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Pleometrotic colony foundation in the ant *Crematogaster scutellaris* (Hymenoptera: Formicidae): better be alone than in bad company

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Abstract

In this study, we investigated whether pleometrosis occurs in *Crematogaster scutellaris* (OLIVIER, 1792) and which advantages it may provide. A survey of *C. scutellaris* queens in aphid galls revealed that 19% of the occupied galls contained two or more queens (max. five). Moreover, the observed distribution of the number of queens per gall was not statistically different from that expected from a null model based on random allocation. Microsatellite analysis on naturally associated queens showed no evidence of relatedness among them. We then investigated the possible advantages of pleometrotic foundation, comparing the survival and brood production of queens spontaneously nesting alone or in pairs, in small and large nests. Paired queens never showed any form of cooperation in brood care and interacted aggressively or tried to avoid each other. Mortality of paired queens was always greater than that of solitary queens, and mortality was higher in small than large nests. Finally, the larger queen in a pair was more likely to survive the fights. The total number of eggs produced by queens in pairs was nearly twice that produced by isolated queens, although the total progeny production (larvae, pupae, and workers) was the same. This suggests significantly higher brood attrition in paired queens than in solitary ones, probably due to an increase in egg cannibalism or trophic egg production. Further, the time until first worker eclosion was the same for pleometrotic and haplometrotic queens. These results suggest that associative foundation in *C. scutellaris* is more likely to be the result of a random process, probably triggered by the need to escape from predation and abiotic stresses after the nuptial flight, than an active choice of queens. Additionally, the possible advantages of associative foundation, if any, should be very limited.

Key words: Cooperative founding, acrobat ants, aphid galls, microsatellite.

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Introduction

In ants, the foundation phase represents a critical stage in the life cycle of a colony (HÖLLDOBLER & WILSON 1990). In most species, newly mated queens start new colonies independently, and each queen rears the first brood in complete isolation. This mechanism, termed independent colony foundation or haplometrosis, leads to the formation of primarily monogynous colonies. Sometimes, however, two or more queens may join after the nuptial flight and cooperate in nest construction and brood care, a behavior known as pleometrosis (HÖLLDOBLER & WILSON 1977, BERNASCONI & STRASSMANN 1999). These aggregations are often temporary and do not persist long after the foundation phase (TRUNZER & al. 1998, HEINZE & al. 2001, but see JOHNSON 2004). The eclosion of the first workers usually leads to a general increase of aggressiveness both among queens (URBANI & SOULIÈ 1962, RISSING & POLLOCK 1987) and between the newly eclosed workers and queens (BARTZ & HÖLLDOBLER 1982, BALAS & ADAMS 1996, BALAS 2005). This results in only one queen surviving in the newly founded colony.

The advantages of a pleometrotic foundation are still a matter of debate. Most of the studies on this subject reported some form of cooperation between the queens involved and showed that these queens were on average better off than when founding alone (HÖLLDOBLER & WILSON 1990, BERNASCONI & STRASSMANN 1999). Available information also shows that in the majority of cases pleometrosis involves unrelated queens, so that indirect fitness benefits are unlikely to be important in this kind of foundation (HEINZE & al. 2001, KELLNER & al. 2007, but see NONACS 1990). The first important direct benefit is represented by a larger initial worker force (all other things being equal, two queens produce more offsprings than a single one), and this may in turn enhance colony survival either increasing its foraging efficiency or providing better defence against predation and brood raiding by other ants (BERNASCONI & STRASSMANN 1999, HELMS CAHAN 2001, SASAKI & al. 2005, ARON & al. 2009). Furthermore, queens nesting in association often suffer lower weight loss during the claustral stage, and their brood develop faster (WALOFF 1957, RISSING & POLLOCK 1991, ADAMS & TSCHINKEL 1995). In some habitats where nest availability is limited and the risk of predation, brood raiding, or abiotic stress are high, all these benefits may be sufficient to favor a shift

from independent to cooperative colony foundation (TSCHIN-KEL & HOWARD 1983, HERBERS 1986, RISSING & POLLOCK 1987, HELMS CAHAN & FEWELL 2004, SHAFFER & al. 2016). Additionally, associations may also lead to some social immunity benefits against nest parasites, for both the cooperating queens in the early stage of the founda-tions and for the developing colony (PULL & al. 2013, BRÜTSCH & al. 2014). However, queens may be simply attracted by the safety provided by a closed nesting place without any cooperative aim, and the scarcity of such types of chambers may easily trigger collective nesting (PFEN-NIG 1995, TSCHINKEL 1992). Whatever the advantage, how-ever, all these possible benefits need to be traded-off against the costs of competition between foundresses. In fact, given that usually only one queen survives and no sexual indi-viduals are produced during this phase, only the surviving queen would benefit from cooperation.

