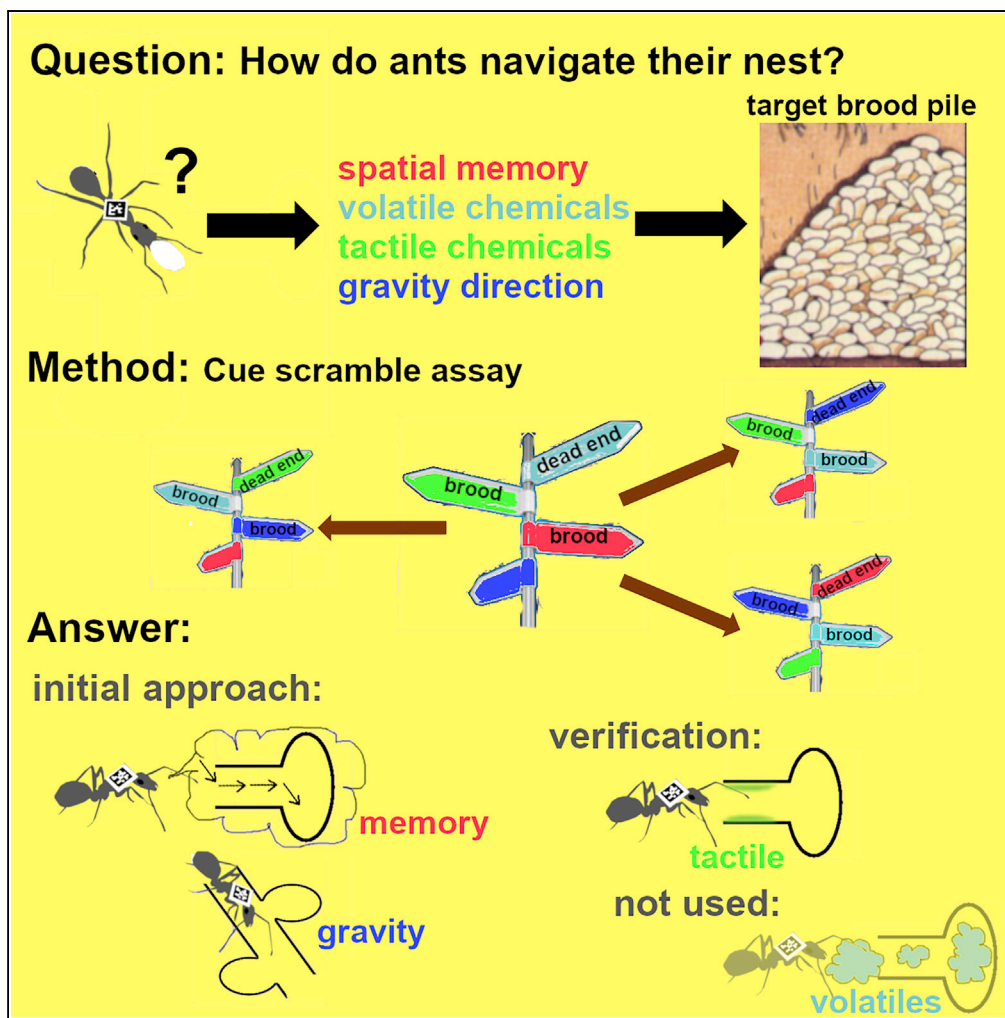


Article

Ants Use Multiple Spatial Memories and Chemical Pointers to Navigate Their Nest



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HIGHLIGHTS

We combine multiple technologies to study how ants navigate within their dark nest

Ants substitute visual cues with gravity, chemical cues, and multi-target memories

Following a catastrophe, ants quickly readjust the relative importance of cues

Heyman et al., iScience 14, 264–276
April 26, 2019 © 2019 The Authors.
<https://doi.org/10.1016/j.isci.2019.04.003>



Article

Ants Use Multiple Spatial Memories and Chemical Pointers to Navigate Their Nest

Yael Heyman,^{1,2} Yael Vilk,^{1,2} and Ofer Feinerman^{1,3,*}**SUMMARY**

Animal navigation relies on the available environmental cues and, where present, visual cues typically dominate. While much is known about vision-assisted navigation, knowledge of navigation in the dark is scarce. Here, we combine individual tracking, dynamic modular nest structures, and spatially resolved chemical profiling to study how *Camponotus fellah* ants navigate within the dark labyrinth of their nest. We find that, contrary to ant navigation above ground, underground navigation cannot rely on long-range information. This limitation emphasizes the ants' capabilities associated with other navigational strategies. Indeed, apart from gravity, underground navigation relies on self-referenced memories of multiple locations and on socially generated chemical cues placed at decision points away from the target. Moreover, the ants quickly readjust the weights attributed to these information sources in response to environmental changes. Generally, studying well-known behaviors in a variety of environmental contexts holds the potential of revealing new insights into animal cognition.

