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The effect of nest topology on spatial organization and recruitment in the red ant *Myrmica rubra*

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Abstract

Nests of social insects are an important area for the exchange of food and information among workers. We investigated how the topology of nest chambers (as opposed to nest size or environmental factors) affects the spatial distribution of nestmates and the foraging behavior of *Myrmica rubra* ant colonies*. Colonies were housed in artificial nests, each with same-sized chambers differing in the spatial arrangement of galleries. A highly connected central chamber favored higher occupancy rates and a more homogeneous distribution of ants across chambers. In contrast, a chain of successive chambers led to a more heterogeneous distribution of ants, with the occupancy of a chamber chiefly mediated by its distance to the entrance. Irrespective of nest topology, the entrance chamber housed the largest proportion of ants, often including the queen, which exhibited a preference for staying in densely populated chambers. Finally, we investigated how nest topology influenced nestmate recruitment. Surprisingly, a highly connected chamber in the center of the nest did not promote a greater recruitment nor activation of ants. At the onset of foraging, the largest number of moving ants was reached in the topology where the most connected chamber was the nest entrance. Later in the process, we found that a chain of successive chambers was the best topology for promoting ant's mobilization. Our work demonstrates that nest topology can shape the spatial organization and the collective response of ant colonies, thereby taking part in their adaptative strategies to exploit environmental resources.

Keywords: Ant; Collective foraging; Nest topology; Network; Queen location; Spatial organization

Introduction

Nests of social insects serve multiple functions: they maintain a homogeneous internal environment (e.g., Korb 2003; Turner 2009; King et al. 2015), contribute to organize the division of labor (e.g., Tschinkel 1987; Tschinkel 2004; Tschinkel and Hanley 2017) and act as a protective fortress for the colony (e.g., Noirot and Darlington 2000; reviewed in Perna and Theraulaz 2017). The nest also represents an important area for the exchange of food and information within the colony. Ant colonies are capable of complex forms of collective behavior such as digging large and complex nests (Tschinkel 2015) or taking efficient collective foraging decisions (Detrain & Deneubourg 2008; Jeanson et al. 2012). Such complex collective organization depends on multiple interactions between nestmates (Theraulaz et al. 2003; Detrain & Deneubourg 2006; Gordon 2010). Most importantly, these interactions often take place in the constrained physical space of the nest. By constraining the movement and the interactions of nestmates, the nest becomes more than a passive setting for information sharing and colony activities. Indeed, the form of the nest contributes to determine where and when interactions occur, which work-related stimuli (larvae, wastes, corpses, etc.) become available to each individual ant, and ultimately the organization and behavior of the entire colony (Mersch et al. 2013; Jeanson & Weidenmüller 2014; Lehue & Detrain 2019).

As interactions between workers are related to non-linear, self-amplifying processes, even small variations in interactions at a local scale can potentially generate large differences in colony-level behavior (Camazine et al. 2003; Detrain & Deneubourg 2006). In particular, the chamber closest to the entrance (i.e., 'the entrance chamber') is inevitably the first place visited by returning foragers and thus appears as a crucial location for information-sharing and initiation of collective recruitment. Some studies (Pinter-Wollman et al. 2013; Davidson & Gordon 2017) showed that the number of ants waiting in this particular chamber affects the rate at which outgoing ants will leave the nest. Assuming that the volume of the entrance chamber limits the maximum number of foragers that can be present at any one time, it could also shape the collective dynamics of food exploitation. However, Pinter-Wollman (2015) showed that the volume of the entrance chamber did not influence the speed of recruitment, suggesting that colony-level behavior was more dependent on the topological features than on the carrying capacity of this chamber. This study highlighted for the first time the importance of nest topology on the emergence of collective behavior in ants.

The nests made by different species of ants, and sometimes also by different colonies of the same species, can take many different forms. Examples range from complex network of galleries excavated in soil or wood cavities, to nests made out of leaves woven together (Hölldobler & Wilson 1990). On the one hand, nest size is often related to the size of the colony (Mikheyev & Tschinkel 2004; Tschinkel

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72 1999, 2005). On the other hand, nest shape and topology change depending on season (Hart &

73 Tschinkel 2012), soil type (Toffin et al. 2010), ants' body size (Kwapich et al. 2018) as well as the

74 presence of brood or food (Römer & Roces 2014). The opposite is also true, and some species dig nests

75 with clearly recognizable shapes across a wide range of ecological contexts (Tschinkel 2004, 2013).

76 Regardless of its complexity, the structure of most underground ant nests can be broken down into a

77 set of chambers interconnected by galleries. These structures can easily be described in terms of the

78 tools derived from network theory (Gravish et al. 2012; Pinter-Wollman 2015, Kwapich et al. 2018). In

79 this case, the chambers are the nodes of the network, and the galleries are the edges. A node (i.e., a

80 chamber) can be characterized by its connectivity degree, which corresponds to the number of edges

81 (i.e., galleries) directly connected to it. A node can also be characterized by its distance to another

82 specific node, called the "path length" or by its average distance to all other nodes in the network,

83 called the "average path length". Because of the importance of the entrance chamber for foraging

84 recruitment and exchanges with the external environment, one can assume that nodes closer to the

85 entrance -having a shorter path length to the entrance chamber -are more likely to be influenced by

86 the external environment.

87 Many underground ant nests, particularly those excavated by small-sized colonies, are tree-like

88 networks meaning that they have no cycles (Antonialli-Junior & Giannotti 1997; Lévieux 1976;

89 Tschinkel 2003). Likely, this is because cycles are only formed when the growing tip of one gallery

90 encounters another gallery, an event that is relatively unlikely at low gallery density in three-

91 dimensional soil. Conversely, larger colonies build nests of higher complexity with a high number of

92 cycles (Gautrais et al. 2014), a factor that could facilitate the rapid mobilization of foraging ants (Pinter-

93 Wollman 2015). Tree-like networks are simpler to characterize because some of their topological

94 properties are fixed: the number of edges is always equal to the number of nodes minus one, and the

95 time that it takes to move between two chambers is always proportional to the path length,

96 independently of the type of movement of ants, e.g. if they follow a completely random walk or they

97 move directly to a destination.

98 Network theory has proved to be a powerful tool to describe and compare the nests of social insects.

99 For example, a network analysis of nests constructed by termites of the genus *Cubitermes* enabled

100 Perna et al. (2008) to highlight the highly efficient network of galleries that created effective

101 communication and transportation paths. In addition to acting as a tool to describe complex structures,

102 network analysis allows researchers to explore relationships between specific properties of the

103 network and collective behavior. In order to isolate the effects of nest topology, the behavior of a

104 colony should be compared across nests with different topologies but otherwise similar characteristics.

For example, in the Argentine ant, *Linepithema humile*, the partial obstruction of nest exits counterintuitively enhanced the outgoing flows of alarmed ants (Burd et al. 2010). In the harvester ant, *Veromessor andrei*, a study by Pinter-Wollman (2015) showed that the speed of recruitment to food increased in relation to the degree of connectivity of the entrance chamber and with the 'meshedness' of the nest network. Such field studies have paved the way for establishing a causative relationship between nest structure and collective behavior.

Some nest casting techniques allow researchers to identify the ants' location within the nest when the cast is dissolved/melted (Tschinkel 2010). However, in field studies it is not always possible to accurately relate the colony-level behavior to its underlying mechanisms because the spatial distribution of workers, their mobility and their interaction patterns are usually not known (but see Pinter-Wollman et al. 2013). Even when the distribution of ants inside the nest can be observed, it remains difficult to discern if it is determined by the size and topology of different nest parts, or instead it matches environmental gradients of temperature, humidity and other physico-chemical parameters. The same limitations apply to the study of ant queens. Ant queens have primarily been observed deep down in the nest and/or in areas of high density of workers (Tschinkel 1993; Walin et al. 2001), but observations of queen location are relatively scarce and do not allow to tell apart the effect of environmental gradients and nest topology.

Laboratory studies represent an interesting alternative to field studies as ant colonies can be housed in artificial nests designed to control directly nest properties such as the size, number of chambers, and the spatial arrangement of chambers and corridors. Many laboratory studies have focused on ant nest occupancy and behavior inside the nest (Moreau et al. 2011; Pinter-Wollman et al. 2011; Mersch et al. 2013), but most of these studies have focused on relatively simple nests comprising only one or very few chambers. One recent exception is Tschinkel (2018), who compared colony growth and worker production for colonies housed in either artificial nests of a single chamber or nests of equal total volume but made up of multiple small chambers.

In our study, we want to specifically address the question of how nest topology affects the nest occupation and the collective recruitment to food in the red ant *Myrmica rubra.* This ant species is widespread in temperate regions, nesting from Europe to Western Asia but also North America where it is considered as invasive. We know that this species uses various types of microhabitats for nesting; it is mostly found under or inside dead wood as well as beneath leaf litter. Brood is stored in curled leaves but also under rocks and even in moss tussocks (Groden et al. 2005). Nest relocation has been observed (Dobrzańska & Dobrzański 1976; Bernard 2014) but remains understudied (Abraham &

137 Pasteels 1980). The variety of nest structures dug/occupied by the red ant makes it an ideal species for

138 studying the effect of different nest topologies.

139 Here, we address the question how nest topology shapes the spatial distribution of the ant population

140 and thus the emergent collective behavior of the colony. We characterized the spatial organization of

141 workers inside the nest as well as the process of recruitment to food in colonies of the red ant *M. rubra*

142 which were housed in artificial nests with three different topologies. Each nest had unique topological

143 properties, while all nests had identical size and number of chambers. Specifically, we compared the

144 dynamics of nest occupancy over a week focusing on the distribution of workers across the chambers.