Crematogaster scutellaris (OLIVIER, 1972) is a domin-ant tree-nesting myrmicine ant widely distributed in the Mediterranean basin (BERNARD 1968). As for other domi-nant species, *C. scutellaris* plays an important role in the structuring of ant communities in both natural and managed habitats (RETANA & CERDÁ 2000, SANTINI & al. 2007). After the nuptial flight, newly mated queens take shelter under tree bark or in tree holes and form new monogynous colonies by independent claustral foundation (SOULIÈ 1960, 1961, CASEVITZ-WEULERSSE 1972, 1991, FRIZZI & al. 2015). At the end of the mating period (late summer to early aut-umn in our study area) it is not uncommon to find mated queens inside cynipidae or aphid galls on different species of trees such as *Quercus* sp. and *Populus* sp. (TOROSSIAN 1971, ESPADALER & NIEVES-ALDREY 1983, CASEVITZ-WEULERSSE 1991). These galls, which lignify after para-site eclosion, contain a small internal chamber connected to a narrow surface opening and represent a safe refuge for many different insect species. The genus *Crematogaster* comprises mostly tropical species with different types of colony structure and foundation strategies ranging from simple monogyny to polygyny (HEINZE & al. 2000, QUI-NET & al. 2009, BLAIMER 2012). Additionally, the ability to shift from haplometrosis to pleometrosis under different environmental conditions has been observed in some spe-cies of the subgenus *Decacrema* (FELDHAAR & al. 2005). The ant *C. scutellaris* has been described to be monogy-nous, and aggressive behavior has been documented be-tween foundresses (URBANI & SOULIÈ 1962). However, during the hibernation period, it is not uncommon to find more than one *C. scutellaris* foundress within the same gall, suggesting that some sort of pleometrosis may be present in this species.

The aim of this study was to investigate whether pleo-metrosis occurs in *Crematogaster scutellaris* and which advantages it may provide. We first determined the fre-quency of multiple queens within galls in a population of this species. Using nuclear DNA microsatellite analysis, we then assessed their degree of relatedness. Finally, we compared survival and brood production for queens spon-taneously nesting alone or in pairs.

Materials and methods

Study area and sampling: We sampled 1952 lignified aphid (*Pemphigus* sp.) galls from *Populus nigra* trees in public parks near Florence (Tuscany, Italy). Galls were col-lected on tree branches at a height of up to four meters from the ground, after the end of the nuptial flight of *Crematogaster scutellaris* (October - November 2013). Each gall was placed in a plastic bag and carried to the labora-tory within an hour after collection. The galls were care-fully opened and ants found inside were placed in Petri dishes (one dish per gall, maintaining together naturally associated queens). In total we obtained 626 *C. scutellaris* queens (439 alive and 187 dead). *Crematogaster scutella-ris* queens were weighed (± 0.1 mg, accuracy) and marked on the thorax with a small dot of non-toxic water-based paint (UniPosca marker pen, OSAMA). Ants were pro-vided with water but no food. Air humidity was controlled by adding a moistened cotton flock, which was replaced every week to avoid molding. Before experiments (see below), queens were kept in the dark in a thermostatic chamber for three months at a 6 ± 1 °C in order to repro-duce the natural hibernation period.

We used a null model to test whether the frequency distribution of the number of queens associated in a gall could be due to random assortment. The model parameters were the number of available galls (total collected galls minus those galls hosting a complete colony, a colony frag-ment or the colony of a different species) and the total number of queens found in the galls. Each queen was then randomly assigned to a gall. The random allocation was repeated 10000 times, and each time the frequency distri-bution of the number of queens per gall under the null model was compared to the observed frequency distribution using a χ^2 test. We then calculated the number of times the computed χ^2 statistic was higher than the expected the-oretical value for the same number of frequency classes at $\alpha = 0.05$.