INTRODUCTION

Navigation is a major component in the adaptive and ecological success of any animal species. Different environments demand different navigational strategies as they vary in their resource distribution, the sensory cues they offer, and their topological structure. The vast majority of current knowledge concerns navigation above ground, which heavily relies on visual cues and often takes place in environments, either two- or three-dimensional, that allow for relatively unconstrained motion. Life, however, also inhabits subterranean environments. Navigation in these dark constrained conditions (Tschinkel, 2005; Kimchi et al., 2004; Chittka et al., 1999) is far less understood.

Ants have attracted special attention in the study of navigation. Different ant species exhibit exceptional navigational skills despite an extremely small brain size (Wehner, 2003; Knaden and Graham, 2016). This has allowed for an extensive study of ant navigational strategies, of the mechanisms that underlie ant navigation, and of its ecological costs and benefits (Knaden and Graham, 2016; Wehner, 2003; Collett et al., 1998; Müller and Wehner, 1988; Merkle and Wehner, 2008). Similar to other species, ants depend on visual cues for navigation to a great extent (Merkle and Wehner, 2008), even when walking along pheromone trails (Czaczkes and Beckwith, 2018; Aron et al., 1993) or during nocturnal activity (Warrant and Dacke, 2011; Narendra et al., 2017). Correspondingly, the vast majority of research on ant navigation concerns movement on the surface of the ground. This stands at odds with the fact that ants spend a considerable fraction of their lives within their nests (Heyman et al., 2017).

The navigational capabilities that ants display above ground do not stop at the nest entrance: ants have preferred locations within the nest (Sendova-Franks and Franks, 1995; Mersch et al., 2013) to which they return repeatedly (Heyman et al., 2017). However, many of the navigation strategies that ants employ above the ground cannot be expected to carry over to intranidal navigation. Light does not penetrate underground. This renders the prevalent strategies of visual beaconing (Wehner et al., 1996; Graham et al., 2003; McLeman et al., 2002) and image matching (Lent et al., 2010) useless. Moreover, celestial bodies, often used as global positioning cues in various navigation mechanisms, are inaccessible. Here, we study the cues that are available underground and the ways in which ants integrate them into their navigational decisions.

What sources of navigational information are accessible inside the ant nest? Gravitational signals may account for an ant colony's organization along the vertical axis (Tschinkel 1999, 2003, 2005; Tschinkel and Hanley, 2017), whereas magnetic sensation (Anderson and Vander Meer, 1993) could play a similar role in the horizontal direction. Chemical-encoded information is another possible source of navigational cues within the nest. Above ground such cues come in the form of pheromone trails (Holldobler and Wilson, 1990;

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<https://doi.org/10.1016/j.isci.2019.04.003>



David Morgan, 2009; Czaczkes et al., 2015), hydrocarbon gradients (Sturgis et al., 2011), and volatile chemical gradients (Steck et al., 2011; Buehlmann et al., 2012). The role of CO₂ soil gradients in colony organization was studied within natural nests (Tschinkel, 2013). Recently, it was shown that chemical navigational cues within the nest allow the ants to distinguish between different nest chambers (Heyman et al., 2017).

Spatial memory may also be useful within the dark confines of the nest. An appealing mechanism in this respect is path integration, a prevalent navigational strategy that was studied mostly above ground but could potentially remain efficient under it (Kimchi et al., 2004) because ants were shown to perform path integration, which includes vertical components (Wohlge-muth et al., 2001). Another possible mechanism is motor learning, wherein movement sequences are memorized (Stamps, 1995; Srinivasan and Zhang, 2004). Ants were shown to apply motor learning while navigating in mazes with no visual landmarks (Macquart et al., 2008). Such self-referenced mechanisms reduce the dependence on external reference points, which may be unavailable within the nest (Collett and Collett, 2000; Wehner, 2003; Jeffery, 2003). However, independence from external references has its limitations: path integration must be accompanied by other navigational mechanisms to avoid runaway errors (Merkle et al., 2006; Merkle and Wehner, 2009; Müller and Wehner, 1988), whereas motor learning requires practicing the same route many times (Stamps, 1995).