145 We investigated whether the connectivity of a chamber or its distance to the entrance influenced the

146 level of occupancy by the ants. We paid particular attention to whether the queen had a higher

147 tendency to occupy chambers located far from the entrance and/or chambers characterized by high

148 connectivity and/or densely occupied by workers. Eventually, we compared the intensity of nestmate

149 recruitment in the different nests to see if some topological features facilitated the spreading of

150 recruiting stimuli to the whole colony.

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Methods

The species used in this study is the red ant, *M. rubra* (Linnaeus, 1758) (Hymenoptera: Formicidae). A total of nine ant colonies were collected in the summer of 2016, 2017 or 2018, in woodlands located at Sambreville (Namur district: N 50°25.210'; E 004°37.878') and Aiseau-Presles (Hainaut district: N 50°25.657'; E 004°35.764') in Belgium. *M. rubra* nests are found in a variety of substrates such as dead wood, leaf litter or soil and are typically composed of 100 up to 1500 workers and a highly variable number of queens from one up to 20 (based on our personal observation and Elmes 1973). For the nests that were dug under stones or under wood logs, the superficial nest chambers covered a total area of 20 to 50 cm², housing a few hundred individuals and consisting of a large single chamber or of multiple chambers of a few square-centimeters each, separated by loose walls or well- defined ridges (personal observations). The colonies were kept under controlled laboratory conditions (21°C ± 1°C; 50% ± 5% humidity and 12L – 12D daylight cycle) for a minimum duration of 30 days before any experiment. We provided colonies with water, sucrose solution (0.3M) and freshly killed mealworms (*Tenebrio molitor*) ad libitum.

Each experiment took place in a 15x25cm plastic tray with sides covered with Fluon® (polytetrafluoroethylene) to prevent the ants from escaping. The nests were made from 2mm thick, laser-cut Plexiglas discs of 6.5cm diameter (see Online Resource 1 for nest pictures). Each nest chamber was 1.5cm in diameter and each gallery connecting two adjacent chambers was 0.5cm long and 0.2cm wide. The nest had access to the outside through a single opening connected to the so-called "entrance chamber". This agrees with previous results (Lehue & Detrain 2019) showing that *M. rubra* ants located within a 2-centimeter radius from the nest opening were oriented towards the entrance and were the most likely to interact with nestmates coming back from the outside. The overall nest size was chosen in order to be sufficiently large to comfortably host the entire colony as well as to have chamber dimensions that were broadly in the same range of sizes as those observed in natural *M. rubra* nests. The nest ceiling consisted of another Plexiglas disc of the same size as the nest (diameter 6.5cm) and was covered by a red filter paper to create darkness inside the nest. The nest floor was made from plaster and sat over a 3mm thick, highly water-absorbing synthetic fabric to ensure homogeneous humidity across the whole nest area. This absorbent plaster base and the entire foraging arena were watered daily (4mL/day). The nests were off-centered in the tray and placed 2cm away from one of the short sides with the entrance facing the large foraging area.

184   Nest topologies
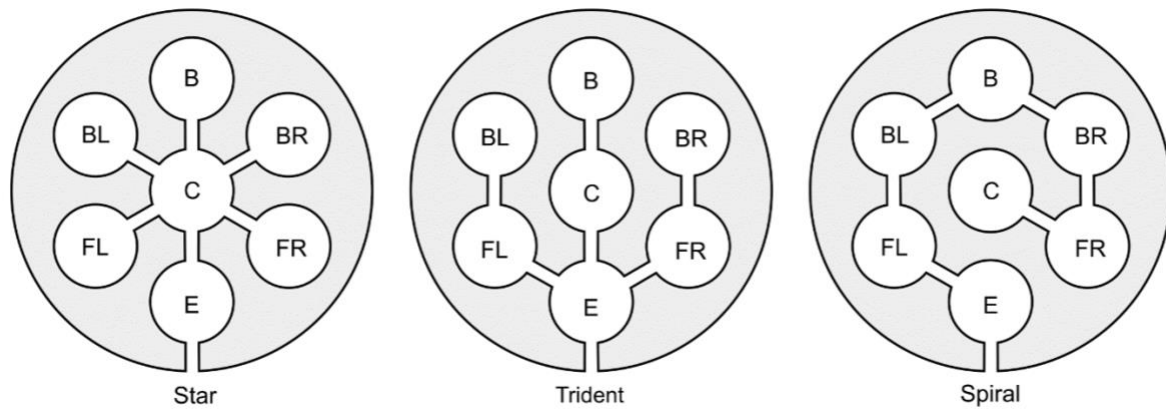
188   The three topologies studied will be referred henceforth as: the "star", the "trident" and the "spiral"
189   (Fig. 1). Several characteristics were shared by these three topologies. The first common feature is that
190   all nests were "tree" networks and were open to the outside environment by a single entrance. All
191   three topologies contained the same number of equally sized chambers (seven chambers), thus
192   offering a similar volume to host nestmates. Finally, all nests had the same number of galleries (i.e., six
193   galleries, plus one entrance gallery) with an average connectivity per nest chamber of 1.7. Although
194   the three nests had the same number of chambers, they markedly differed in the way these chambers
195   were interconnected by galleries leading to different topological properties (Tab. 1).

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	Star	Trident	Spiral
Average Path Length (n nodes)	1.71	2.29	2.67
Average Distance to Entrance (n nodes)	1.57	1.29	3.00
Maximum Distance to Entrance (n nodes)	2	2	6
Maximum Connectivity (n nodes)	6	3	2

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The "star" nest had the lowest average path length of all three topologies. The central chamber was highly connected with a degree of connectivity of 6, while every other chamber was little connected with a degree of connectivity of 1. This feature created a nest topology with five chambers that were dead-ends and that inevitably went through a central hub before reaching the entrance chamber.

The "spiral" nest had the highest average path length of all three topologies. The chambers were connected in series, and the degree of connectivity of the chambers was 2 except for the entrance chamber and the terminal chamber where the degree was 1. By consequence, the distance to the entrance gradually increased until a maximum of 6.

The "trident" nest had an intermediate average path length. It was the only topology where the entrance chamber was connected to more than one chamber, with a degree of connectivity of 3. In this network, the entrance was the most connected chamber in a way that is reminiscent of the top-heavy connectivity of natural ant nests (Tschinkel 2015). In terms of connectivity and average path length, the "trident" was intermediate between the two other topologies but provided a unique feature with the highly connected entrance chamber and the lowest average distance to the entrance.

Experimental procedure

The experiments lasted for nine weeks, and three colonies were tested simultaneously (Fig. 2). This process was repeated three times to reach a total of nine tested colonies. Each experiment consisted of the same consecutive events, starting by the settlement of ant workers in one of the three nest topologies, followed by a week of data collection on the spatial organization of the colony. After a resting period of eight days, the colony was starved for 72 hours prior to the recording of the food recruitment process. After two days, we removed the artificial nest and replaced it with a new artificial

nest characterized by another topology. The procedure was repeated until every colony was tested in

each of the three nest topologies. The testing order of nest topologies was randomized.
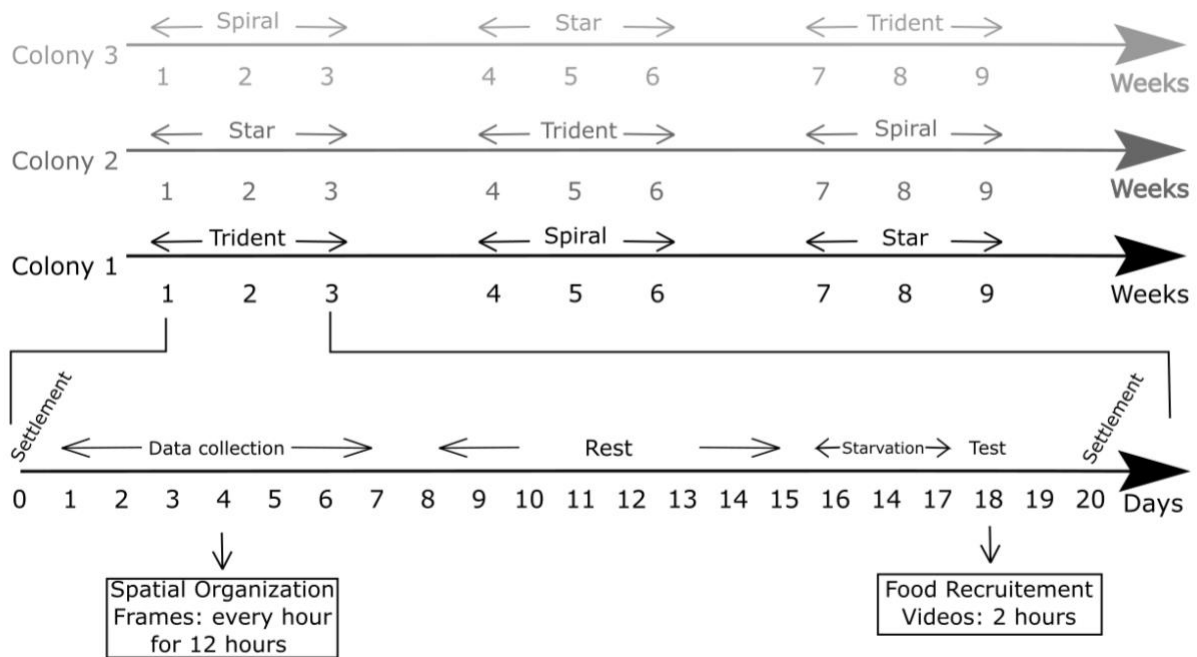
Fig. 2 Timeline of the experiments