Pleometrosis experiments: To investigate the effect of pleometrosis on survival and brood production we used spontaneously associated (i.e., two queens found together in a same gall) and non-associated queens (i.e., one so-litary queen found in a gall). Queen number was never manipulated and all queen pairs used in the experiments corresponded to spontaneous aggregations found in the galls. Moreover, to assess the role of nest size, queens were randomly allocated to small or large nests. Small nests were Petri dishes of 2 cm diameter, similar in size to a natural gall, whereas large nests consisted of 9 cm Petri dishes, comparable in size to natural nests commonly found under tree bark (A. Masoni, M. Mattioli, S. Turillazzi, C. Ciofi, G. Santini & F. Frizzi, unpubl.). A total of 39 queens (13 natural pairs and 13 single individuals) were placed in small nests (small nest experiment), and the same number was placed in large nests (large nest experiment). Tests were carried out from February to May 2014. After the hiber-nation period queens were gradually acclimated to the ex-perimental temperature by keeping the temperature at 20 °C for one week followed by a gradual increase to 25 °C. This would trigger the foundation phase. During all experi-ments ants were maintained in the dark at 25 °C for 12 hours and 18 °C for an additional 12 hours in order to si-mulate day-night cycle. Queens were kept with no food until the eclosion of the first worker. Each colony was then fed once a week with a few drops of honey and three dead *Drosophila melanogaster* adults. For each experi-mental treatment (small or large nest), observations ended 28 days after first worker emergence, or when at least one
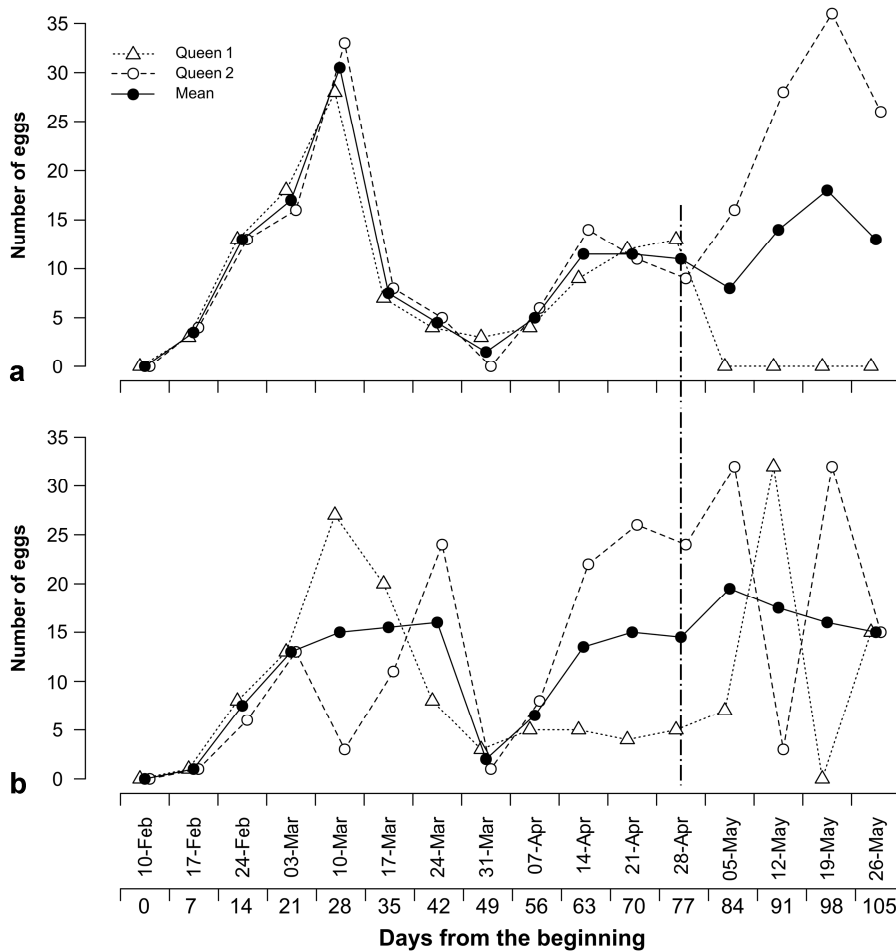
Fig. 1: Examples of interactions between queens in a pair, within large nests. (a) Each queen rears her own brood (eggs) without any interaction with the nest-mate until eclosion of the first workers (vertical dot-dashed line). (b) Queens interact during the whole test period, performing reciprocal brood raiding.

queen in all the pairs assigned to that treatment died (ARON & al. 2009). During the experiments we observed the colonies twice a week and recorded brood production and queen mortality. We also counted the total amount of individuals in each brood class (number of eggs, larvae, pupae, and workers) without taking into account different developmental stages for each class.