Ants combine private and social cues in a variety of contexts (Cronin, 2013; Robinson et al., 2009; Czaczkes et al., 2011). Social information, which is formed by the combined knowledge of many individuals, is often reliable and stable (Galton, 1907) yet slow to respond to environmental changes (Feldman et al., 1996). In contrast, private information, which is based on individual learning, has shorter update times but is error-prone (Merkle et al., 2006; Merkle and Wehner, 2009; Müller and Wehner, 1988). The latter source of information becomes crucial in situations of rapid environmental changes where social information is either missing or misleading (Harrison et al., 1989). These two information sources therefore complement one another to allow for organized and adaptive behaviors (Rieucan and Giraldeau, 2011; Templeton and Giraldeau, 1995).

In this article, we use the brood-retrieval behavior of the species *Camponotus fellah*, to study how ants navigate their nest. We do this by tracking the trajectories of ants as they move from a misplaced brood pile outside the nest to a target chamber within the nest. We analyze which cues play important roles in the different parts of this trajectory. We find that, to navigate within the nest, the ants combine three independent sources of information. First are self-referenced cues where the ants memorize multiple target locations and orient toward them with no requirement for any visual or olfactory cues. Second are socially generated chemical cues that are placed at decision points located away from the destination and mark the route toward it. Third, we show that ant navigation is assisted by global gravitational cues. We go on to show how ants combine these different information sources and how individuals can adjust the weight attributed to conflicting cues in a way that allows them to adopt new routes while abandoning unrewarding ones. This fast individual learning process leads to global, stable improvement in the collective performance of the colony.

RESULTS

Manipulating Nest Structure to Identify Relevant Navigational Cues

The ants' navigational capabilities were evaluated by following their performance in a brood-retrieval task. Experiments were initiated by placing a single pile of (≈ 50) brood items at a random location on the perimeter of the arena, outside the nest. Workers who encountered this misplaced brood tend to carry it into a nest chamber. To get from the arena to the nest chambers workers had to walk on the nest roof and climb down the entrance as the chamber section was embedded under the arena (see [Methods](#) section "Planar nest structure," [Supplemental Information](#) section "Nest setup," and [Figure S1](#)). To simulate the dark underground environment, the experimental setup was specifically designed to prevent the use of visual cues: all lights were in the infrared spectrum and nest corridors were sharply curved to block the line of sight. To verify that the ants carry the brood toward a designated goal within the nest we used an artificial nest that contains four symmetric corridors, three of which lead to identical chambers and one that leads to a dead end (see [Figure 1A](#) and [Methods](#) section "Planar nest structure"). Entries to the dead-end corridor by brood-carrying ants are defined as errors. We find that, in the absence of any manipulation, the proportion of errors in the brood retrieval task is extremely low $\left(\frac{\text{number of errors}}{\text{number of retrievals}} < 9 \times 10^{-3}, N = 794 \text{ retrievals}, p < 1 \times 10^{-100}, \text{ by the tail of the binomial distribution} \right)$. This establishes that brood-carrying