Spatial organization of nest population

Each colony was placed in an experimental tray and could freely occupy the artificial nest characterized

by one of the tested topologies. Ants started to settle inside the nest as it was the only place offering

them suitable conditions of humidity and darkness. Because some colonies were slower than others

to discover the nest, we started recording data as soon as at least 30% of the workers' population was

settled in. Once this threshold was reached, video frames of the nests were taken every hour for 12

hours, during seven consecutive days. We then followed the hourly and daily location of the queen as

well as the number of workers per nest chamber. The population size of each colony was standardized

to approximatively 100 randomly picked workers, one queen and 15 larvae (first to third instars). We

counted and removed every ant that died during the experiment and did not replace them. Throughout

the experiment, the daily mortality remained very low, with an average of 0.22 ant dying per day. The

highest and lowest mortality values observed in a colony were 0.42 and 0.10 dead ants per day

respectively. In order to assess the impact of nest topology on the dynamics of ants' settlement, a daily

occupancy index was calculated by averaging the total number of ants inside the nest for each day and

each topology. The dynamics of daily occupancy indices were then compared between topologies to

see if one type of nest structure promoted a faster/larger settlement of ant colonies during the seven

days of experiment. Then, we observed the location of workers to see if the distribution of ants was homogeneous across all chambers (same percentage of ants in the chambers) and if it was influenced by the properties of the chambers (for instance if its occupancy changed with distance to the entrance or connectivity degree).

Furthermore, we tracked the queen's movements between successive frames and measured, for each chamber, its level of occupancy by the queen. This was done by counting how many times a queen was observed in a chamber for each day and each topology. This chamber's level of occupancy was then related to the topological properties of the chamber. We also recorded the frequency at which the queen moved from a chamber to another (assessed as its mobility) as a function of the number of workers locally occupying the abandoned chamber. Information about brood location was also collected on the last day of the experiment. In particular, we checked for possible effects of nest topology on the relocation of brood pile between the first hour and the last hour of observation.

Recruitment to food

Before carrying out the experiments on food recruitment, the colonies were starved for 72h. Then, a single food source of 600µL of sucrose solution (1M) was placed at 10 cm from the nest entrance, and the whole tray was video recorded for 2 hours.

We noted the exact moment the first ant entered the entrance chamber right after drinking at the food source, that is, the moment when the recruitment process started. We counted the resulting flow of workers leaving the nest after the arrival of the first recruiter. To measure the outgoing flow, we counted the total number of ants leaving the nest during a period of either 30 seconds or 3 minutes. We also assessed ants' mobility in each nest chamber as a proxy for the spread of recruiting stimuli, before and during the recruitment to food. To this aim, during short periods of 10 seconds, we counted the number of ants that were moving in each chamber, 20 seconds before the arrival of the first recruiter ant as well as 30 seconds, and 3 minutes after its arrival. An ant had to walk for a minimum distance of half of its body length during that 10 seconds period to be considered as "moving".

Data analyses

Spatial organization of the colony

All statistical tests were done using the R software v3.3.1. (R Core Team 2018).

Data were analyzed on the seven colonies whose workers were well-settled in the artificial nest, with more than 30% of the worker population staying inside the nest. Two colonies that showed an abnormally low occupancy were discarded from the analysis. In order to investigate the effect of the

272   nest topology, time, and colony on the occupancy of chambers, we used the R package lme4 (*v1.1-21*;

273   Bates et al. 2015) to fit a linear mixed model (LMM). In this model, the colony and the topology were

274   treated as categorical variables, whereas the day was treated as a continuous variable. The colony was

275   specified as random effect whereas the topology and the day were specified as fixed effects. In order

276   to detect a possibly faster occupancy for certain nest topologies, we added the interaction effect

277   between the day and the nest topology to our model. The significance of our variables was tested using

278   the two-tailed Wald X^2-tests. When significant, pairwise comparisons were made by using the Tukey

279   test.

280   To compare the homogeneity of nest occupancy, we carried out a permutation test on the values of

281   chamber occupancy after the ants had settled in the nest (the average daily occupancy index at day 7).

282   In the test, the empirical values of chamber occupancy for each nest and for each colony were

283   randomly permuted across the chambers of the same nest and the same colony to generate a new set

284   of values in which the total number of ants and their heterogeneous distribution across different

285   chambers were preserved, but the identity of the chambers was lost. We extracted 1000 such

286   permutation samples that were used to calculate chamber occupancy indices, averaged across all

287   samples for each particular nest topology. The distribution of these average values gave us the 95%

288   confidence intervals (CI) that allowed us to spot chambers with significantly different daily occupancy

289   indices. Furthermore, by comparing the width of the CI between the three nest topologies, we were

290   able to determine which topology favored a homogeneous occupancy of its chambers. Finally,

291   Spearman tests were performed in order to detect a correlation between the chamber's level of

292   occupancy by the ants and the distance to the entrance value as well as the connectivity degree of

293   these chambers.

294   Spearman tests were also performed between the level of chamber occupancy by the queen and the

295   distance to the entrance or the connectivity degree of that chamber. We used generalized linear mixed

296   models (GLMM, R package lme4) with binomial distribution to assess whether the probability of the

297   queen to leave a chamber was influenced by nest topology and by the number of workers in this

298   chamber. In the model, the colony and the topology were treated as categorical variables, whereas

299   the number of workers staying in the chamber with the queen was treated as a discrete variable. The

300   colony was specified as a random effect, while the topology and the number of workers were specified

301   as fixed effects. Fixed effect significance was calculated using the two-tailed Wald X^2-tests, and the

302   pairwise comparisons were done with the Tukey method. The impact of nest topology on the

303   distribution of brood across chambers as well as the relative occurrence of brood relocation were

304   analyzed using X^2 tests.

305   Recruitment of food

306   Data analyses on food recruitment were performed on eight of the nine colonies since one colony
307   showed an abnormally low nest occupancy at the time of food recruitment, with less than 30% of the
308   worker population staying inside the nest. By using a Spearman correlation test, we related the number
309   of ants in the entrance chamber to the flow of workers exiting the nest after the arrival of the first
310   recruiter. In order to see if nest topology influenced the flow of outgoing ants, we used a GLMM with
311   a Poisson distribution. In this model, the nest topology and the colony were both treated as categorical
312   variables. The colony was specified as a random effect, whereas the topology was set as a fixed effect
313   whose significance was calculated using the two-tailed Wald X^2-tests followed by pairwise comparisons
314   using Tukey method. Similarly, the effect of nest topology on the proportion of moving ants inside the
315   nest was assessed using a GLMM with binomial distribution.

316   Finally, for each nest topology, we identified the chambers in which workers were significantly
317   activated by the recruitment signal. To this aim, we used a GLMM with a binomial distribution
318   considering the chamber as a fixed effect and the colony as a random effect. If the "chamber" had a
319   significant effect (two-tailed Wald X^2-tests), we then performed pairwise comparisons with the Tukey
320   method to spot chambers within a significantly different proportion of moving workers.

321

322

323 Results

324 Spatial organization of workers inside the nest

325 The topology of nest chambers had a significant impact on the overall level of nest occupancy by ant

326 colonies (LMM: X^2_2=20.58, P<0.0001). Indeed, when looking at the whole week of experimentation,

327 the "star" topology with a central hub housed on average more ants than the "spiral" and the "trident"

328 topologies (Post hoc comparisons with Tukey method: star-trident P=0.0001 | star-spiral P=0.0047 |

329 trident-spiral P=0.47) (Fig. 3). Moreover, the occupation of the nest was influenced by the number of

330 days that the colonies spent in the setup, as gradually more ants were counted inside as time passed

331 (LMM: X^2_1=50.47, P<0.0001). However, no topology promoted a significantly faster occupancy of the

332 nest as there was no interaction effect between nest topology and time on the nest occupancy (LMM:

333 X^2_2=1.91, P=0.38).
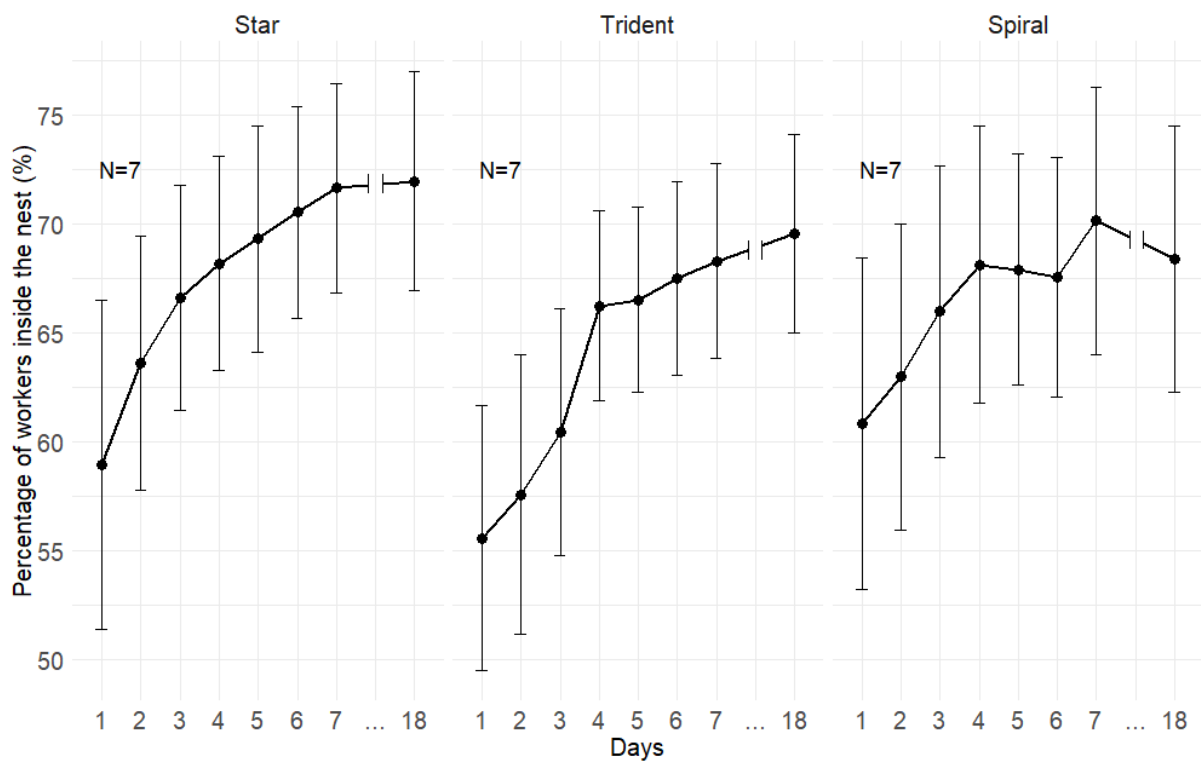
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335 **Fig. 3** Occupancy dynamics of *Myrmica rubra* colonies in the three different nest topologies for 7 days and on the 18th day.

336 Each point is a mean percentage of nest occupancy, based on the daily occupancy indices of seven replicates (N=7). The

337 represented error bars are the standard error associated with each mean percentage

338 On day 7, the settlement of ants was stabilized since for each topology the percentage of housed

339 workers did not differ from the ones observed on day 18 (Paired Wilcoxon-test: star: W = 12, *N*=7,

340 *P*=0.81 |trident: W = 14, *N*=7, *P*=1.0|spiral: W= 24, *N*=7, *P*=0.11). The average percentages of ants

341 occupying each nest chamber were compared to the expected value of 14.3%, this latter value

corresponding to a perfectly homogeneous distribution of all the ants being equally split between the seven chambers (Fig. 4). On the last day of the occupancy dynamics (day 7), we observed the most homogeneous distribution of ants across all the chambers in the "star" topology, as shown by the width of its 95% confidence intervals (Permutation test-star: CI-= 12.2, CI+= 16.7, Width-CI= 4.6, see Online Resource 2 for the results in a table). The most heterogeneous occupancy of nest chambers was observed for the "spiral" topology (Permutation test-spiral: CI-= 10.2, CI+= 18.2, Width-CI= 8.0). In particular, the average occupancy of the entrance chamber in the "spiral" topology was four times greater than the average occupancy of the most remote chamber (i.e., chamber C for "spiral" topology on Fig. 1). The spatial distribution of the workers in the "trident" topology showed an intermediate level of heterogeneity across nest chambers (Permutation test-trident: CI-= 10.9, CI+= 17.5, Width-CI= 6.5).
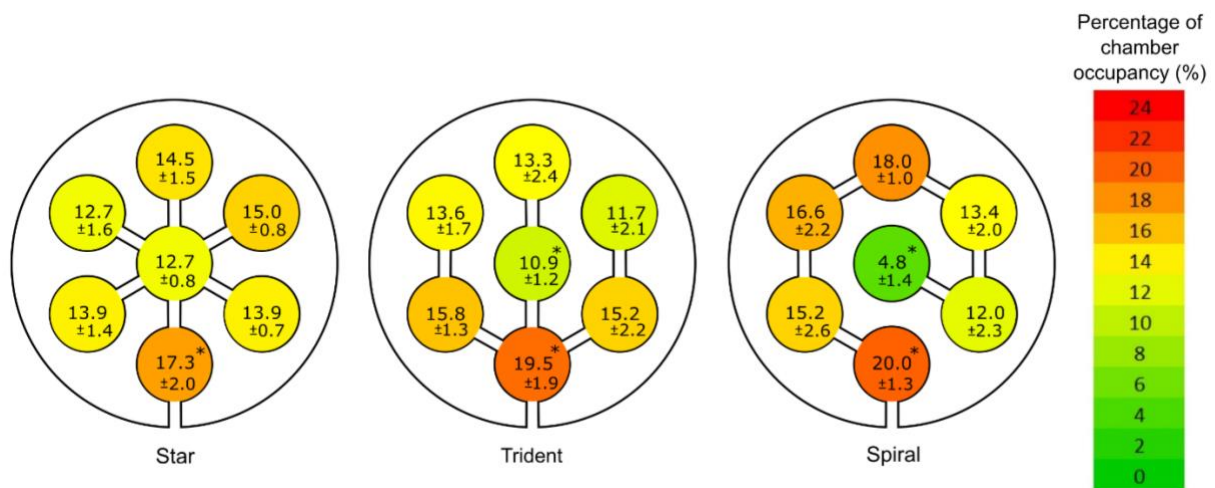

Fig. 4 Percentage (mean ± standard error) of *Myrmica rubra* workers located in a given chamber over the total ant population hosted inside the nest on day 7. The color-scale enables to visualize how ants are distributed inside the nest. In the case of a perfectly homogenous distribution, the average expected occupancy per chamber is 14.3% as there are 7 chambers (100% / 7 = 14.3%). Proportions colored in yellow are close to this expected average whereas proportions colored in red and green are higher and lower respectively. Mean numbers with (*) are values outside of the 95% CI of the permutation test

For all the tested topologies, the mean percentage of workers standing in the entrance chambers (E chamber Fig. 1) on day 7 were always significantly higher than the upper confidence interval obtained from the permutation test (Fig. 4) (star: mean=17.3+2.0, CI+=16.7 |trident: mean=19.5+1.9, CI+=17.5 |spiral: mean=20.0+1.3, CI+=18.2). On the contrary, the central chamber from the "spiral" topology, which was the most distant from the entrance, as well as the central chamber from the "trident" topology, were significantly less occupied (trident: mean=10.90+1.2; CI-=10.93 |spiral: mean=4.8+1.4; CI-=10.2).

366   In the "spiral" and the "trident" topologies, the number of *M. rubra* workers decreased in chambers

367   that were located further from the nest entrance (Fig. 5). Indeed, when looking at the first day of

368   settlement, we observed a significant negative correlation between proportion of ants in a chamber

369   and its distance to the entrance except for the "star" topology (Spearman test – day 1: star: r_s = -0.24,

370   *N*=49, *P*=0.098 |trident: r_s = -0.40, *N*=49 *P*=0.0048 |spiral: r_s = -0.66, *N*=49, *P*<0.0001). Distance to the

371   entrance still influenced the local occupancy of nest chambers until the end of the experiment for the

372   "spiral" topology but had no longer a significant impact for the "trident" and for the "star" topologies.

373   (Spearman test – day 7: star: r_s = -0.11, *N*=49, *P*=0.46 |trident: r_s = -0.28, *N*=49, *P*=0.053 |spiral: r_s = -

374   0.59, *N*=49, *P*<0.0001). The "star" topology showed the less steep decrease of chamber occupation

375   with distance to the entrance (Fig. 5) suggesting that this topology facilitated ants shifting across

376   chambers. Throughout the week of experiment, the influence of the distance to the entrance on the

377   distribution of workers was most important in the "spiral" topology which is the topology that offers

378   the largest possible values of distances to the entrance.

379   We found no relation between the connectivity degree of the chambers and the proportion of workers

380   residing in them except for the "trident" topology which showed a positive correlation on the first day

381   of the experiment (Spearman test – day 1: star: r_s = -0.09, *N*=49, *P*=0.53 |trident: r_s = 0.40, *N*=49,

382   *P*=0.005|spiral: r_s = 0.04, *N*=49, *P*=0.76). On the last day, that tendency was no longer significant for

383   the "trident" (Spearman test – day 7: star: r_s = -0.24, *N*=49, *P*=0.092 |trident: r_s = 0.28, *N*=49,

384   *P*=0.053|spiral: r_s = 0.09, *N*=49, *P*=0.54). It is worth noticing here that in the "trident" topology the

385   connectivity degree of a chamber is inversely proportional to the distance to the entrance (Fig. 1).