Survival probability of single vs. paired queens from both nest types was inferred using the Kaplan-Meier estimator (KLEIN & MOESCHBERGER 2003). Differences in brood production (eggs, larvae, pupae, and workers) and initial body weight of single and associated queens for each nest and between nest types were modeled using a Generalized Linear Model (GLM) with Poisson error term. Larval (A_l) and pupal (A_p) attritions were evaluated as follows:

$$A_l = (1 - \frac{N_l}{N_e})$$ [1]

$$A_p = (1 - \frac{N_p}{N_l})$$ [2]

where N_e, N_l, N_p are the number of eggs, larvae, and pupae, respectively, counted the day before the first individual of the next stage emerged. Difference between single and paired queens was tested using the Wilcoxon test. No attrition was considered after the pupal stage (TSCHINKEL 1993).

Death probability was modeled as a function of body size using a GLM with binomial error term. Significance was tested by a likelihood ratio test with reference to a
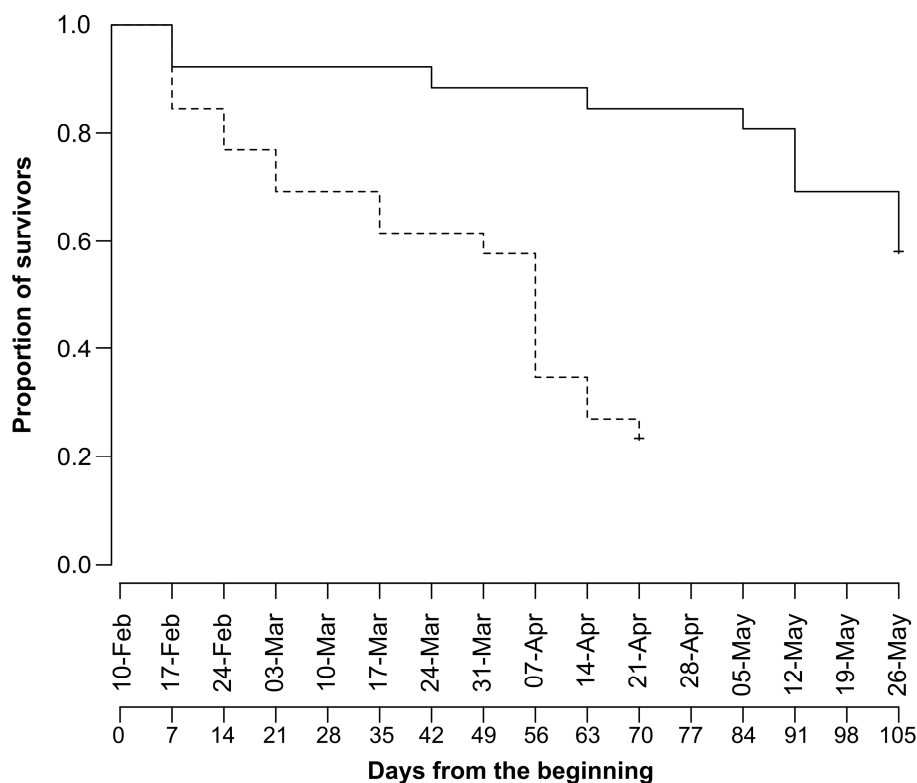
null model (intercept only). All analyses were carried out using the R 3.2 statistical package and the "multcomp" R library 1.3 for multiple comparisons.

Genetic Analyses: Kinship analysis was performed for 26 queen pairs using seven microsatellite loci (crem16, crem19, crem21, crem23, crem24, crem45, and crem46) developed for *Crematogaster scutellaris* by FRIZZI & al. (2009). For each queen, DNA was extracted by grinding one leg in a microcentrifuge tube with 100 µl of 10% CHELEX 100 resin (BIO-RAD). The mixture was vortexed for 15 s, then centrifuged for 15 s at 13.000 rpm, and subsequently incubated at 95 °C for 20 min. Each sample was then vortexed, centrifuged, and 1 µl supernatant was used as template for polymerase chain reaction (PCR) amplification as described in FRIZZI & al. (2009). PCR products were resolved by capillary electrophoresis in an ABI 3130xl genetic analyzer and alleles scored using Gene-Mapper 5.0 against a GeneScan 500 ROX size standard (Thermo Fisher Scientific, USA). We estimated pairwise relatedness (*r*) among associated queens using a maximum-likelihood (ML) estimator (KONOVALOV & HEG 2008) and the regression interpretation of *r* of ROUSSET (2002) implemented in Kingroup 2.0 (KONOVALOV & al. 2004).

