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POTENTIAL FOR TRANSITION TO A POLYGYNOUS-SUPERCOLONIAL DEMOGRAPHY IN *LASIUS NIGER* (HYMENOPTERA, FORMICIDAE)

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Potential for Transition to a Polygynous-Supercolonial Demography in *Lasius niger* (Hymenoptera, Formicidae). Stukalyuk, S. — The formicine ant, *Lasius niger* (Linnaeus, 1758), is one of the most abundant and intensively studied Palaearctic ant species, and was previously thought to form exclusively monogynous colonies, spread by single-queen dispersal, and occur claustrally. Two closely neighboring nest complexes of *L. niger* were observed in 2017–2020 in an abandoned field near Kyiv / Ukraine. Nest complex A contained 14 650 nest mounds on an area of 11.8 ha and nest complex B contained 15600 mounds on an area of 13.3 ha. Data were collected by measuring the height and diameter of nest mounds and counting the number of workers in each nest. In addition, worker movements between nests were observed and tests for aggressive behaviour between ants from different nests and sub-complexes were carried out. In the pleometrosis experiments, young mated gynes collected from the territory of nest complex B showed mutual tolerance after the first workers emerged from pupae. In contrast, in pleometroses with gynes from a distant monodomous population, the gynes began a deadly fight after workers emerged, with only a single queen surviving. Nest mounds within nest complexes are connected by a well-developed network of trails. The results of the analysis showed a positive correlation between nest size and population size. The coefficient of determination r2 for nest mound volume was 0.68415, indicating that 68.4% of the variation in population size was explained by changes in nest mound volume. In comparison, r2 for nest mound diameter was 0.7872, meaning that 78.7% of the variation in population size was explained by changes in nest mound diameter, while r2 for nest mound height was 0.42734, meaning that only 42.7% of the variation in population size was explained by changes in nest mound height. Observations of worker movements revealed a high degree of organisation and specialisation, helping to maintain connections between nest mounds. Aggression tests revealed low levels of aggression between workers from different zones of the same nest complex, but increased aggression in confrontations between workers from a nest complex with workers from a remote monodomous population and with workers from a second nest complex. Ple-

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ometrosis and reduced aggression between workers facilitate the integrity of the nest complex and its rapid growth. Based on the data obtained, it was concluded that the low aggressiveness of *L. niger* ants allows them to coexist over large areas in the form of large nest complexes containing thousands and tens of thousands of nest mounds. Additional auxiliary nests were identified that may become new full-scale nest mounds in the future, indicating a potentially higher settlement density than if only nest mounds were considered.

Key words: Lasius *niger*, trails, monogyny, nest complex, aggression tests, pleometrosis.

Introduction

Polydomy in ants is a form of social organization in which multiple physically separated nests are socially connected by the exchange of workers, males, queens, brood and food. The alternative condition is called monodomy in which the whole ant colony is housed in a single nest and behaves aggressively against other conspecific monodomous colonies. Polydomous systems occur more rarely in colonies with a single reproductive queen (monogyny) and more frequently in those with multiple queens (polygyny) (e. g., Debout et al., 2007; Seifert, 2018). Depending on the species' potential and environmental conditions, polydomous colonies can reach an enormous population size and cover a large area. The extreme expression of this life form is called a nest complex and was defined by Seifert (2018) as "a large to huge polydomous-polygynous colony with permanent connections between the nests and workers not showing mutual aggression even if originating from nests kilometers apart".

Supercoloniality is a typical trait of many invasive (neozootic) ant species and is one reason for their success in the newly colonized territories. In Europe north of the Alps ten invasive ant=species have occupied urban and rural outdoor habitats and nine of these are supercolonial and became notorious pest species (Seifert. 2020, a, b). In extreme cases, nest complexes of invasive ants contain millions of nests (Giraud et al., 2002; Sunamura et al., 2009; Van Wilgenburg et al., 2010; Moffet, 2012). The number of nests can range from thousands in *Crematogaster subdentata* Mayr, 1877 (Stukalyuk & Netsvetov, 2018) to millions in *Linepithema humile* (Mayr, 1868) or the invasive garden ant *Lasius neglectus* Van Loon et al. 1990 (Tartally, 2006, reviewed in Seifert, 2018).

Nest complexes of non-invasive, autochthonous ants can also reach impressive size. Examples of very big nest complexes in the temperate to subboreal zones of the Palaearctic are found in particular in the ant genus *Formica*: *Formica yessensis* Wheeler, W. M., 1913 in Japan with 45000 nests on an area of 270 ha (Higashi & Yamauchi, 1979), *Formica exsecta* Nylander, 1846 in Romania with 3347 nests on 21.8 ha (Markó et al., 2012), *Formica foreli* Bondroit, 1918 in Germany with 2550 nests on 6.2 ha (Bönsel & Busch, 2003) and *Formica paralugubris* Seifert, 1996 in Switzerland with 1200 nests on 70 ha (Cherix & Bourne, 1980).

Ants of the genus *Lasius* are considered to be rarely polygynous and thus the likelihood to form polydomous structures is low. Among the 56 Palaearctic species of subgenus *Lasius* s. str. (Seifert, 2020, c) four facultatively to obligatory polygynous species are known so far. *Lasius austriacus* Schlick-Steiner, 2003 is weakly polygynous or oligogynous and forms polydomous subterranean colonies (Pedersen & Cremer, 2008). *L. sakagamii* Yamauchi & Hayashida, 1970 and *Lasius neglectus* are highly polygynous and may form true nest complexes (Yamauchi et al., 1981; Espadaler et al., 2004; Tartally, 2006; Stukalyuk & Radchenko, 2018). *Lasius precursor* Seifert, 2020 is moderately polygynous and polydomous (Cremer et al., 2008) and was considered by Seifert (2020 c) as a model for transition from a largely monogynous-monodomous social type (exemplified by the sister species *Lasius turcicus* Santschi, 1921) to a supercolonial type (exemplified by the closely related species *L. neglectus*).

Lasius niger (Linnaeus, 1758), as the most abundant and best-studied member of the subgenus *Lasius* s. str., shows a continuous distribution from West Europe (10° W) to the eastern Baikal region (108 °E). It is by natural distribution mainly an element of the moderately moist meadows in wood-and-steppe and forest zones but following the spread of human culture there was a strong range expansion into rural and urban habitats even of the south boreal zone (Seifert, 2018, 2020 c). Plenty of studies on diverse aspects of biology have been conducted in this ant. Swarming flight and mating scenarios were intensively studied (by: Boomsma and Leusink, 1981; Boomsma & van der Have, 1998; Fjerdingstad et al., 2002; Van der Have et al., 2011). Colony foundation and colony demography also were investigated (by Mrazek, 1906; Eidmann, 1928; Goetsch, 1936; Sommer and Hölldobler, 1992, 1995; Aron and Passera, 1999; Aron et al., 2009). The observations of these authors resulted in the following consistent picture: after mass swarming and aerial mating *Lasius niger* gynes perform claustral colony foundation either alone (in haplometrosis) or in temporary cooperation of several gynes (in pleometrosis). The last mentioned seven authors agree in stating that pleometroses are terminated by mortal fighting between foundresses after the first workers have eclosed from pupae resulting in the survival of only one queen. The most fertile queen is more likely to be fed by the workers and thus to survive the fight (Sommer and Hölldobler, 1992, 1995; Aron et al., 2009). As a result, a monodomous-monogynous colony structure is established with workers of different nests behaving mutually aggressive. This presented the generally accepted view on this ant so far.