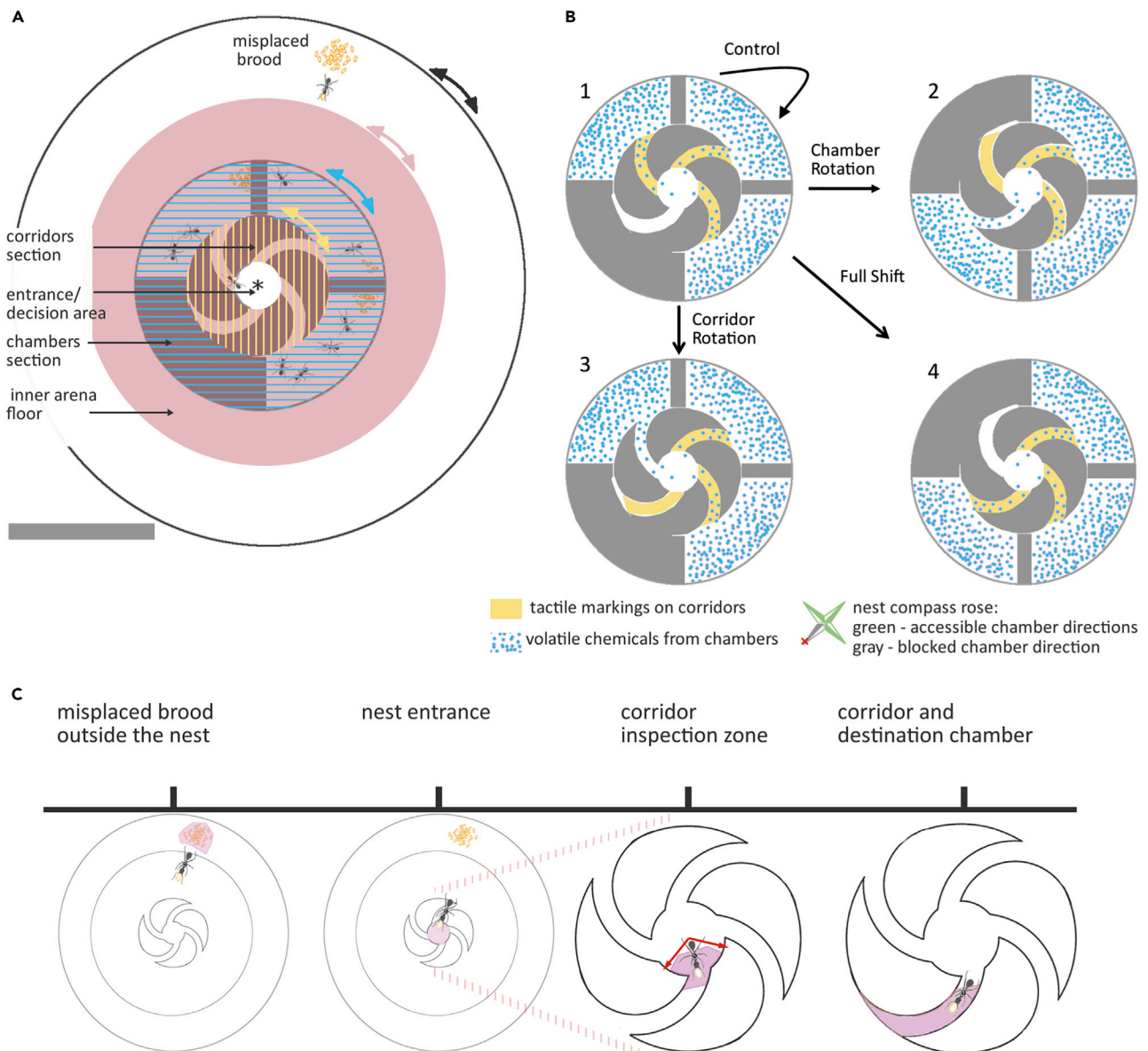


Figure 1. Experimental Setup and Manipulations

(A) Nest structure scheme. The nest is composed of four identical corridors that lead to three identical chambers and one blocked chamber. The chamber unit is marked with blue stripes; the corridor unit is marked with yellow stripes; the entrance to the nest, which is included in the corridor unit, is marked by a small white circle; and the decision point is labeled with an asterisk. The corridors and chambers were covered by an infrared (IR) filter top (marked in pink). The chamber unit, the corridor unit, and the IR filter, which forms the center of the arena floor can rotate with respect to each other and to the foraging arena (marked by yellow, blue, and pink arrows; see [Methods](#) section “Planar nest structure,” [Figure S1](#), and [Video S1](#)). The entire arena can also be rotated with respect to the laboratory frame of reference (marked by black arrow). Scale bar, 6.5 cm.