386   Consequently, for this nest it is not possible to disentangle the impact of these two network

387   parameters on chamber occupancy.
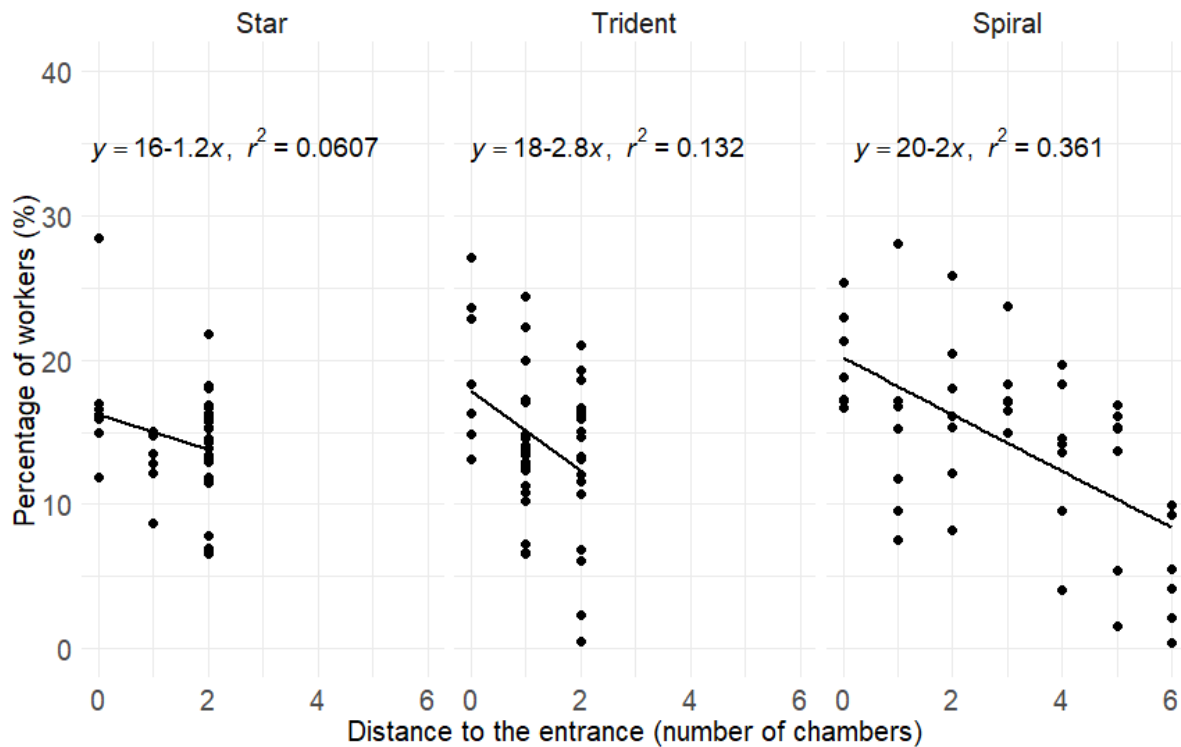
Fig. 5 Percentage of *Myrmica rubra* workers inside a nest chamber on day 7 as a function of its relative distance to the entrance. Each dot represents the percentage of ants in a given chamber over the total ant population inside the nest that was averaged over the 12 observations made at day 7. The trend line is the linear regression of the data with its corresponding equation. The distance of the chamber to the entrance (expressed as number of nodes) ranges from 0 to 2 for the "trident" and the "star" topologies and from 0 to 6 for the "spiral" topology

Spatial localization of the queen and larvae inside the nest

For each nest topology, every chamber was occupied at least once by the queen when considering all the observations made on the seven tested colonies (Fig. 6). The least occupied chamber by the queen was the terminal chamber of the "spiral" nest where the queen was recorded on 1.7% of all observations for that nest (N=588). The chamber in which the queen was found most often was the entrance chamber of the "trident" nest where the queen was recorded on 40.8% of all observations.

We found no correlation between the distance to the entrance of a chamber and its level of occupancy by the queen, on the first day of experiment (Spearman test – day 1: star: r_s = -0.03, N=49, P=0.86 |trident: r_s = -0.18, N=49, P=0.20|spiral: r_s = -0.26, N=49, P=0.066). On the last day, however, we found a negative correlation in the "trident" topology (Spearman test – day 7: star: r_s = 0.13, N=49, P=0.36 |trident: r_s = -0.47, N=49, P=0.0007|spiral: r_s = -0.26, N=49, P=0.07).

We found no correlation between the connectivity of a chamber and its level of occupancy by the queen, on the first day of experiment (Spearman test – day 1: star: r_s = -0.13, N=49, P=0.38 |trident: r_s = 0.18, N=49, P=0.20|spiral: r_s = -0.03, N=49, P=0.81). On the last day, however, we found a negative

17

correlation in the "trident" topology (Spearman test – day 7: star: r_s = -0.12, N=49, P=0.41 | trident: r_s = 0.47, N=49, P=0.0007 | spiral: r_s = 0.12, N=49, P=0.42).
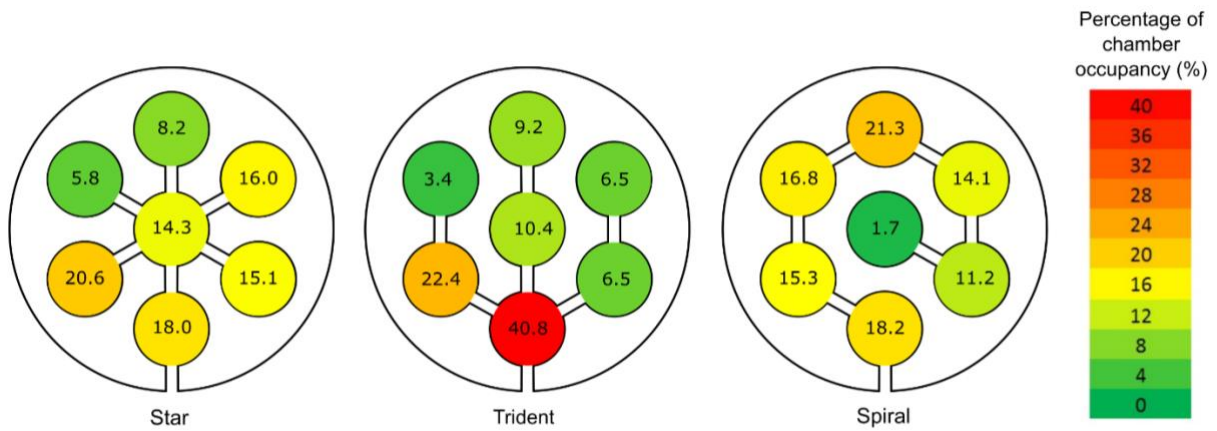

Fig. 6 Percentage of chamber occupancy by the *Myrmica rubra* queen for each nest topology. The percentage is given by the number of occurrences of queen presence in a chamber over 588 observations (12 observations per day for 7 days and for 7 colonies)

In each colony, the queen frequently moved from one chamber to another throughout the day. The probability for the queen to relocate significantly differed between nest topologies (GLMM: X^2_2=11.68, P=0.0029). The queen's position was less stable in the "star" topology as she moved from one chamber to another on average 2.7 ± 2.1 times per day, compared to 1.7 ± 2.1 in the "trident" and 2.0 ± 1.8 in the "spiral" (Post hoc comparisons with Tukey method: star-trident P=0.004 | star-spiral P=0.036 | trident-spiral P=0.72). Furthermore, we found that the number of workers standing in the chamber was the explanatory variable from the generalized linear mixed model (GLMM: X^2_1=15.61, P<0.0001), the queen being more likely to leave sparsely populated chambers (Fig. 7). When staying in a chamber with very few workers (at most 4 workers), the queen had a probability of 0.4 to be observed in another chamber the next hour whereas this probability decreased to less than 0.05 in a populous chamber (with 17 to 20 workers).

With respect to larvae, on the last day of the experiment, brood could be located in a single chamber (5 out of 21 experiments) but was most often split between two chambers (8 out of 21 experiments) or even between three to five chambers (8 out 21 experiments). The nest topology did not influence the number of chambers in which larvae were staying (Khi^2 test: X^2_2=1.4, P=0.497). Moreover, in each of the three tested nest designs, the queen was observed in the same chamber as larvae in four out of the seven replicates. The occurrence of brood relocation was not influenced by the nest topology (Khi^2 test: X^2_2=1.376, P=0.503). Over the course of the last day, larvae were scarcely moved from one chamber to another. In the "star", "trident," and "spiral" topology, we observed respectively only 4, 5

433   and 2 relocation events among the 49 observed chambers (seven nest chambers observed during

434   seven replicates).
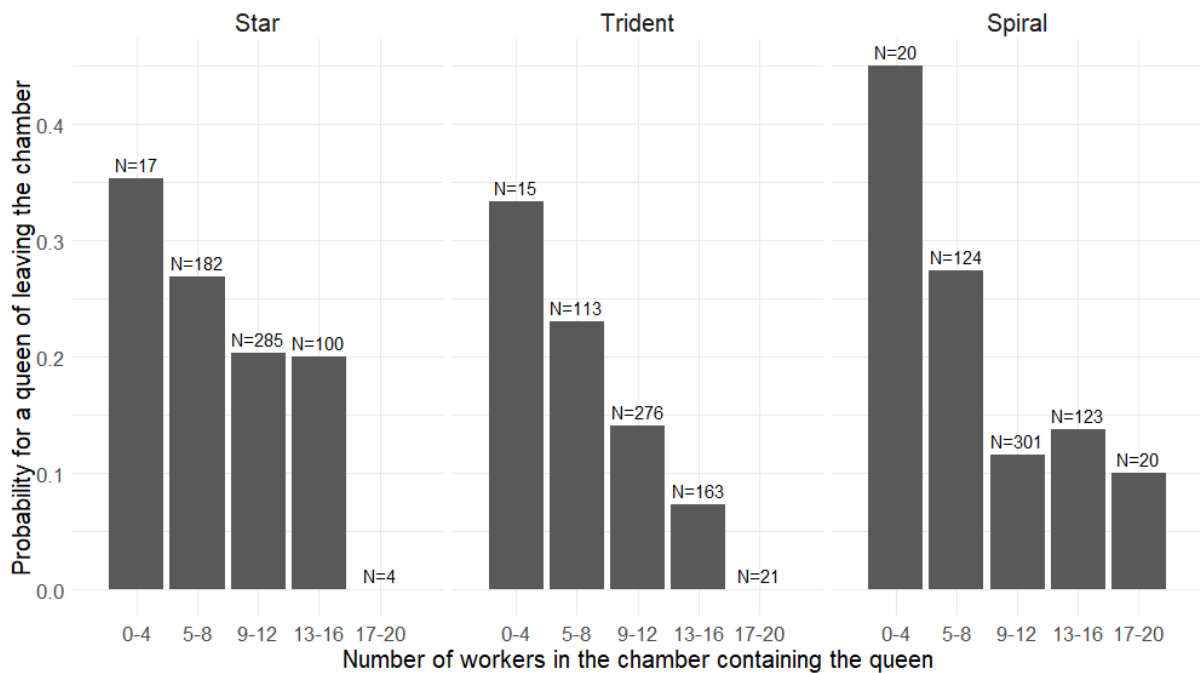
435

436   **Fig. 7** Probability for *Myrmica rubra* queen of leaving a nest chamber as a function of the number of workers in this chamber

Recruitment to food

438   We found a significant influence of the nest topology on the outgoing flow of ants from the nest during

439   the 30 seconds that followed the arrival of a recruiter ant inside the nest (GLMM: X^2_2=6.64, P=0.036).