In this paper, we describe for the first time the spatial structure, connectivity and intraspecific behavior in a huge nest aggregation of *Lasius niger* discovered near Kyiv (Ukraine). We argue that this nest aggregation meets the following criteria: (1) nests are interconnected by a network of above-ground trails and subterranean tunnels within which workers move freely; (2) workers from close to remote parts of the colony show no or only little aggression to each other; (3) gynes in pleometrotic foundations tolerate each other after eclosion of the first workers.

This study also examined nest density, including auxiliary nests, and tested the likelihood of worker movement along trails between different nests within a polycalic settlement. In addition, population size was examined by fully excavating nest mounds. These additional tasks will help to understand how coherent nest aggregation is (by proving or disproving the hypothesis of worker movement between different nests) and how large the population of each nest mound is.

Material and Methods

Study area. Two closely-neighboring, large nest complexes of *L. niger* were discovered near the city of Vyshneve (Kyiv-Sviatoshyn District, Kyiv Region) in Ukraine (50.3906 °N, 30.3364 °E, 179 m a. s. l.). Including patches with no mounds detected, the nest complexes cover an area of 27 ha (for details, see Stukalyuk et al., 2022; 2023). The site is a flat terrain with small elevations, dry and clayish soil and has been

Fig. 1. The *Lasius niger* nest complex B, sectors 7, 8

arable land in the past. Cultivation has been gradually abandoned since 2010. Today, the area is dominated by segetal, semi-ruderal and ruderal communities of annual, biennial, and perennial plants, namely grass species from classes *Stellarietea mediae* and *Artemisietea vulgaris* (Mosyakin & Fedoronchuk 1999) as well as remnants of the former crop communities (cereals). The vegetation has a mean height of 20 to 30 cm and a surface coverage of 40 to 65%. There are also spots where the grass stand reaches a height of 50 to 70 cm.

Observation and recording methods. Field studies were conducted in 2017 to 2018. To check if this huge complex of *L. niger* nests could represent a true nest complex, we investigated the boundaries of the complex, the network of above-ground trails and subterranean tunnels between, the level of aggression between ants in different zones of the complex and the behavior of gynes in pleometrosis experiments which allows conclusions on the likelihood of coexistence of several queens in the same nest mound (polygyny).

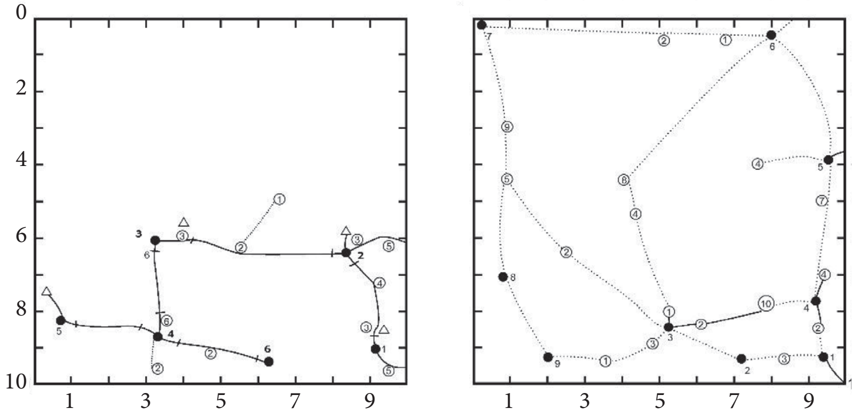
Assessing density and size of nest mounds was done according to the methods of Dlussky (1965) and Zakharov and Goryunov (2009) by walking along 10-m-wide line transects covering the whole area. This was done in April 2017 when the vegetation cover was low and the mounds were clearly visible (Fig. 1).

Additionally, height and diameter of nest mounds were measured. A total of 118 transects were allocated with lengths differing in dependency from the shape of the two nest complex territories. Based on transect data, a mosaic of 23 sectors was established which contain nest mounds from different density classes: Class I with 1 to 5 mounds/100 m^2, Class II with 6 to 10 mounds/100 m^2, Class III with 11 to 15 mounds/100 m^2, and Class IV with ≥16 mounds/100 m^2. Locations of populated sectors and of areas with no mounds were mapped by placing their geographic coordinates on Google Maps. The distinction of two separate nest complexes A and B was based on their complete isolation by unpopulated arable land and on aggression tests. In three 100 m^2 quadrats, detailed mapping of the following features was performed: major nests with clearly visible mounds, underground nests not covered by mounds, auxiliary nests such as soil pavilions around the feeding sites and small and above-ground trails and subterranean tunnels.

Two sites within the nest complex B were selected for research in August 2021. At the first site, detailed mapping was carried out to identify roads between nests. An area of 10 × 10 m in a 10-m square was selected, containing 6 nest mounds (Fig. 2, *a*).

The trails between the anthills in this area were on the surface because the soil was too dense to make tunnels. The location of the roads on the surface made it easier to record ant movements. At the beginning of each road (0.5–1.0 m from the nearest nest), foragers were attracted by bait (boiled fish) placed in a test tube. After a large number of foragers (more than a hundred) had been mobilised into each test tube, the test tube was closed. As a result, 10 test tubes of foragers were collected between five nests. On each trail between two nests, two groups of foragers were selected — one near one nest and one near the other. Distribution: 1 vs. 2 (foragers collected near nest 1), 2 vs. 1 (near nest 2), 3 vs. 2, 2 vs. 3, 3 vs. 4, 4 vs. 3, 4 vs. 5, 5 vs. 4, 6 vs. 4, and 4 vs. 6. The tubes with ants were cooled in a refrigerator at 3–4 C for 1 hour on the same day, after which the ants were marked with paint while they were inactive. Thirty workers were selected for each variant. For each of the 10 variants, an appropriate colour mark was chosen to identify them as belonging to a particular nest mound. 1 vs. 2 — red, 2 vs. 1 — blue, 3 vs. 2 — yellow, 2 vs. 3 — green, 3 vs. 4 — orange, 4 vs. 3 — pink, 4 vs. 5 — purple, 5 vs. 4 — white, 6 vs. 4 — light blue, 4 vs. 6 — light green. The ants were taken to the site and the test tubes were opened to allow the ants to return to their nest mounds. The next morning (11–12 o'clock), 5-minute counts were made of the number of ants with marks on the trails. For each variant, 10 counts were made in a row.