(B) Setup position under the different experimental manipulations and the resulting cue combinations. The blocked chamber is colored gray, and the corridor that originally led to it is white, whereas corridors that led to accessible chambers are colored yellow. Blue dots mark the possible presence of volatile chemicals emanating from accessible chambers into the entrance area. Compass rose signifies the spatial memory of the ants from the learning phase. The initial position of the setup is depicted in (B1). The four manipulations we employed are as follows. (B1) Control: the setup is rotated back and forth, retaining the original orientation of both corridor and chamber units, as well as the cue combinations. (B2) Chamber rotation: the chamber unit is rotated. (B3) Corridor rotation: the corridor unit is rotated. (B4) Full rotation: both the corridor and the chamber units are rotated, whereas their relative orientation is kept fixed. For more details refer to [Figure S2](#).

(C) Timeline of the locations that an ant visits as she retrieves brood into the nest.

ants do not randomly search for their destination chamber within the nest, but rather employ a reliable navigational strategy that takes them into specific corridors.

In our experimental setup, when a brood-carrying ant enters the nest, she immediately arrives at a decision point that is the junction between four corridors (Figure 1A, decision point is marked with an asterisk). Her decision to enter a specific corridor may be guided by one or several of the following cues: chemicals that are adsorbed to the surfaces of the corridor and sensed by direct tactile contact (*tactile*, solid at room temperature); volatile chemicals that diffuse away from a chamber (*volatile*, liquid or gas at room temperature); spatial memory, which reflects the ant's previous experience (*spatial memory*); and external cues such as the earth's gravitational or magnetic fields (*global*). To understand how the ants integrate these available cues toward reliable navigation (Wystrach et al., 2015; Wehner et al., 2016), we employed several confusion assays. We allowed the colony to return approximately half of the misplaced brood undisturbed (baseline phase) before applying one of several structural changes (test phase). These structural manipulations include independent rotations of one or more of the following parts of the setup: the chamber unit, the corridor unit (which includes the nest entrance), a large part of the arena floor, and the entire experimental setup (see Figure 1 and Video S1).

These rotations allowed us to isolate the effects of the aforementioned local cues (tactile, volatile, and spatial memory; Figure 1B, Supplemental Information section "Manipulation types and resulting cue combination," and Figure S2) as well as of the global cues (such as external magnetic fields). Gravitational cues were studied separately by using a vertical setup as described in section "Navigation in Vertical Nests."

To uncover the relevant cues and their relative importance we follow the complete trajectories of ants as they navigate from the misplaced brood to their destination within their nest. The structure of the Results section follows the timeline of this trip (see Figure 1C). Initially, an ant picks up a brood item from the misplaced brood and carries it to the nest entrance. After reaching the nest entrance, the ant approaches one of four identical corridors. Finally, the carrier ant enters one of the corridors and, eventually, the connected chamber where she places the brood.

From the Misplaced Brood Pile to the Nest Entrance

The earth's magnetic field or external air flows are examples of global cues that may assist ant orientation on her return trip to the nest. We tested the importance of global horizontal cues using the "arena rotation" manipulation, in which, halfway through the retrieval process, the entire experimental setup was rotated relative to the laboratory frame of reference ($N = 4$ experiments on two colonies). This manipulation maintains the links between the corridors, chambers, and any landmarks within the arena, but changes the orientation of these relative to the environment outside the setup. The spatial distribution of approaches relative to the arena frame of reference was unaffected by the manipulation ($p = 2.1 \times 10^{-3}$, $N = 4$ experiments, by the tail of the binomial distribution, see Figures 2A and 2B and Supplemental Information section "Arena rotation"). These results indicate that any horizontal directional cues that the ants may be using are confined to the experimental arena.

Arena-confined cues may come in the form of chemical cues, such as a pheromone trail that extends from the misplaced brood area through the nest entrance (Greene and Gordon, 2007; David Morgan, 2009; Czaczkes et al., 2015) and toward a specific direction within the nest. To test for the existence of such a trail, we rotated a large portion of the arena floor ("arena center rotation," shaded area in Figure 2C) so that it lost its initial alignment with the misplaced brood area. This manipulation had no effect on the paths that ants followed on their way back to the nest (Figure 2C), ruling out the use of a pheromone trail on the external part of the route.

By means of elimination our results point toward two potential strategies by which the ants find their way from the misplaced brood pile and back to the nest entrance. These strategies are, indeed, well established for ant navigation outside the nest: following a gradient of volatile chemicals that may emanate from the nest (Buehlmann et al., 2012) and self-referenced spatial memory cues such as path integration (Collett and Collett, 2000; Wehner, 2003; Jeffery, 2003).