440   The mean outgoing flow was the lowest in the "star" topology and was significantly different from the

441   one observed in the "spiral" nest (Fig. 8; Pairwise–test - Tukey method: star-spiral P=0.039 | star-

442   trident P=0.71 | trident-spiral P=0.21). Intermediate values of ant flows were observed in the "trident"

443   nest. After 3 minutes, the outgoing flow of workers tended to even out as no nest topology significantly

444   differed (GLMM: X^2_2=0.38, P=0.82). One can however notice that the average outgoing flow of ants

445   was the lowest in the "star" topology at all times. Furthermore, a higher number of workers in the

446   entrance chamber did not result in higher numbers of recruited ants. Indeed, we found that the

447   number of ants standing in the entrance chamber did not influence the outgoing flow of ants 30

448   seconds after the arrival of the first recruiter ant (Spearman test: r_s = -0.17, N=24, P=0.44) nor 3

449   minutes after (Spearman test: r_s = -0.10, N=24, P=0.64).

450
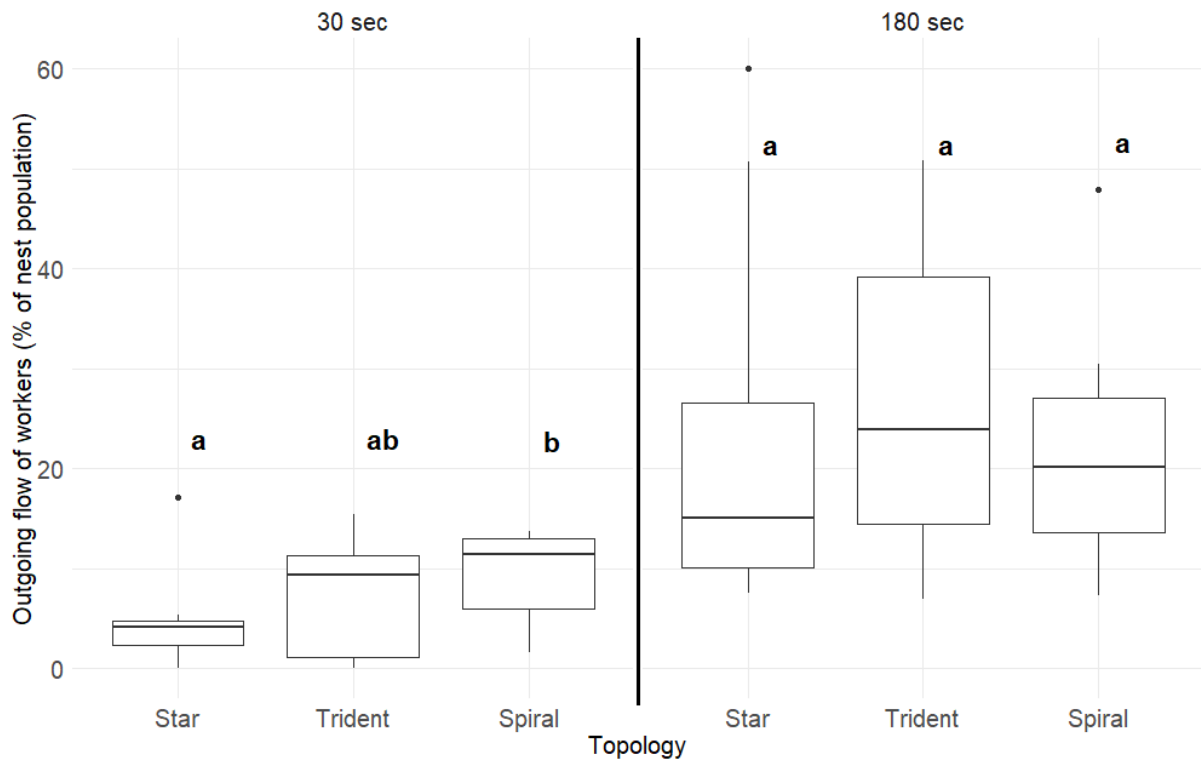
451

452 **Fig. 8** Cumulative outgoing flow of *Myrmica rubra* ants, 30 and 180 seconds after the arrival of the first recruiter. The outgoing
453 flows were expressed in percentages given by the cumulated numbers of outgoing foragers over the total number of ants
454 present inside the nest. N=8 per nest topology. Post-hoc pairwise comparisons were made using the Tukey method. Boxplots
455 sharing a common letter for a given time, are not significantly different

456 Before the arrival of a recruiter, the total percentage of moving ants did not differ between the three
457 topologies (Fig. 9; GLMM: X^2_2=4.67, P=0.097). Furthermore, the percentage of active ants did not differ
458 across chambers from a same nest (GLMM: star: X^2_6=7.03, P=0.32 |trident: X^2_6=4.14, P=0.66 |spiral:
459 X^2_6=2.87, P=0.82). However, the arrival of a recruiter ant induced, after 30 seconds, an increase in the
460 total percentages of ants in movement, which differed between nest topologies. (Fig. 9; GLMM:
461 X^2_2=7.12, P=0.028). The "star" and the "trident" nests showed respectively the lowest and the highest
462 total percentage of activated ants while the spread of recruiting stimuli was intermediate in the "spiral"
463 nests (Pairwise–test - Tukey method: star-spiral P=0.699 | star-trident P=0.028 | trident-spiral P=0.18).
464 After 180 seconds, the three nest topologies no longer differed by their total percentages of moving
465 ants (GLMM: X^2_2=0.16, P=0.925). Furthermore, in each nest topology, the percentage of moving ants
466 in a given chamber differed across nest chambers at 30 seconds (GLMM: star: X^2_6=46.90, P<0.0001
467 |trident: X^2_6=70.67, P<0.0001 |spiral: X^2_6=69.46, P<0.0001) and at 180 seconds after the arrival of the
468 first recruiter (GLMM: star: X^2_6=37.08, P<0.0001 |trident: X^2_6=31.02, P<0.0001 |spiral: X^2_6=35.18,

20

469   *P*<0.0001). In all cases, the percentage of active ants was higher in the entrance chamber than in the