A detailed mapping of all trails, tunnels, anthills and burrows from which ants emerged was carried out in square 9 (Fig. 2, *b*). This was done by carefully examining the entire territory of square 9. Where necessary, the location of tunnels was determined by excavating around the nest. The soil was excavated to a depth of 0.5 m in thin slices. Tunnels were usually found in the top layer of soil to a depth of 0.1 m. Once a tunnel had been found, the soil layer was opened with a knife every 10 cm to



a *b*

Fig. 2. Maps of squares 9 and 10: *a* — Square 10 for recording worker movements between different nests; *b* — Square 9 for recording central and auxiliary nests

determine the direction of the tunnel. This process was repeated until it was clear where the tunnel led — to another nest mound, to a food plant or to an auxiliary nest. To identify nest entrances separate from the nest mounds, the whole site was divided into 1m wide sections, which were carefully searched until the top layer of turf had been removed. To account for the size of the population in sub-complex B, 41 nest mounds were excavated in late April–May 2021. This time of year was chosen because in spring the ants leave the underground passages under the nest mound and move to the above-ground part of the nest, where their brood develops. Each day 1–2 nests were excavated. All nesting material from each nest was placed in plastic bags. In the laboratory, the number of workers was extracted from the material and counted by sorting the pieces of soil on the day of collection. Each count was completed when all the nesting material from each nest mound had been examined. For each nest mound, the height and two diameters (at the narrowest and widest points) were measured.

The Pearson correlation between linear dimensions (height, mean diameter, volume) and population size was then calculated.

The formula to calculate the volume of a cone when the diameter (*d*) and height (h) are known is as follows:

$$V = \tfrac{1}{3}\pi r^2 \text{h},$$

where *r* is radius (half of the diameter).

Twelve aggression tests between workers from mounds from different zones of nest complex B were conducted in late May 2018. Nest mounds from the center of the nest complex were tested against nests distant from the center by 30, 250, 500 and 1000 meters, with three tests performed in each the four distance categories. In a control experiment, three tests between nests from the center of nest complex B and nests from a monodomous population located in a garden 2 km away (50.3957 °N, 30.3449 °E) were performed.

The test samples were collected by placing open test tubes with bait (freshly killed European June beetles *Amphimallon* sp.) next to a mound. The worker ants were allowed to feed on the bait until the tube was filled with around 100 workers. The tubes were then clogged with cotton wool and taken to the laboratory. This mode of sampling was considered to cause less stress than collecting the workers directly from the mound. In the pairwise confrontation tests in the laboratory, the tubes were tightly connected via their opened ends and the interaction between ants was videotaped during two minutes. This method requires much lower observation time and less technical expense as video recording has to monitor only a confined space instead of a large observation arena. However, this mode of encapsulated sudden confrontation is suspected to result in some aggression where under other circumstances no aggression would occur (see discussion). In September 2020, three aggression tests were carried out to measure potential aggression between members of subcomplexes A and B. Each test consisted of ants from different nests. In general, 6 different nests were enrolled.

Similar to Wallis (1962), Batchelor & Brifa (2011), we classified the ants' responses into five categories: swift thusts and drawbacks (1); threatening by mandible opening (2) or gaster flexion (3); seizing or clenching an enemy's appendages (4) and attack with spraying of gaster secretions (5). Additionally, we counted the number of workers killed during confrontation. The aggressive behaviors of ants were scored from 1 to 4 with escalating intensity and then summed to a Total Agonistic Index (TAI). Swoops, bounces and threats were each scored 1, seizing was scored 2, attacks with apparent spraying of gaster secretions scored 3 and the number of killed ants scored 4. We calculated an index TAI_3 considering only behavioral acts scored 1 to 3 and an index TAI_4 additionally considering killed ants. Only observations of aggressive reactions are considered in this study whereas non-aggressive encounters such as friendly mutual antennation or trophallaxis were not recorded.

Laboratory experiments to determine the level of aggression between young queens during pleometrotic founding were performed as follows. Thirty-seven (most probably inseminated) dealate gynes were collected from ground surface within the territory of nest complex B 10 July 2019 immediately after nuptial flight. On the same day, a control group of 21 dealate gynes was collected from ground in a territory with scattered monodomous *L. niger* nest mounds in the Feofaniya Park (50.3408 °N, 30.4869 °E) in the suburb of Kyiv, about 12 km ESE from the nest complex B. The collected gynes were placed in test-tubes 25 cm long and 4 cm in diameter. One third of the tubes were filled with a moist cotton plug. The gynes from the examined nest complex B (UK group) were placed in seven test tubes with six tubes containing five gynes each and the seventh tube seven gynes. The control gynes (F group) were placed in four tubes with three tubes containing five gynes and one tube six gynes. The tubes were stored in the dark at a temperature ranging from 20 to 21 °C and inspected every five days at noon with the help of a magnifying glass (magnification 10×). The following variables were taken: (1) the number of gynes alive; (2) the number of gynes that probably died of natural causes (those whose dead bodies appeared intact); (3) the number of gynes killed (those whose dead bodies showed sign of damage); (4) the number of egg packages; (5) the number of larvae; (6) the number of pupae, and the number of workers dead (7) and killed (8). The gynes were also monitored for keeping together or staying apart. After the first workers arrived, the incipient colonies were fed after each inspection with sugar syrup and small fruit flies (*Drosophila* sp.) killed by freezing. When all but one gynes in the tube died, the tube was excluded from further observation. The experiment was planned to go until the second generation of workers in tubes. However, due to the COVID-19 related lockdown, the experiment was forcibly brought to an end on 27th February 2020. Author had no access to colonies because strict quarantine measures were imposed on the city of Kyiv. Yet, this was no crucial constraint as the deciding criterion, behavior after eclosion of first workers, was under full observation. Overall, tubes from the area of the nest complex B underwent 298 inspections and the control tubes 74 inspections.

Species determination. Ants were determined according to the key of Radchenko (2016) and Seifert (2018).

Statistical analysis. We evaluated the Total Agonistic Index (TAI) data with one-way ANOVA in comparing the TAI data of each of the five aggression test settings pairwise. Changes over time in the queen number in the pleometrosis experiments were evaluated by Fisher's two-tailed exact test run with the software package R (R Development Core Team 2012). ANOVA tests and principal component analysis were performed with the software package SPSS 15.0.