Results

Approximately 28.5% (557) of a total of 1952 aphid galls were occupied by *Crematogaster scutellaris.* Of these, 70.9 % contained a single queen (320 queens were alive and 75 were dead), 15.4% hosted two queens (26 galls had

Fig. 2: Survival of paired queens in large (continuous line) and small (dotted line) nests.

both queens alive, in 28 galls both queens were dead, and 32 galls had one queen dead and one alive) and 3.3% contained more than two queens (up to a maximum of five queens per gall). Eighteen galls (3.2%) contained an incipient colony (i.e., a one-year colony formed by a queen and a maximum of 30 workers), and 40 galls (7.2%) had a colony fragment (workers only with no queens). Of all the collected galls, 43 (2.2%, n = 1952) contained colonies of other ant species, including *Temnothorax* spp., *Colobopsis truncatus* and, more rarely, *Camponotus lateralis*. The observed distribution of the number of queens per gall was not statistically different from that expected from the null model ($P = 0.57$). Probability of death varied depending on the number of queens per gall. Risk of death for queen pairs was more than four times higher than for queens found alone in a gall (odds ratio = 4.47). This difference was statistically significant ($\chi^2 = 59$, $df = 1$, $P < 0.0001$).

At the beginning of the foundation phase, most of the associated queens in small and large nests remained at the opposite sides of the Petri dish trying to avoid each other while rearing their own brood. As the incipient colony developed, queens showed a sharp increase in aggressiveness that varied with nest size. In large nests, interactions between queens were mainly limited to antennation, aggressive displays (e.g., mandible gaping upon encounter) and occasional mutual raiding of the brood. Fights leading to the death of one queen occurred in approximately one third of queen pairs. Queens that were paired in small nests interacted more frequently and more aggressively right after the start of the experiment. Aggressiveness consisted in repeated and frequent biting, brood raiding or egg cannibalism, and often in direct fights ending with the death of one or both queens. Two examples of such behavior are shown in Figure 1. In a number of cases, each queen kept its own brood until workers were born and limited inter-

actions with the nest mate to occasional events of egg cannibalism (Fig. 1a). In other cases, queens reciprocally and alternately raided the whole brood (Fig. 1b). For this reason the number of eggs tended by each queen fluctuated significantly from one observation to the next.

Single founding queens had a high (> 90%) probability to survive to the end of the experiment irrespective of nest size. Only one queen died from fungal infection. On the other hand, survival probability of paired queens was lower and changed with nest type ($\chi^2 = 40.6$, $df = 3$, $P < 0.001$; Fig. 2). In large nests, each paired queen had 57% average survival probability, and this was due to the death of one queen in almost all pairs. In only four cases both queens survived to the end of the experiment, and only in two tests both queens died. Paired queens in small nests showed a considerably higher mortality rate (~ 73%) and no queen pair survived to the end of the experiment. Out of the 13 pairs tested, seven small nests had one queen that survived, while in the remaining six nests both queens died due to the injuries sustained during fights. In small nests, at least one queen in each pair died before the first worker eclosion and we therefore decided to stop the experiment and compare mortality rate and brood production at day 70 (referred to as Day70). On the contrary, the large nest experiment ended at day 105 (referred to as Day105), i.e., 28 days after first worker eclosion. Brood production of single and paired queens was compared only for pairs where at least one queen survived to the end of the test. Brood production of large and small nests was instead compared at Day70.