470   other ones (see Online Resource 3 for each pairwise comparisons across all chambers).
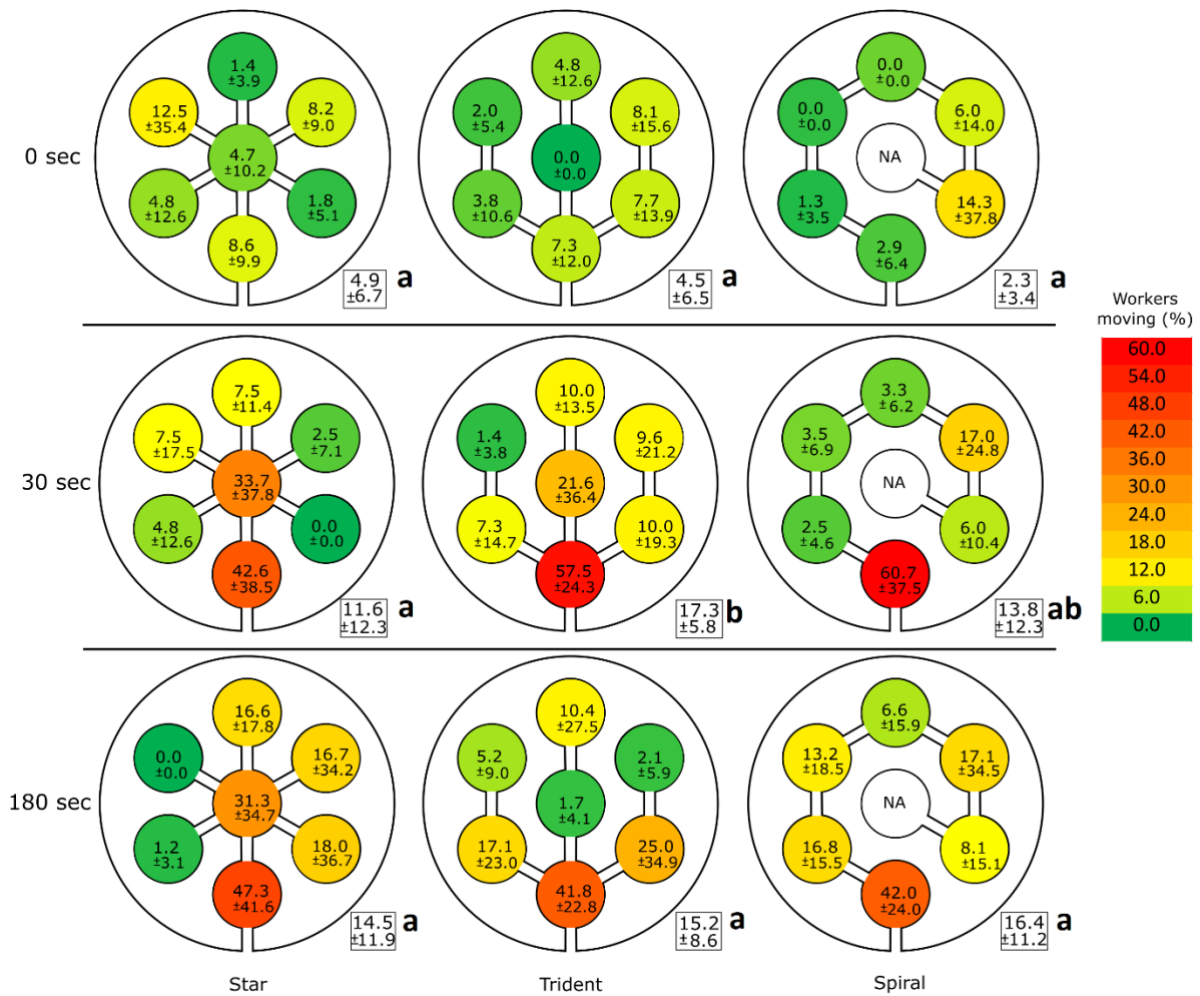
Fig. 9 Percentage of moving *Myrmica rubra* ants observed 0, 30 and 180 seconds after the arrival of the first recruiter ant. The values in each nest chamber are the percentages of moving ants ± SE out of the number of workers present in this chamber. Percentages of moving ants are calculated only when at least two ants were present in a chamber. NA: Non-available data when less than two ants were present in the chamber for more than two replicates. N=8 replicates per nest topology. The total percentages of moving ants in the whole nest are written in the square boxes at the bottom right of each nest and post-hoc pairwise comparisons were made using the Tukey method. The percentages of moving ants in the whole nest are not significantly different if they share a common letter at a given time

Discussion

The spatial organization of *M. rubra* colonies within a nest is highly dependent on its topology. Environmental parameters such as the availability of food (Mailleux et al. 2011) or the weather/seasonality (Porter & Tschinkel 1993; Caut et al. 2013; Murdock & Tschinkel 2015) are already known to impact the localization of workers inside and outside the nest. However, our study indicates that, even in a homogeneous environment, the nest topology alone is enough to influence the level of nest occupancy as well as the distribution of workers among nest chambers.

A nest topology with a highly connected chamber acting as a hub (i.e. the "star" topology) promoted a significantly greater occupancy of the whole nest. However, at a local scale, the connectivity degree of one chamber was not correlated to the number of ants housed in the chamber. In fact, the highly connected central hub of the "star" topology had a very high connectivity degree of 6 and yet it housed the same number of ants as the five terminal chambers with a lower connectivity degree of 1. The "star" topology favored a more homogeneous distribution of the workers across all the chambers. Furthermore, the little impact of distance to the entrance on the occupation of nest chambers as well as the high mobility of the queen observed in the "star-shaped" nest suggests that this topology favors exchanges and movements of nestmates between chambers. Interestingly, this homogeneous distribution of workers parallels the homogeneous distribution of network parameters: five of the seven chambers in the "star" topology were characterized by the same connectivity degree as well as the same distance to the entrance or to the other nodes.

In contrast, the two nest topologies that showed series of successive chambers (i.e. one series for the "spiral" and three series for the "trident" topology) led to more heterogeneous patterns of chambers' occupancy. In the case of the "trident" topology, the occupancy of one chamber in each branch seemed to depend not only on its topological location but also on the geometry of the whole nest structure: ants were more likely to occupy the lateral branches of the "trident" topology. This geometric effect could possibly result from the thigmotactic tendency of ants to walk along the walls to reach deeper nest parts.

Our data also revealed that a single series of successive chambers, as for the "spiral" topology, favored a heterogeneous spatial distribution of workers, with the number of ants decreasing in chambers that were the most remote from the nest entrance. These results differed from field data on the vertical distribution of ants inside natural nests as it can be obtained from wax or dental plaster casts (Tschinkel 2010)-that showed lower ant densities in the upper chambers close to the nest openings (Tschinkel 2004; Murdock & Tschinkel 2015). These lower ant densities could, however, be partially explained by the top-heavy structure of the studied ant nests, which were characterized by larger and more

numerous chambers excavated in the upper nest regions. Furthermore, when considering the number of workers (instead of their density), the upper half part of the nest hosted the majority of the ant population. Finally, in these field studies, ant workers positionned themselves not only according to nest topology but also in response to temperature (Ceusters 1986), light (Pamminger et al. 2014) or even carbon dioxide levels (Hangartner 1969; Kleineidam 1999 but see Tschinkel 2013), which vary from the entrance until deeper nest chambers. Because of the small size of artificial nests used in our study and the strictly controlled laboratory conditions, variations in temperature, humidity or CO_2 level were limited inside the nest interior and unlikely to play a major role on the spatial distribution of workers nor on their clear-cut preference for the entrance chamber in all nest topologies. This suggests that workers tend to aggregate near the entrance, and indirectly, that workers can detect proximity to the entrance without necessarily relying on sensing environmental gradients. The distance that they cover inside the nest when returning from foraging or their rate of encounters with returning foragers are some of the possible cues that they could use.

As regards the impact of nest topology on the location of the ant queen, she was most frequently present in the chamber close to the entrance. The entrance chamber was the most occupied by the queen in the "trident" topology and the second most occupied chamber in the "star" and the "spiral" topologies. This is quite surprising knowing that selection for disease control is believed to have shaped the spatial organization of insect societies in a way that mitigates risks for pathogens' transmission (Stroeymeyt et al. 2014). This "organizational immunity" implies that key-individuals and susceptible colony members such as the queen or the larvae should stay further from the entrance (Varoudis et al. 2018) so as to reduce encounters with potential pathogen vectors like foragers (Mersch et al. 2013). In natural nests, queens were often observed in deeper parts of the nest and/or in areas of high density of workers (Tschinkel 1993; Walin et al. 2001). The preferred location of the queen in the entrance chamber of artificial nests suggests that a high density of nestmates for grooming and humidity conservation could outweigh the higher sanitary risks associated with interactions with foragers.

The queen was very mobile and ended up visiting chambers in response to the presence of workers. Indeed, queen relocation from one chamber to another were more frequent in the "star" topology. This may be a consequence of the homogeneous distribution of workers throughout the chambers for this nest topology, leading to evenly attractive chambers from the queen's perspective. Furthermore, we found that the queen preferred chambers that were highly populated regardless of their topological properties. The tendency of a queen to stay in the most occupied chambers relates to a well-known phenomenon in gregarious insects where the probability of leaving a group decreases non-linearly with the group size (e.g. in cockroaches (Jeanson et al. 2005), crickets (Sword et al. 2005) or ants (Deneubourg et al. 2002; Tennenbaum et al. 2016). This decision rule adds to the ability of ant

547   queens, including queens of *M. rubra*, to produce attractant pheromones (Vienne et al. 1998), that

548   trigger and maintain the aggregation of ant workers around them. A high density of workers also

549   implies more mutual grooming as well as a more thorough sanitization of the chamber, which both

550   reduce transmission rates of pathogens (e.g. in leaf-cutting ants, Hughes et al. 2002).

551   Because spatial proximity is a prerequisite for ants to interact, the entrance chamber that harbors most

552   encounters between outside foragers and inner-nest workers plays a key role in the emergence of

553   collective foraging behavior (Greenee et al. 2013; Pinter-Wollman et al. 2013; Davidson & Gordon

554   2017). On the one hand, we found that the number of *M. rubra* workers in the entrance chamber did

555   not influence the speed of recruitment. On the other hand, we noticed that the highest number of

556   moving ants 30 seconds after the return of a recruiter was observed in the "trident" topology for which

557   the entrance chamber showed the highest level of connectivity. These two findings somehow

558   corroborate previous studies made on the harvester ant *Veromessor andrei* (Pinter-Wollman 2015)

559   where the connectivity of the entrance chamber had a greater impact on the recruitment speed than

560   the number of workers this chamber can potentially hold. Indeed, as the connectivity of the entrance

561   chamber increases, so does the number of locations from which ants can be aroused and mobilized

562   for foraging. As regards the average distance of nest chambers to the entrance, it did not influence the

563   recruitment intensity. Indeed, neither the outgoing flow of foragers nor the total number of moving

564   ants differed between the "trident" and the "spiral" nest, even though these two topologies showed

565   respectively the lowest and the highest average distance to the entrance. This finding could be

566   explained by a propagation of information mostly limited to the entrance chamber which showed the

567   highest percentage of moving ants stimulated by the arrival of successful recruiting ants.