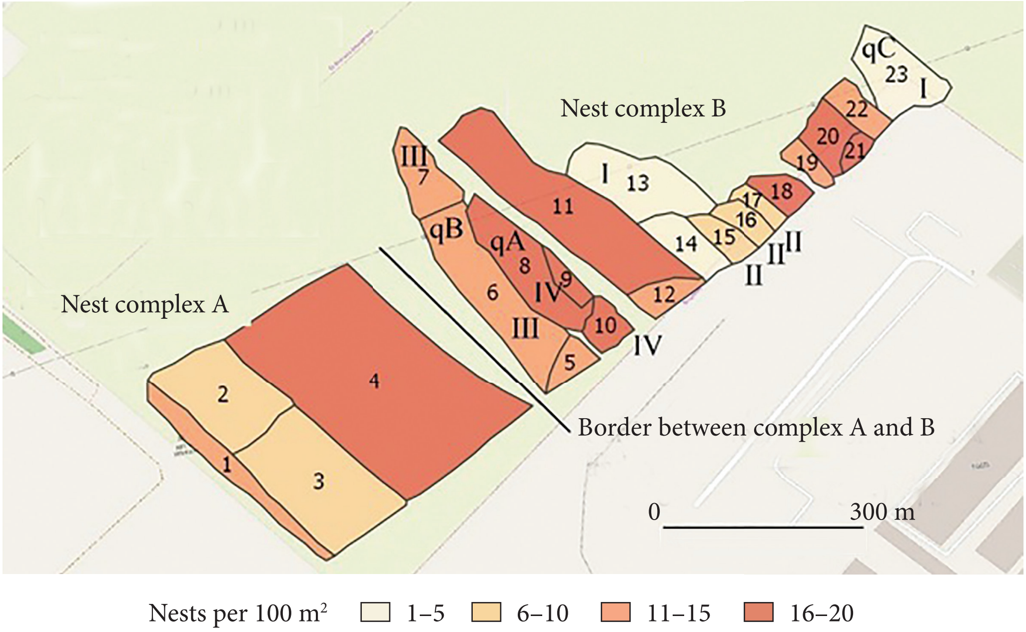


Fig. 3. The map showing *Lasius niger* nest complexes *a* and *b* with sections of variable density (taken from Stukalyuk et al., 2023)

We used Kruskal-Wallis test, a non-parametric alternative to the one-way ANOVA test, to assess the difference in PCAI in contests between workers from subcomplex A vs subcomplex B, and sub complex B vs garden mounds we employed Kruskal-Wallis test and then Wilcoxon test for post-hoc analysis. We chose this index (PCAI) because the aggression tests between subcomplexes A and B involved fewer workers (10 vs 10) than the other tests (100 vs 100) and it was necessary to compare the results obtained. The PCAI index is calculated using the formula PCAI = TAI/total number of workers involved in the experiment. To analyse the effect of the linear dimensions of the anthill (height, average diameter) on the size of its population, we used a bivariate regression model, the corresponding calculations being carried out in the Past program (version 3.14).

Results

Species determination. Ants from both the nest complex are clearly determined as *Lasius niger*.

Topography and size of the nest complexes.

The examined nest complexes stretch over about 1200 m in southwest-northeast direction with a width ranging between 86 and 450 m (Fig. 3).

The nest complexes are located on overgrown fields, which began to regenerate approximately 7–10 years ago after the cessation of plowing. These complexes are separated by a strip of plowed land, with a width ranging from 20 to 120 meters. Additionally, within this dividing area, there is an unplowed section that has existed for at least 24–31 years. This unplowed section is not the physical barrier itself, but its age indicates the duration of isolation between

Table 1. **Main characteristics of the *Lasius niger* nest complex B**

Density class	Number of mounds per 100 m^2, x ± SE	Average mound diameter, cm, x ± SE	Average mound height, cm, x ± SE
I	15.9 ± 1.2	31.6 ± 0.6	25.6 ± 0.6
II	11.9 ± 1.1	39.0 ± 1.0	23.5 ± 0.7
III	11.5 ± 0.7	32.8 ± 0.7	20.2 ± 0.7
IV	4.4 ± 1.0	32.4 ± 1.5	21.7 ± 4.7
Total	11.1 ± 0.8	34.1 ± 0.5	22.9 ± 0.4

the two complexes, as it predates the overgrown fields where the complexes formed. Thus, the primary divider is the plowed land, while the unplowed part reflects the historical context of this separation. The southwestern nest complex A with sectors 1–4 is square-shaped, occupies an area of 11.8 ha and includes 14 655 nest mounds resulting in a nest density of 12.4 nest mounds/100 m^2. The corresponding data of the more elongated and irregularly shaped nest complex B with sectors 5–23 are 13.3 ha, 15 599 nest mounds and 11.7 nest mounds/100 m^2. These nest density data are most probably an under-recording as the transect walks counted only the clearly visible mounds. As reported by Seifert (2017, a) true nest densities are only recordable by carefully probing the whole ground. Seifert found a mean and maximum density of 17.6 and 108 nests 100 m^2 on 68 test plots in Central Europe belonging to a multitude of habitat types. According to the B. Seifert observations about the situation in Central Europe, the dimensions of mounds from the investigated nest complex, with a mean basal diameter of 35 cm and mean height of 24 cm (Table 1), correspond to the average situation in *Lasius niger.*

The spatial distribution of nests is also no conclusive argument for the presence of a nest complex. The two high-density zones in nest complex B (Fig. 3) might possibly indicate origins of colony extension by nest-fission. Yet, this is speculative as density and absolute size of nest mounds strongly depend from environmental factors and do not necessarily reflect the spreading history of a colony.

Connections between the mounds. Each nest mound represents a separate nest.

Figure 4 shows the topography of above-ground trails and subterranean tunnels, connecting mounds within nest complex B.

Direct observation of above-ground trails showed an intense traffic of workers. These multiple connections linking both major and auxiliary nests can be seen in different zones of the nest complex. Auxiliary nests are usually initiated by constructions of mineral soil covering aphid colonies, both subterranean or at base of plant stems, and may develop in the long term to major nests. Figure 4, *a* shows an example of a trail network connecting 13 major mounds in a 100 m^2 plot plus connections to seven auxiliary nests. A medium-sized *L. niger* nest with a basal mound diameter of 35 cm as found in our study (table 1) is estimated to contain a population of 14 000 workers and the maximum population maintained in a monogynous nest seems to be approximately 60 000 (Boomsma et al., 1982). This maximum size is limited by the laying capacity of the single queen. The whole population of 13 major and 7 auxiliary nests mapped in the 100 m^2 plot in figure 3, A should be 250 000 workers at least. It appears most unlikely that such a big population can be maintained by a single queen.