Single and associated queens started to lay eggs at approximately the same days in both nest types (Fig. 3a). At Day70, the amount of eggs produced per nest did not significantly differ between single and associated queens in small nests ($z = -1.304$, $P > 0.05$). On the other hand, queens in large nests had significantly more eggs than single
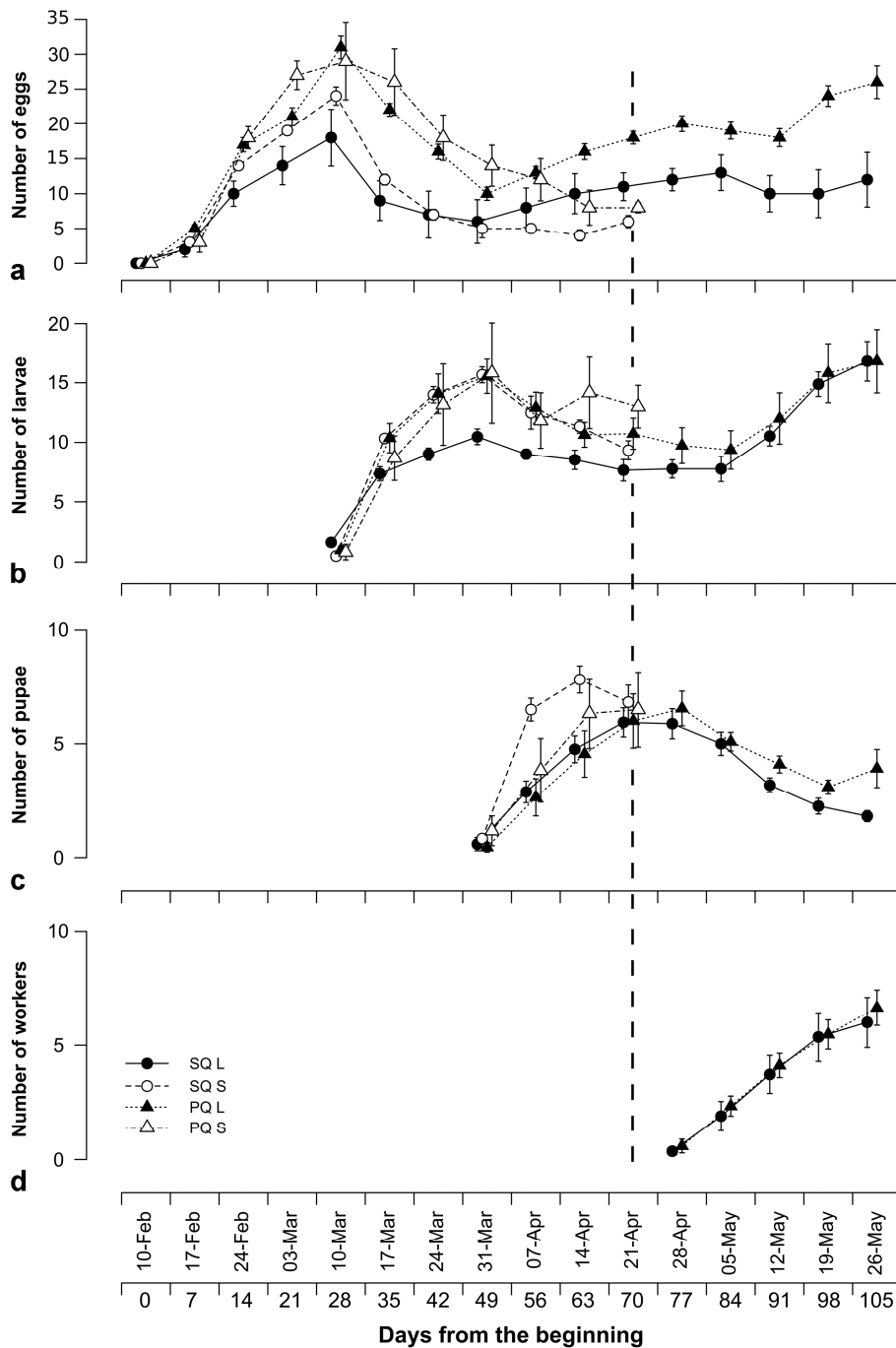
Fig. 3: Mean brood production of single and paired queens in both nest types. (a) Eggs, (b) larvae, (c) pupae, (d) workers (absent in small nests). Bars represent standard error. SQ L = single queens in large nests, SQ S = single queens in small nests, PQ L = paired queens in large nests, PQ S = paired queens in small nests. The dashed line represents the time at which the small nest experiment was terminated (Day70).

foundresses (z = -4.088, P < 0.001). An even more pronounced difference was observed at Day105 (z = -3.152, P < 0.01), when the amount of brood produced by pairs was nearly twice as high as that produced by single queens. This indicated a lack of difference in per-capita egg production. Additionally, single queens produced larger amounts of eggs in large than in small nests (z = -3.469, P < 0.01). A similar trend was recorded in paired queens, although comparisons were difficult because of the early death of one queen often observed in small nests.

Larval production did not reflect the differences observed in eggs (Fig. 3b). The number of larvae produced at Day70 and Day105 were similar (χ^2 = 9.746, df = 3, P > 0.05; χ^2 = 0.634, df = 1, P > 0.05, respectively) regardless of the number of queens or nest size. This was a similar trend to that observed for pupae production (Fig. 3c), where no differences between small and large nests were observed at Day70 (χ^2 = 0.377, df = 3, P > 0.05). However, at Day105, paired queens in large nests produced a larger amount of pupae than single queens (χ^2 = 12.204, df = 1, P < 0.01). Each paired queen produced approximately 50% fewer larvae and pupae than single queens. Finally, single and associated queens did not differ in the number of workers produced at the end of the large nest experiment (Day105) (χ^2 = 0.450, df = 1, P > 0.05; Fig. 3d).