Preliminary Orientation within the Nest Relies on Spatial Memory

When ants first enter the nest they do not simply continue on the straight path they took from the brood pile to the nest entrance (contrary to Macquart et al., 2006, see Supplemental Information section "Approach

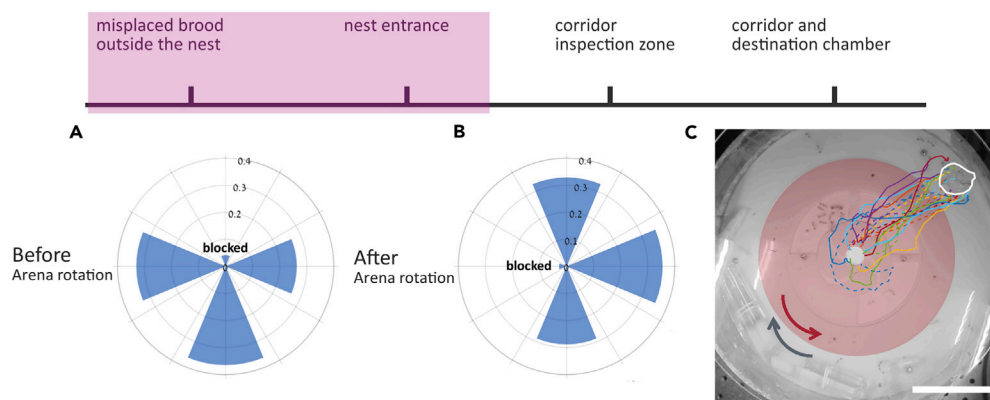


Figure 2. Navigating from the Misplaced Brood to the Nest Entrance

(A) Histogram of the directions of initial approaches before the manipulation of the “arena rotation” experiments. Data is normalized by the number of retrievals. The direction pointing up is that of the blocked chamber before the rotation. Only the first corridor approach on the first retrieval of each ant per experimental phase is included. All experiments are pooled together.

(B) A similar histogram of the directions of initial approaches after the “arena rotation” manipulation.

(C) Trajectories of ants that are retrieving brood from the brood pile (marked white) to the nest entrance before (solid trajectories) and after (dashed trajectories) an “arena center rotation” manipulation that rotated the central part of the arena floor. Scale bar, 9 cm.

direction has low correlation with entry angle” and [Figure S3](#)) but rather choose between one of four structurally identical corridors. At this point the ants cannot use the location of the external brood pile for orientation due to the dark conditions. Any deviation from random choice may rely on volatile chemicals that potentially emanate from inhabited chambers, tactile cues adhered to specific corridors, or spatial memory.

To study the relative importance of these cues, we analyzed the ants’ response to rotational manipulations that either shifted the overall nest orientation or disrupted the connection between internal nest units (see [Figure 1B](#)). We find that the spatial distribution of initial approaches (relative to the laboratory frame of reference) before and after a manipulation is remarkably similar regardless of the type of manipulation ([Figure 3A](#)). As manipulations alter tactile and volatile cues, this result raises the possibility that ants use prior spatial information when deciding which corridor to approach. To test this, we divided the corridors, from all possible manipulations, into two groups by their orientation before the manipulation: those that were oriented in a direction that led to an accessible chamber and those that were oriented in a direction that led to a dead end (directions are taken with respect to the laboratory frame of reference, see [Supplemental Information](#) section “Manipulation types and resulting cue combination”). We find that the approach rate to the first group is significantly higher ([Figure 3B](#), $p = 1.2 \times 10^{-2}$, z-test). Repeating a similar analysis for tactile and volatile cues (see [Methods](#) section “Rating cue importance”) did not yield any significant results implying that the ants do not rely on chemical cues when initially approaching a corridor. When considering combinations of cues, we also did not find a significant additive effect (see [Supplemental information](#) section “Additive effect of navigational cues”). This lack of dependence on environmental cues supports an assumption that the ants’ initial direction of approach is guided by self-referential, idiothetic mechanisms.