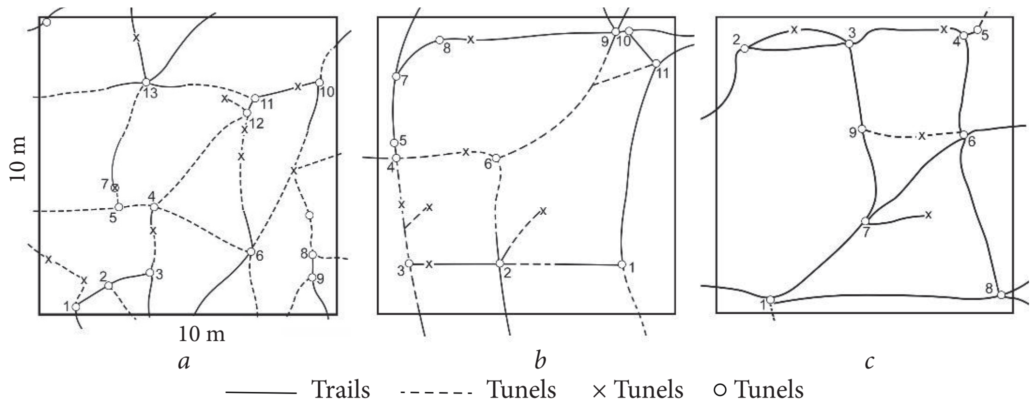


Fig. 4. Nest mounds and trails between nest mounds in *Lasius niger* nest complex B: *a*— zone IV, *b* — zone III, *c*— zone I

Ant marking. Marked ants were found to move evenly between nest mounds. At the same time, foragers tied to one nest did not leave the path where their main number was recorded. For example, between nest mounds 1 and 2, an average of 1 red marked ant and 0.6 blue marked ants were found in 5 minutes of recording, between nest mounds 2 and 1–1.1 red marked ants and 0.9 blue marked ants. Between nest mounds 6 and 4 there were 1.4 ants with a blue mark, 1.1 ants with a light green mark and between nest mounds 4 and 6–1.7 ants with a blue mark and 0.9 ants with a light green mark. This means that the ants move along a trail between two nest mounds without visiting the trails leading to other nest mounds. Cases where ants with different colour markings were found on the trails are isolated. For example, between nest mounds 4 and 6 one ant with a white mark was found in 10 counts, between nest mounds 4 and 5 one ant with a light green mark, between nest mounds 2 and 3 one ant with a red mark. So ants from one nest mound are bound to a trail and move between their nest and the neighbouring nest to which the trail leads. In some cases, the trail may branch out and lead to a food source (Fig. 2) — young aspen trees (*Populus tremula* L.). There is always a food nest at or near the base of the tree (the numbers inside the circles in the figures indicate the number of entrances).

Population size. The minimum population among the 41 nest mounds studied is 132 workers, the maximum is 11,995 workers, the average is 4,164 ± 446 (median 3,719) workers. The distribution of anthill population size by category is as follows: up to 1,000 workers — 8 nest mounds, from 1,000 to 5,000 workers — 23 nest mounds, from 5,000 to 10,000 workers — 9 nest mounds, more than 10,000 workers — 1 nest mound. Thus, most nest mounds have a population in the range of 1,000 to 5,000 workers.

Based on the data obtained and correlation analysis, patterns were identified between the size of the anthill (height and diameter) and its population. A positive correlation was also found between nest mound height and population size, but it was moderate, with a correlation coefficient of 0.65371. A strong positive correlation was observed between nest mound diameter and population size, with a Pearson correlation coefficient of 0.88724, indicating that the larger the worker ant population, the larger the nest mound diameter,

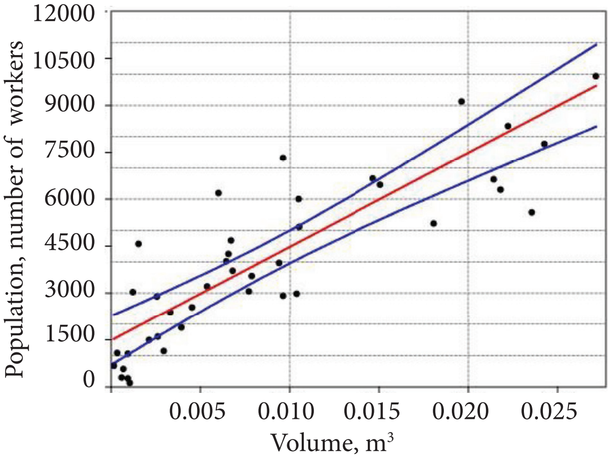


Fig. 5. Bivariate regression model between nest mound volume and nest mound population

Based on statistical analysis, a significant correlation was found between the volume of the nest mound and the population size (number of workers). The results indicate that larger populations are associated with greater nest mound volumes (Fig. 5, *b*). Furthermore, the analysis revealed that linear dimensions of the nest mound, primarily the diameter and, to a lesser extent, the height, also increase with population size.

The correlation coefficient between nest mound volume and population size $r = 0.82713$ and $r^2 = 0.68415$, with a t-value of 9.191 and *p*-value 2.64×10^{-11}, indicate a strong and statistically significant relationship (Fig. 5). A permutation test confirmed this relationship ($p = 0.0001$).

Thus, nest mound size is positively correlated with population size, with the strongest correlation observed for nest mound volume and diameter.

Based on the regression and correlation assessment of the relationship between nest population size and nest size, the following results were obtained.

The results indicate that population size significantly influences the structural dimensions of nests:

The coefficient of determination r^2 for nest height is 0.42734, meaning that 42.7% of the variation in nest height can be explained by changes in population size.

For nest diameter, r^2 is 0.7872, indicating that 78.7% of the variation in nest diameter is influenced by population size.

When considering nest volume (calculated as that of a cone using height and diameter), the r^2 value is 0.68415, showing that 68.4% of the variation in nest volume is determined by population size.

These results demonstrate that larger populations tend to construct larger nests in all dimensions, with the strongest influence seen on diameter, followed by volume and height.

Aggression between workers.The results of the aggression tests are presented in Table 2.

If tested in a one-way ANOVA, the aggression observed between nests within nest complex B did not significantly differ in each of the six pair wise comparisons of the four different test settings. The biggest within-nest complex difference, that of the test setting “center vs. 500 m” and that of the setting “center vs. 1000 m” was also not significant (TAI$_3$: F$_{1,4}$ 5.28, p = 0.083; TAI$_4$: F$_{1,4}$ 5.74, p = 0.075). Furthermore, no increase of within-nest complex aggression with growing distance of the nests could be shown in linear regressions of TAI$_3$ ($r^2 = 0.167$, n = 12, p = 0.187) and TAI$_4$ data ($r^2 = 0.203$, n = 12, p = 0.142).