Development time from egg to worker was approximately 77 days, considering that this estimation may be affected by some days of variation due to the fact that observations on brood development were conducted twice a week. From the transition from eggs to larvae 21 days oc-

curred and a similar time length was observed for transition from larvae to pupae. Worker eclosion, that is the development time from first pupae to the first worker, was instead 28 days. Larval attrition was significantly higher for paired than single queens (49% and 34% larvae loss, respectively; w = 86 P < 0.01), however, no difference was recovered between nest types (w = 25 P > 0.05 for single queens; w = 32.5 P > 0.05 for queen pairs). Similarly, pupal attrition was significantly higher in paired (53%) than single (40%) queens (w = 182.5 P < 0.01) and no effect of nest size was recorded (w = 13 P > 0.05 and w = 23 P > 0.05 for within-single and within-pair comparisons, respectively).

Body size had a strong influence on probability of death. Death of at least one queen occurred in 85% of pairs. Among these, death of the lighter queen was the most frequent outcome (55% of cases), followed by death of both queens (36% of cases). On the other hand, the death of the heavier queen in a pair was a rare event (9%). Therefore, the lighter foundress in a pair had a ~ 77% probability to die while the death probability of the heavier queen was 38%. The death probability in a pair significantly decreased as body size increased (χ^2 = 5.879, df = 1, P < 0.01).

No significant departure from Hardy-Weinberg equilibrium for any of the loci analyzed was detected (P > 0.05). Number of alleles per locus ranged from 10 to 15. Mean observed and expected heterozygosity were 0.785 (range: 0.635 - 0.937) and 0.850 (range: 0.821 - 0.891), respectively. Relatedness between queen pairs was very low, between -0.141 and 0.122, with an average value of r = -0.018 ± 0.021 (mean ± S.E). No evidence of relatedness was found between queens forming pairs (likelihood ratio test with respect to unrelated condition r = 0, P > 0.05).

Discussion

This study evaluated the role of pleometrosis in colony foundation in the dominant ant *Crematogaster scutellaris*. The spontaneous association of two or more queens before hibernation, a phase that anticipates the foundation of a new colony, is not a rare event and was recorded in 19% of all aphid galls colonized by *C. scutellaris* (considering associations of both alive and dead queens). The results of our study, however, question the actual benefits a queen may obtain by such an association.

The frequency distribution of the number of foundresses per gall did not differ from that expected by random allocation, suggesting that pleometrotic colony foundation may not be the result of an active choice by newly mated queens. The simple model we used may be questionable to some extent and more realistic variants could be proposed and tested, for instance, by including spatial variation in gall availability, relaxing the assumption that ants nest only inside galls, or adding information on dispersal ability of queens. However, in our study area, very few options existed for ants to establish a nest in places other than galls. Our model shows that a preference of ant queens for occupied galls is not needed to explain the observed foundress distribution and that associations of up to five queens per gall can be expected by mere chance. Ant queens nesting in association had a significantly higher mortality risk than that faced by single foundresses, suggesting that

association is on average less rewarding. In both small and large nests, however, mortality occurred at different times of the founding stage. In the naturally associated queens it was hard to ascertain when death occurred exactly, either soon after entering the gall or during hibernation, and what was the cause of death, whether a fight or infection. On the other hand, during our experiments, associated queens' death was rare during hibernation and mortality was almost entirely due to the injuries inflicted during fights, and particularly after the eclosion of the first workers. Interestingly, death rate was inversely related to the size of the experimental nest. In small nests, comparable in size to a gall, aggressive behavior was relatively frequent since the beginning of the foundation phase. On the contrary, in larger nests queen-queen aggression became relevant only after the eclosion of workers, a pattern similar to that described in *Lasius niger* (LINNAEUS, 1758) by SOMMER & HÖLLDOBLER (1995).

Contrary to most behavioral observations of pleometrotic associations (BERNASCONI & STRASSMANN 1999, HELMS CAHAN & FEWELL 2004), no clear cooperative behavior such as brood sharing, reciprocal grooming or trophallaxis was observed in paired queens (PULL & al. 2013). Queens avoided each other for as long as possible by remaining in different sections of the Petri dish. However, they also raided each other's brood and interacted aggressively when they met. Queen-queen aggression was clearly triggered by the presence of workers (see also SOMMER & HÖLLDOBLER 1995). The workers were never aggressive toward any of the queens, although they did contribute to brood raiding. Brood raiding eventually triggered a fight between queens. In particular, the queen that had lost the brood attempted to take the brood of the winner (see FOWLER & ROBERTS 1983 for a similar finding).

