation, but mutual attraction and active co-founding could occur with large queen density\(^\text{45}\). In the tree-nesting ant *Crematogaster scutellaris*, the number of groups formed by queens under natural conditions did not differ from that expected based on random allocation\(^\text{46}\), suggesting that newly mated queens were not actively co-founding colonies. However, this study did not take into account spatial variation in nest-site availability or the density of newly mated queens.

Here, we examined whether colony co-founding could result from queens actively grouping together. We used the black garden ant, *Lasius niger*, as a model system (Fig. 1a,b). In this species, mating occurs during large-scale nuptial flights, where thousands of sexuals from many colonies gather for a few hours. Once mated, queens land in an unknown environment, lose their wings, and quickly find a nesting site (small burrows in the open soil or under stones). In about 18–25% of cases, groups of 2–5 unrelated queens co-found colonies\(^\text{47}\). However, after the first workers emerge, queens start fighting with each other. Ultimately, only one queen survives, and she alone benefits from the colony’s future reproductive success. An experimental study of colony founding in *L. niger* offered newly mated queens an asymmetrical binary choice of nesting chambers: queens could settle either in an empty chamber or in a chamber containing another newly mated queen. The study found that queens did not display a preference for either scenario, supporting the conclusion that colony co-founding was likely a random process.
random process promoted by high queen densities. To better understand the forces driving colony co-founding, we explored whether newly mated queens actively nested in groups. To this end, we presented newly mated queens with a symmetrical binary choice between two nesting chambers that were both initially unoccupied. We investigated how queen number affected the grouping patterns of queens across the two chambers by carrying out experimental trials involving two, four, and eight queens. Queens were allowed to move freely between the two chambers. We compared the observed grouping patterns of the queens across the two chambers after 24 h with the expected grouping patterns given random allocation based on stochastic simulations. Under conditions of random allocation, there would be no attraction among queens, and the queens would have an equal probability of ending up in either chamber (p = 0.5). If queens were actively grouping together, frequencies of larger groups of queens would be higher than expected based on random allocation. Conversely, if queens were actively avoiding each other, frequencies of larger groups of queens would be lower than expected based on random allocation.

### Results

In the three types of experimental trials (N = 2, 4, or 8 queens), the vast majority of queens ended up in one of the two chambers (Table 1 and Supplementary Table S1). The mean proportion of sheltered queens did not differ among trials (Kruskal–Wallis one-way analysis of variance, P > 0.05).

First, we evaluated the results for the trials in which all the queens were sheltered (2 queens: 34/38 trials [89%]; 4 queens: 23/38 trials [61%]; 8 queens: 25/41 trials [61%]; Table 1). Remarkably, queens grouped together in a single chamber in 91% of the trials with 2 queens (n = 34), in 78% of the trials with 4 queens (n = 23), and in 76% of the trials with 8 queens (n = 25) (Table 1). The mean proportion of sheltered queens found in the largest group was significantly greater than that expected given random assortment (P < 0.0001 for all three trial types; Fig. 1d).

Second, we evaluated the results for the trials in which some queens remained in the arena. After excluding trials with 0–1 sheltered queens, we were left with two situations: trials with 4 queens in which there were 2–3 sheltered queens (7/38 trials [18%]) and trials with 8 queens in which there were 2–7 sheltered queens (15/41 [37%]) (Table 1). As previously, the mean proportion of sheltered queens found in the largest group was significantly greater than that expected given random assortment (P < 0.034 and P < 0.0001 for trials with 4 and 8 queens, respectively).