Table. 2. **Worker aggression tests between nests from the center of nest complex B and from nests of nest complex B located 30, 250, 500 and 1000 m away from the center (lines 1–12) and aggression tests between nest complex B nests and nests from a remote monodomous garden population (lines 13–15)**

Test setting	Score 1	Score 2	Score 3	Score 4	TAI_3	TAI_4	PCAI
Within nest complex B: center vs. 30 m	75	6	2	0	93	93	0.42
Within nest complex B: center vs. 30 m	37	0	0	0	37	37	0.56
Within nest complex B: center vs. 30 m	28	3	1	0	37	37	0.46
Within nest complex B: center vs. 250 m	60	4	2	2	74	82	0.11
Within nest complex B: center vs. 250 m	77	4	4	2	97	105	0.27
Within nest complex B: center vs. 250 m	63	5	5	2	88	96	0.41
Within nest complex B: center vs. 500 m	22	0	0	0	22	22	0.37
Within nest complex B: center vs. 500 m	41	3	2	1	53	57	0.49
Within nest complex B: center vs. 500 m	63	6	2	1	81	85	0.44
Within nest complex B: center vs. 1000 m	66	9	0	1	84	88	0.47
Within nest complex B: center vs. 1000 m	61	21	3	3	112	124	0.19
Within nest complex B: center vs. 1000 m	65	10	2	3	91	103	0.19
Nest complex B vs. garden	108	11	5	9	145	181	0.73
Nest complex B vs. garden	144	12	7	12	189	237	0.95
Nest complex B vs. garden	103	9	4	8	133	165	0.67
Nest complex A vs nest complex B (PCAI)	14	1	1	1	19	–	0.95
Nest complex A vs nest complex B (PCAI)	12	2	1	1	19	–	0.95
Nest complex A vs nest complex B (PCAI)	18	4	2	2	32	–	1.60

Note. Increasing scores indicate increasingly aggressive behavior. The Total Agonistic Index TAI_3 considers only aggressive behavior (scores 1–3) and TAI_4 additionally the number of killed ants (score 4). Hundred workers from each nest were confronted.

Tests for aggressiveness between garden populations have not been carried out. Aggression between nest complex nests and those of the monodomous garden population was significantly larger than in any within-nest complex test. Even the closest data, that of the setting “nest complex vs. garden” and that of the setting “center vs. 1000 m” differed significantly (TAI_3: $F_{1,4}$ 9.98, $p = 0.034$; TAI_4: $F_{1,4}$ 13.63, $p = 0.021$). These data give a clear indication that worker aggression within the nest complex is reduced whereas increased agonistic behavior occurs in confron-

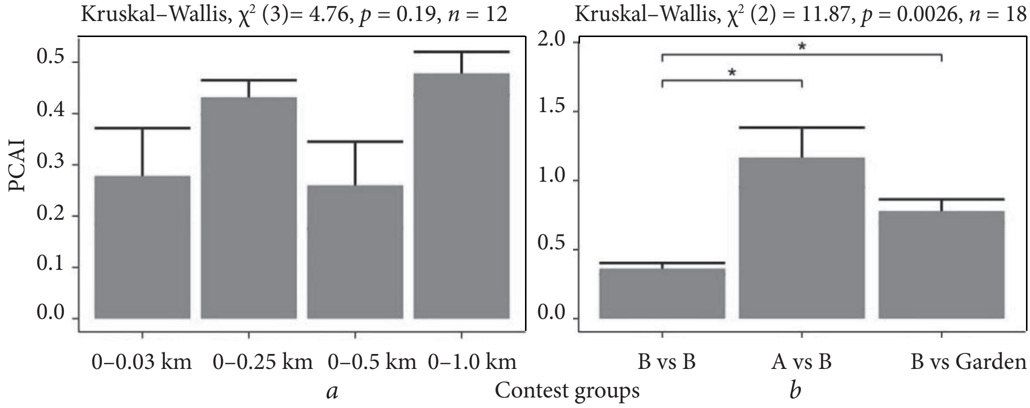


Fig. 6. Per capita agonistic index (PCAI) (mean ± s. e.) reached in confrontations of workers from mounds located at various distances from each other within the subcomplex B (6, *a*); and between subcomplexes B and A; B and garden mounds (6, *b*). Statistical comparisons were performed using Kruskal-Wallis test and Wilcoxon test. Asterisks indicate 0.5 significance level after Bonferroni correction for multiple comparisons. pwc: Wilcoxon test; p. adjust: Bonferroni

tations of nest complex workers with those from the monodomous (supposedly monogynous) population.

Aggression tests found a low overall level of aggression within the same subcomplex with no significant differences in the PCAI index between the sectors (Fig. 6, *a*). In contrast, a more than three times larger PCAI index was found in confrontations of workers from the subcomplexes A and B (Fig. 6, *b*). This indicates the microgeographic isolation of the two subcomplexes. Given that the uncultivated area is older than the two fields where the subcomplexes formed, it is likely that both subcomplexes emerged simultaneously after the fields were abandoned. They may have originated from nest mounds previously located in the uncultivated area, which could explain the relatively low aggressiveness observed between workers from these subcomplexes compared to the tests with the garden population. Furthermore, aggression between workers of the subcomplex B and ants from the remote monodomous garden population was more than twice as large as mean aggression between workers of different sectors of subcomplex B.

Our observations of moderate aggression in within-nest complex confrontations is suspected to be a consequence of the experimental setting. The encapsulated confrontation within the small space of two tubes suddenly put together with their open ends is likely to raise some initial aggression which would possibly not occur under other conditions. Each ant student who has divided a group of workers in the laboratory, has kept them in separate vials for some days and reunified these by releasing them suddenly into a petri dish will observe initial disorientation accompanied by aggressive behavior which will settle after a while. The same occurs in the field when for instance a *Formica rufa* group nest is massively disturbed and the excited workers attack each moving object including their own nest-mates. The possible boosting of aggression data in our test system does not question our conclusion of a significantly reduced aggression within the nest complex as each test was affected in the same way.