We found no evidence of relatedness among paired queens, and this would exclude higher inclusive fitness resulting from pleometrotic foundation. This finding corroborates previous studies where low relatedness values were found between pleometrotic foundresses (RISSING & POLLOCK 1988, HÖLLDOBLER & WILSON 1990) and related queens were hardly more successful in rearing workers than unrelated individuals (e.g., *Lasius pallitarsis* (PROVANCHER, 1881), see NONACS 1990, *Gnamptogenys striatula* MAYR, 1884, see GIRAUD & al. 2001). In principle, the surviving foundress acquires all brood and hence it may have a potential advantage during the first stage of colony development when both competition and risk of predation are high (TSCHINKEL 1992, 1993). However, although egg production in paired queens was approximately twice as much as in solitary ones, neither the number of workers nor the number of larvae or pupae produced at the end of the test appeared to provide a substantial advantage to paired queens. On the contrary, given that we observed no difference in brood production between paired and solitary queens, the per-capita production of associated queens was half of that of single queens, suggesting that association would represent an impediment rather than a benefit. Similarly, REBER & al. (2010) found that the increase in brood productivity associated with a pleometrotic foundation may be transient and does not represent any real benefit after the early stage of the foundation. These findings contradict previous studies on pleometrotic foundation where associated queens were reported to produce more work-

ers than single foundresses (e.g., OFFENBERG & al. 2012). The paired queens showed significantly higher brood attrition than solitary foundresses given that the total number of larvae, pupae and workers was lower than the number of eggs produced. Whether this attrition is due to differences in trophic egg production or increased egg cannibalism, both frequent in many ant species (HÖLLDOBLER & WILSON 1990), is not known and deserves further investigation. If brood attrition is mainly due to egg cannibalism or egg laying inhibition due to pheromone (VARGO 1992), the surviving queen of a pair may have a higher prospective number of workers than single queens, provided all the eggs develop into adults. Such a potential advantage would be delayed because of the time needed for an egg to develop into an adult worker (approximately 77 days from egg laying to worker eclosion). It is also interesting that the benefits (if any) and costs of associative foundation are related to body mass. In fact, the smaller foundresses in a pair had a significantly higher probability to die than their larger counterpart. This finding is not surprising because fighting ability is known to be greater in larger individuals (e.g., BERNASCONI & KELLER 1996, 1999, BALAS & ADAMS 1996), and suggests that a foundress may prefer a nest occupied by a smaller conspecific rather than one attended by a larger queen. An interesting framework to interpret these data is that recently proposed by SHAFFER & al. (2016) who studied the evolution of cooperative nest founding in the harvester ant *Pogonomyrmex californicus*, and showed that pleometrosis can have clear effects in some populations but not others. In this species, in fact, when the between-group competition is intense the evolution of cooperative breeding may be driven by group-selection. We have no information to understand whether this mechanism also applies to *Crematogaster scutellaris* or other species, but this perspective will certainly occupy a central place in future research on pleometrosis. To conclude, it should be considered that we compared spontaneously associated queens to solitary ones, and did not manipulate their number. Therefore, we cannot exclude the possibility that the queens that associated in the field differ from the ones founding singly, and that this does not affect the results.

A final consideration, not strictly related to pleometrosis, involves the significant difference between the number of foundresses found in the galls and the number of incipient, one year old colonies. We found 396 galls that hosted at least one queen alive and 18 that contained an incipient colony. Under the hypothesis that the yearly number of founding queens in the galls is constant, we may conclude that colony mortality is as high as 90%, as also previously suggested by SOULIÈ (1961) and CASEVITZ-WEULERESSE (1991). Premature death of founding queens and young colonies is due to a diversity of factors including disease, starvation, predation or competition between incipient colonies and brood raiding activity, and it is quite common in ants (BARTS & HÖLLDOBLER 1982, RISSING & al. 1986, HÖLLDOBLER & WILSON 1990, TSCHINKEL 1992, WIERNASZ & COLE 1995, BALDACCI & TSCHINKEL 1999). The observed mortality rate in *Crematogaster scutellaris* is comparable to that observed in *C. ashmeadi* MAYR, 1886, a dominant tree nesting ant inhabiting the longleaf pine forest of northern Florida, where only 7.6% of incipient colonies survived after the first year (HAHN

& TSCHINKEL 1997). Finally, since galls can host colonies of limited size only, it may be that after a short initial phase a colony migrates to find a more suitable nesting site (TOROSSIAN 1972).

In conclusion, the results of this study suggest that associative foundation in galls by *Crematogaster scutellaris* may be the result of a random allocation process rather than an active search by foundresses for nesting sites already occupied by a queen. Moreover, we found no evidence of an immediate benefit from associative nest foundation.

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