| Experimental trial | n | # sheltered queens (S) (# trials) | Proportion of sheltered queens ± SD across all trials | % trials where queens sheltered in the same chamber | MWSE ± SD | P |
|-------------------|---|---------------------------------|---------------------------------------------------|-------------------------------------------------|-----------|---|
| 2 queens          | 38| 2 (34)                          | 0.92 ± 0.24                                       | 91%                                              | 1.91 ± 0.28 | <0.0001 |
|                   |   | 1 (2)                           |                                                   |                                                 | –         | –       |
|                   |   | 0 (2)                           |                                                   |                                                 | –         | –       |
| 4 queens          | 38| 4 (23)                          | 0.76 ± 0.36                                       | 78%                                              | 3.78 ± 0.41 | <0.0001 |
|                   |   | 3 (5)                           |                                                   | 60%                                              | 2.6 ± 0.49 | <0.034  |
|                   |   | 2 (2)                           |                                                   | 100%                                             | 2.0 ± 0.0  |         |
|                   |   | 1 (4)                           |                                                   |                                                 | –         | –       |
|                   |   | 0 (4)                           |                                                   |                                                 | –         | –       |
| 8 queens          | 41| 8 (25)                          | 0.84 ± 0.26                                       | 76%                                              | 7.76 ± 0.43 | <0.0001 |
|                   |   | 7 (5)                           |                                                   | 80%                                              | 6.40 ± 1.20 |         |
|                   |   | 6 (0)                           |                                                   |                                                 | –         | –       |
|                   |   | 5 (2)                           |                                                   | 50%                                              | 4.50 ± 0.50 |         |
|                   |   | 4 (5)                           |                                                   | 60%                                              | 3.20 ± 0.98 |         |
|                   |   | 3 (3)                           |                                                   | 66%                                              | 2.66 ± 0.47 |         |
|                   |   | 2 (0)                           |                                                   |                                                 | –         | –       |
|                   |   | 1 (0)                           |                                                   |                                                 | –         | –       |
|                   |   | 0 (1)                           |                                                   |                                                 | –         | –       |

Table 1. Description of experiment and queen grouping patterns. The table indicates the results of the different types of experimental trials (N = 2, 4, or 8 queens released in the arena at the start of the trial); the total number of trials of each type (n); the total number of sheltered queens (S) and the number of trials in which different values of S occurred (# trials); the mean proportion of sheltered queens ± SD across all the experimental trials of a certain type; the percentage of experimental trials in which sheltered queens were all in the same chamber; the mean number of sheltered queens in the largest group (MWSE) ± SD; and the probability of obtaining MWSE by chance, based on 10,000 simulations of random allocation outcomes. Experimental trials in which S = 0 or S = 1 were excluded when calculating MWSE and P.
Queen grouping patterns. To define the expected frequencies of grouping patterns under conditions of random allocation, we performed stochastic simulations in which queens were distributed across the two chambers (see Supplementary Methods for a detailed description of statistical procedure). We were interested in the variable $S$, which was the number of sheltered queens (defined as queens that had taken shelter in a chamber).
For example, in an experimental trial with 4 queens, S could be equal to 4 (all 4 queens were sheltered), 3 (3 queens sheltered, 1 queen in the arena), or 2 (2 queens sheltered, 2 queens in the arena) (Table 1). Experimental trials where S = 1 or S = 0 were discarded since they were not informative. These simulations made it possible to calculate the mean number of queens expected under random allocation in the larger group (this group can be in the left or right chamber) of sheltered queens, for the experimental trials with N = 2, 4 or 8 queens. Using this approach, we examined two sets of data. First, we considered the data from experimental trials in which all the queens were sheltered (S = N). For each sample (N = 2, 4 or 8 queens), we calculated the mean number of queens in the larger group. Then, 10,000 simulations with random allocation of queens across the two chambers were performed, and we calculated the proportion of means simulated that were equal to or higher than the experimental mean. The null hypothesis that the queens’ grouping patterns arose from random allocation was rejected when this proportion was ≤ 0.05. Second, we considered the data from the experimental trials in which some of the queens remained in the arena (S < N). In this case, the small sample sizes for each grouping pattern (see Table 1) made it impossible to compare observed and expected queen grouping patterns. We summed the number of queens in the larger group of sheltered queens across the experimental trials for the different values of S. Therefore, simulations were weighted based on the number of sheltered queens observed (S) (Supplementary Methods). Comparisons between the results of the simulations and those of the experimental trials were realized as described above.

Ethics. Research on the species in this study does not require ethical licences. All experiments were performed in accordance with relevant guidelines and regulations in Belgium.

Data availability
All the datasets supporting this article are included in the present work and the Supplementary Information.

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Author contributions
S.A. and J.L.D. designed the study, carried out the work, the data analysis and the statistical analysis, and wrote the manuscript. Both authors gave final approval for the publication.

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