The pleometrosis experiments. The situation with gyne material from the remote monodomous population in Feofaniya Park (control group F) was the same as in the pleometroses observed by Sommer and Hölldobler (1995) with material collected near Würzburg/Germany. Soon after eclosion of workers from pupae, gynes began a deadly fight ending with the survival of only one queen. Sommer and Hölldobler also found in a series of experiments that gyne fighting did not occur when daily removing each hatched worker from the pleometrosis associations while the adding of workers to a workerless situation or increasing their numbers had the opposite effect. Hence it is definitely the presence of workers which induces gyne fighting. Based on the fact that, in our setting, pleometrosis experiments in which no workers hatched are useless for indicating behavioral differences between different populations, we excluded these experiments from consideration. As a consequence, we only evaluated five experiments with gynes collected from the area of the nest complex B (group UK) and three experiments with gynes from the remote monodomous population (group F). The results in group UK differed strikingly (Fig. 7, *a*). In none of the five experiments with four to seven gynes living before eclosion of workers (mean value 4.8 gynes), the number of surviving queens was reduced to a single one within 35 days after eclosion of first workers. The mean number of surviving gynes was as large as 4.2. All three pleometroses of the control group F with six, four

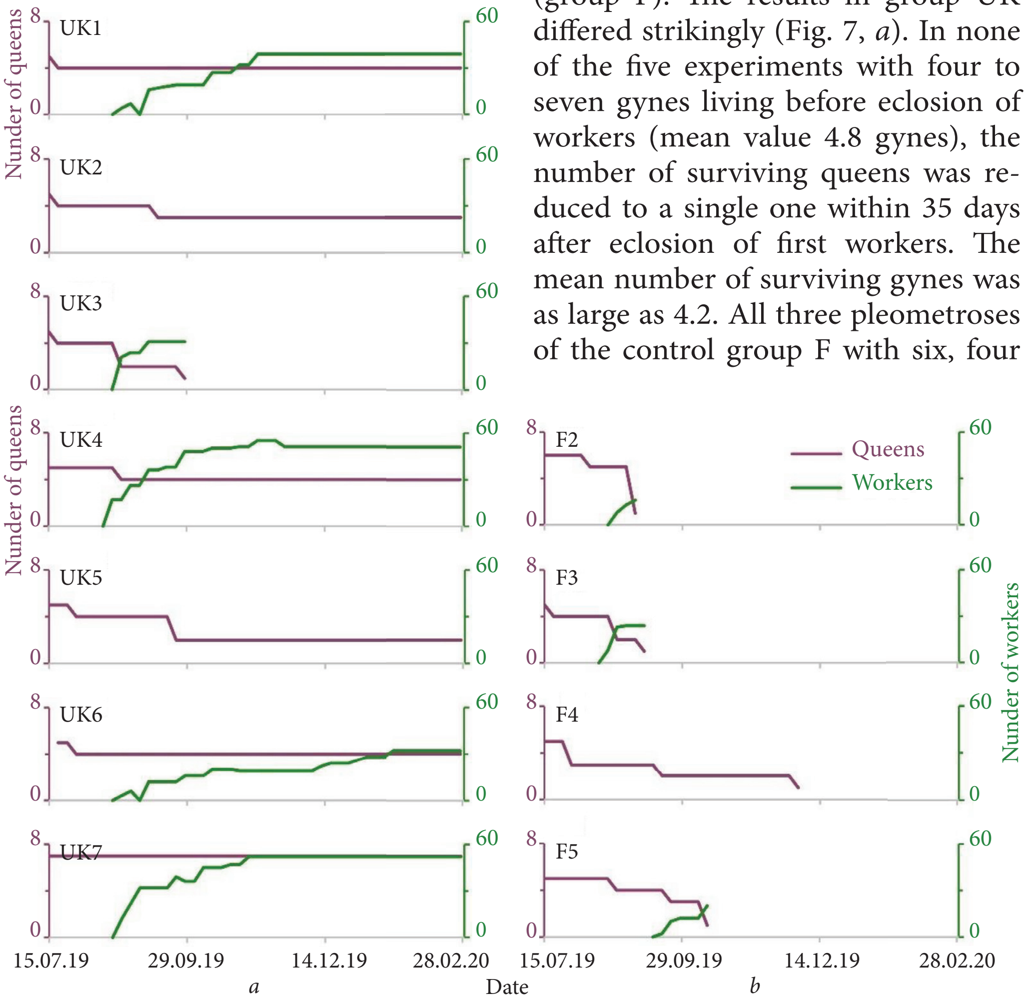


Fig. 7. Temporal changes in queen and worker number in pleometrosis experiments with foundresses from the nest complex B UK group (*a*) and the monodomous F group (*b*)

and four gynes living before eclosion of workers (mean value 4.7 gynes) were reduced to a single surviving queen within 23 days after the first workers appeared (Fig. 7, *b*).

Despite the low sample size available, statistical tests show significant differences between the UK and F groups. Five experiments with no reduction to a single queen (UK group) and three experiments always ending with a single queen (F group) differ with $p < 0.018$ according to Fisher's exact test which is the most adequate test when cells with zero numbers are in the data set. This discrete YES/NO consideration is confirmed by continuous data when gyne reduction ratios within the 35-days interval are tested. The reduction of gyne number to 22% of the pre-hatching figure in group F and to 86% in group UK represents a significant difference (ANOVA, F1,6 23.26, $p < 0.003$). These pleometrosis experiments indicate no aggression between mated gynes from the nest complex B in the presence of workers.

Discussion

A study of the relationship between nest size and population size revealed important patterns. It was found that nest mound population size is positively correlated with nest mound volume, diameter and height. At the same time, population size has a more significant effect on nest mound volume and diameter than height.

Observations of the movement of workers showed that they moved evenly between nest mounds, preferring certain trails and rarely leaving them. This suggests a high degree of organisation and specialisation in their movement, allowing them to effectively maintain links between nest mounds and optimise foraging.

An analysis of nest mound population size showed that most nest mounds have a population in the range of 1,000 to 5,000 workers.

A study of the aggressive behaviour of the ants showed a low level of aggression within a sub-complex and a significantly higher level of aggression between different sub-complexes and populations (such as garden population).

In fact, intensive investigations of the relations between nests on study plots with high nest density of *Lasius niger* have been missing up to the present. What we only have are casual observations of aggressive interactions between nests. According to Czechowski (1984) and Seifert (2018), these typically start with tournaments at territorial borders. Seifert (2018) wrote: "Conflicts between mature colonies of approximately the same size are mainly conducted by ritualized encounters with low mortality: Hundreds of opponents stand face to face along a front line and perform jerky, high-frequent back and forth movements towards the alien ants. This may last one hour or more and is apparently a means of assessing the strength of the opponent. Only in case of strong numeric superiority of one party, the encounter may finally go over to mortal fighting and raiding of the weaker nest". It is obvious in these cases that the workers of the engaged parties had a disparate pattern of cuticular odor cues and that they most probably belonged to monogynous colonies.

Humans are alerted by aggressive acts and not by peace. Hence it is an expected psychological phenomenon that aggressive encounters between ant societies attract the attention of ant students much more than workers peacefully running along trails. The ecological dominance and biomass of this ant across its Palaearctic range from Western Europe to Siberia is enormous. The situation in Central Europe was examined by Seifert (2017, b). Among 79 study plots on which *Lasius niger* was present, this ant showed densities > 30 nests / 100 m^2 on ten plots, with a maximum of 108 nests / 100 m^2 found on a 32 m^2 plot. He reported the highest densities for grasslands in landscapes with strong anthropogenic impact — often in rural and suburban regions with fertile and moderately dry tschernosem or alluvial soils. In none of these high-density plots, the colony status was checked. Extreme local frequency is also observed in similar habitats in the Ukraine in the Kyiv, Zhytomyr and Chernihiv regions where large associations with hundreds of nest mounds are ubiquitous in rural fields, gardens or initial states of tree plantations. The high abundance of *L. niger* within the city zone of the urban gradient is well known for a long time — for Russia and the Ukraine this has been recently reported for Moscow (Putiatina, 2011) and Kyiv (Stukalyuk, 2017).