568   Interestingly, the "spiral" topology was the one that most facilitated the activation of deeper nest

569   chambers with at least 5% of the workers moving in every chamber 3 minutes after the first

570   recruitment events. This may result from the serial arrangement of chambers that canalizes the

571   recruitment signals along a single axis extending from the entrance chamber to the deepest one. The

572   "spiral" topology could promote a delayed, yet more efficient sharing of information and food

573   throughout the whole nest. Further studies should investigate the differential timescales at which a

574   given nest structure shows its optimal functionality. Indeed, a topology could be more functional on

575   long time scales because it optimizes food transfer within the whole nest while another topology could

576   be optimal on short time scales because it facilitates the rapid activation of workers and the

577   recruitment of many individuals to a new outside task.

578   Our study confirms that the nest topology alone affects the pattern of nest occupation by workers as

579   well as their activation in the context of foraging. This potentially opens new questions about the

580 adaptive value of different topologies. One can hypothesize that ant species relying on collective
581 foraging and on a strategy of food monopolization could benefit from a high level of connectivity of
582 near-surface nest chambers. Conversely, for small-sized population and/or ant species using signals of
583 poor recruiting efficiency, high connectivity between chambers may no longer be beneficial or may
584 even hamper any recruitment process to take place. Previous research on social insect nests has mainly
585 focused on the mechanisms of nest digging (Theraulaz et al. 2003; Buhl et al. 2006) and of nest size
586 regulation (Rasse & Deneubourg 2001; Buhl et al. 2004). Topology characterizes a whole set of nest
587 properties that are not captured by size descriptors alone. Our study confirms that topological features
588 of nests are important for understanding not only the mechanisms but also the function of insect-built
589 architecture. Together with previous studies, our work is now casting light on how the topology of nest
590 structures can shape the spatial organization of workers and can thus affect the efficiency of
591 information flow inside the nest. By extending our approach to the study of other ant species with
592 different social organizations and different ecology we will gain a better understanding of the
593 functional value of different nest architectures in relation to the species-specific modes of food
594 exploitation.

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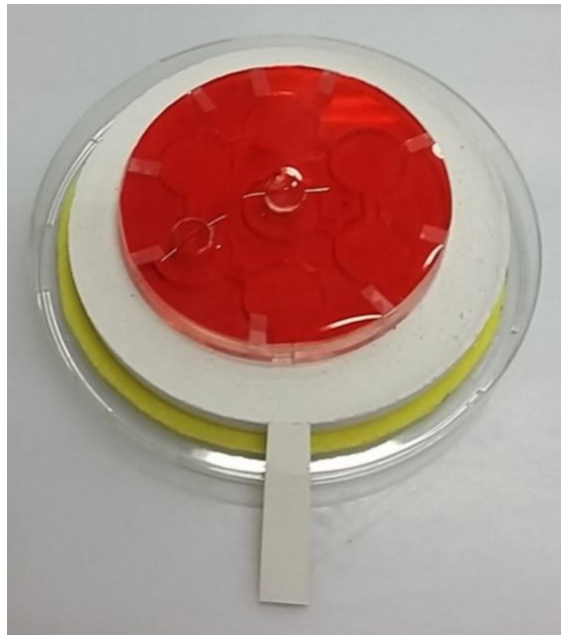
785   Supplementary

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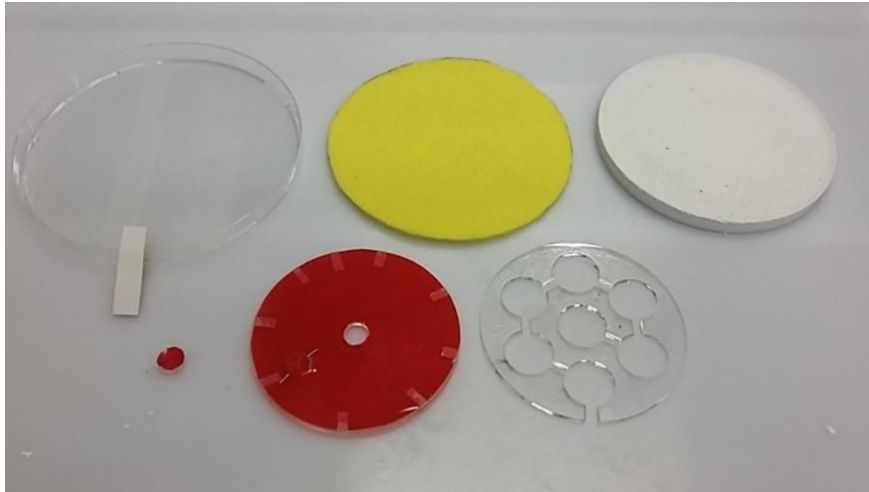
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788   Supplementary 1a: Separated components of the artificial ant nest. From Left to Right and Top to Bottom: Petri dish with
789   access ramp, water-absorbing fabric, plaster disc, ceiling plug, ceiling with red filter paper, Plexiglas nest.

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791

792   Supplementary 1b: Artificial ant nest with the spiral topology.

793

794

795 Supplementary 1c: Artificial nest with the star topology, occupied by *Myrmica rubra* ants. The queen, indicated by a black
796 arrow and characterized by a larger thorax, can be observed in a chamber.

797

Topology	95% Confidence Interval		
	Lower	Upper	Difference
Star	12.2	16.7	4.6
Trident	10.9	17.5	6.5
Spiral	10.2	18.2	8.0

798

799 Supplementary 2: Results from the permutation test performed on the occupation of the seven nest chambers by *Myrmica*
800 *rubra* ants for each nest topology separately, on day 7, for 1000 samples. The numbers express the expected percentage of
801 ants in each chamber.

802

| 30 seconds | | | | | | 180 seconds | | | | | |
| Star | | Trident | | Spiral | | Star | | Trident | | Spiral | |
pairs	p.value	pairs	p.value	pairs	p.value	pairs	p.value	pairs	p.value	pairs	p.value
B - BL	0.9859	B - BL	0.8005	B - BL	0.9998	B - BL	1.0000	B - BL	0.4087	B - BL	0.9606
B - BR	0.6450	B - BR	0.9999	B - BR	0.2120	B - BR	0.9999	B - BR	0.2233	B - BR	0.9989
B - C	0.0064	B - C	0.7890	B - C	1.0000	B - C	0.2276	B - C	0.5722	B - C	1.0000
B - F	**0.0003**	**B - F**	**0.0001**	**B - F**	**<.0001**	**B - F**	**0.0014**	B - F	0.2948	**B - F**	**0.0002**
B - FL	0.7896	B - FL	0.9859	B - FL	1.0000	B - FL	0.2553	B - FL	0.9971	B - FL	0.5235
B - FR	1.0000	B - FR	0.9988	B - FR	0.9963	B - FR	0.9989	B - FR	0.9981	B - FR	1.0000
BL - BR	0.9694	BL - BR	0.8970	BL - BR	0.4683	BL - BR	1.0000	BL - BR	0.9700	BL - BR	0.9995
BL - C	0.0116	BL - C	0.2237	BL - C	1.0000	BL - C	1.0000	BL - C	0.9996	BL - C	1.0000
BL - F	**0.0025**	**BL - F**	**0.0009**	**BL - F**	**<.0001**	BL - F	1.0000	**BL - F**	**0.0025**	**BL - F**	**0.0057**
BL - FL	0.9914	BL - FL	0.9731	BL - FL	0.9987	BL - FL	1.0000	BL - FL	0.6256	BL - FL	0.9798
BL - FR	1.0000	BL - FR	0.5616	BL - FR	1.0000	BL - FR	1.0000	BL - FR	0.1472	BL - FR	0.9821
BR - C	0.0053	BR - C	0.5381	BR - C	1.0000	BR - C	0.2968	BR - C	0.9996	BR - C	1.0000
BR - F	**0.0017**	**BR - F**	**<.0001**	**BR - F**	**0.0002**	**BR - F**	**0.0013**	**BR - F**	**0.0093**	**BR - F**	**0.0030**
BR - FL	1.0000	BR - FL	0.9993	BR - FL	0.2624	BR - FL	0.1629	BR - FL	0.3500	BR - FL	0.8747
BR - FR	1.0000	BR - FR	0.9756	BR - FR	0.6574	BR - FR	0.9881	BR - FR	0.1120	BR - FR	0.9996
C - F	0.9989	C - F	0.0054	C - F	1.0000	C - F	0.6092	C - F	0.0537	C - F	1.0000
C - FL	0.0113	C - FL	0.2278	C - FL	1.0000	**C - FL**	**0.0081**	C - FL	0.7279	C - FL	1.0000
C - FR	1.0000	C - FR	0.9357	C - FR	1.0000	C - FR	0.1696	C - FR	0.3514	C - FR	1.0000
F - FL	**0.0040**	**F - FL**	**<.0001**	**F - FL**	**<.0001**	**F - FL**	**0.0003**	**F - FL**	**0.0244**	**F - FL**	**0.0315**
F - FR	1.0000	**F - FR**	**<.0001**	**F - FR**	**<.0001**	**F - FR**	**0.0031**	F - FR	0.5417	**F - FR**	**0.0035**
FL - FR	1.0000	FL - FR	0.8012	FL - FR	0.9904	FL - FR	0.5050	FL - FR	0.8757	FL - FR	0.7048

Supplementary 3: Results from the pairwise comparison of the percentage of *Myrmica rubra* ants moving between each nest chamber with the Tukey method. In **bold** are the significantly different chambers. Abbreviations for each chamber: F=Front (Entrance); C=Center; B=Back; FL=Front Left; FR=Front Right; BL=Back Left; BR=Back Right.