Our finding of the long-term coexistence of several queens within a pleometrotic group is not the first. As early as 1979, V. E. Kipyatkov noted the long-term (more than 1.5 years) coexistence of such *L. niger* queens with a large number of workers (Kypiatkov, 1979). The author notes that, in this case, the transition to obligate monogyny could be very long. Our data confirms this for 227 days.

Especially interesting to us is the very large difference between the ‘normal’ (solitary nest mound, control group F) and nest complex populations (experimental group UK), given that this species is obligately monogynous. Under natural conditions, pleometrotic groups typically contain between 2 and 4 queens, with a maximum of 9 (Bartz and Hölldobler, 1982), so the group size we used (5) is realistic and close to typical.

L. niger nest complexes are common in Ukraine (Stukalyuk et al., 2022; 2023), as well as in the European part of Russia (Zakharov, 2015). Therefore, it is likely that the nest complex we studied is not unique; it was simply the first to be studied in detail (Stukalyuk et al., 2022, 2023). Unclear is whether other nest complexes also show strong interconnectedness and low intraspecific aggression to members of the nest complex. Under densely populated conditions, pleometrosis is an effective method of establishing new colonies. Given such a dense population, the greater tolerance of pleometrosis we demonstrated should allow newly founded colonies to establish a competitive colony with shared resources quickly. Given the obligate monogyny of *L. niger*, sooner or later, only one queen remains in all such pleometrotic groups. The duration of the transition to monogyny remains open. Still, our results suggest that this period is much longer in pleometrotic groups from nest complexes than in solitary nest mounds, and could potentially last for several years.

Fights between *L. niger* queens can lead to death in pleometrotic groups in 60% of cases (Madsen and Offenberg, 2017), which was confirmed by our experiment, where the number of queens was reduced to one in 3 out of 4 tubes from the group F of single nest mounds.

Interestingly, pleometrotic queens have higher survival rates than haplometrotic queens (Bernasconi and Keller, 1996, 1999). The next peak in queen mortality occurs when workers have emerged from their cocoons (Sommer and Hölldobler, 1995).

L. niger queens investing in high reproduction may experience decreased survival and vice versa (Pamminger et al., 2016). This may be the reason for the high survival rate of queens, at least in the group UK of the nest complex.

Tolerance between queens is key to the possibility of pleometrosis (Overson et al., 2014). We show that the nesting typical for a given ant species in a given part of its range strongly influences tolerance between queens. Another interesting finding of our study is the long transition period from the pleometrotic group to monogyny. However, at the end of the experiment, there were at least four queens in the UK nest complex groups.

The *L. niger* queen ants collected from the nest complex mostly survived when kept in pleometrotic groups, while most queens from the normal single nest mound population did not. What could the mechanism behind this difference be? As the pleometrotic queens from nest complex would not be tolerated by non-pleometrotic'normal' queens, distinct geographical ranges may appear despite mating flights allowing the queens to disperse. This hypothesis would predict that other separate populations of pleometrotic *L. niger* may emerge or already exist.

Alternatively, pleometrotic tolerance may be ontological in origin, either due to developmental changes when developing in a pleometrotic nest or by the virgin queen learning to tolerate a range of workers with reduced relatedness in their natal nest. Uncovering the mechanism behind this pleometrotic tolerance would provide valuable insights into the evolution of pleometrosis.

These nest complex populations also offer a range of other opportunities. Given that workers from this population are also less aggressive to non-nestmates from this population, the mechanisms enforcing heterometrosis may be the same as those involved in nestmate recognition.

Our pleometrotic experiments have several limitations.

1. Each group of queens was confined to a test tube and had no opportunity to disperse. The only option was to disperse along the length of the test tube, but all the queens were always grouped next to the cotton, behind which was a supply of water. In several cases, queens at the exit of the test tube were later found to have died of natural causes. The test tubes were separated from each other, so that queens from different groups were separated from each other. As a result of the coexistence of queens and their brood, the profile of cuticular hydrocarbons may have been homogenised, resulting in a decrease in aggressiveness between queens and between workers. We did not observe any fighting between workers.

2. Another important point is the small sample size. Although the effect sizes observed are very large, repeating the experiment with a larger sample size would be ideal.

3. Some of the queens that died of natural causes may have died as a result of stress — it is impossible to distinguish the two.

4. Another limitation of this study may be that the ant colonies were not overwintered. Overwintering is a prerequisite for colony reactivation in *L. niger* (Kipyatkov, 1995); it was not present under the experimental conditions, but colony growth continued. It may be necessary to repeat the experiment with a larger sample and with the obligatory presence of hibernation.

Conclusions

The study revealed important relationships between nest size and population size in *Lasius niger* ants. Nest mound population size was found to be positively correlated with volume, nest mound diameter and height.

Observations of worker ant movements showed a high degree of organisation and specialisation, allowing effective communication between nests. Workers preferred certain paths and rarely left them, which helps to optimise foraging.

In addition, analysis of ants' aggressive behaviour revealed low levels of aggression within a subcomplex and significantly higher levels of aggression between different subcomplexes and populations. This suggests microgeographic isolation and behavioural specificity between ants from different groups.

L. niger queens, a low-aggression nest complex group UK, survived well under pleometrotic conditions, with at least four queens in each group remaining alive until the end of the experiment. By contrast, *L. niger* queens from the solitary nest group F had low survival, with the number of queens reduced to 1 in each replicate by day 130. Taken together, the results of this study demonstrate that factors intrinsic to the queens, not external environmental factors post-pleometrotic founding, drive survival in pleometrotic groups. However, whether these innate or developmental factors are genetic is unknown.

Key findings of the study include:

1. Confirmation of a positive correlation between nest mound size (volume, diameter) and population size, emphasising the influence of population size on nest mound size.
2. Low levels of aggression within sub-complexes, allowing *Lasius niger* ants to coexist in large nest aggregations of thousands and tens of thousands of nest mounds over large territories.
3. Detection of additional auxiliary nests, which can later become full-fledged anthills, indicating a possible increase in the actual settlement density.
4. *L. niger* queens from the low-aggression nest complex (group UK) exhibited high survival under pleometrotic conditions, with at least four queens per group surviving until the end of the experiment. In contrast, queens from the solitary nest group (group F) showed low survival, with only one queen remaining per group by day 130.

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