A well-established model of self-referential memory is path integration, which, in its most basic form, allows a navigator to calculate the distance and angle between its current position and an origin ([Müller and Wehner, 1988](#)). Under this model ants retrieving brood into the nest can be expected to return to the corridor through which they exited. To explore the possibility that the ants, indeed, apply basic path integration to approach a specific corridor we examined trajectories of brood-retrieving ants in non-manipulated nest structures. We find that on 52% (SEM = 1.8%, N = 763) of return trips, ants initially approach the corridor through which they exited the nest ([Figure 3C](#)). This is significantly higher than the 25% expected for a random approach direction ($p < 10^{-50}$, by the tail of the binomial distribution). This observation is consistent with a basic path integration model with single target memory. However, the remaining 48% of retrieval trips that deviate and approach a different corridor from the one they exited ([Figure 3C](#)) are not

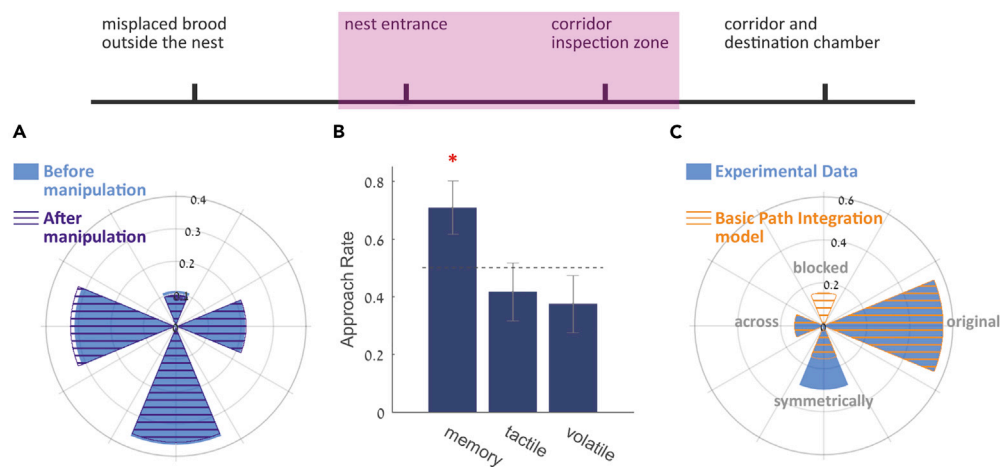


Figure 3. Preliminary Approach Guided by Spatial Memory

(A) Histograms of the directions of initial approaches (see also Figure S3) before (blue) and after (hashed purple) a manipulation for the four manipulation types, normalized to the total number of retrievals. The direction pointing up is that of the blocked chamber before the rotation. Only the first retrievals by ants that were outside the nest during the manipulations are included. All four experiment types are pooled together.

(B) Proportion of approaches to corridors carrying a positive cue, out of all approaches to corridors carrying either one or two positive cues. Only the first approach of the first retrieval of ants that were outside the nest during the manipulation (ants that have no knowledge of the postmanipulation nest structure) is included. * Indicates a proportion significantly different ($p < 0.05$) from the chance level of 0.5 (dotted line). Error bars signify SEMs.

(C) Distribution of angular difference between the direction of the corridor an ant left and the direction of the corridor she approached immediately afterward. Retrievals of ants who had just exited the blocked chamber, or the chamber across from the blocked chamber, were excluded. Approach directions are labeled as the direction of the “original” chamber that the ant had left, the direction directly “across” from this direction, the direction that leads to the “blocked” chamber, and the direction that leads to the accessible chamber that is placed “symmetrically” across from the blocked chamber. Expected random distribution is shown in orange (approaches divide equally between chambers symmetrically distant from the blocked chamber).

symmetrically distributed around the target direction as one would expect if the ant applied basic path integration. We find that deviations toward the blocked chamber were significantly lower than deviations toward a symmetrically placed accessible chamber and, in fact, almost altogether absent (Figure 3C).

Ants Integrate Chemical Cues at Close Range

We next tested which cues are employed in an ant’s decision to enter a corridor once she had approached it. We approximated the probability to enter an approached corridor by the measured ratio of entries to approaches and calculated this probability for every corridor in every experimental phase of the planar experiments depicted in Figure 1B. We averaged the resulting ratios across all corridors that share the same combination of cues (for example, positive tactile and volatile cues but not spatial memory). To rank the importance of the three cues (spatial memory, tactile, and volatile) we repeated the analysis used to create Figure 3B, as described in the previous section. We observed significantly increased probabilities to enter corridors in which the tactile markings are positive (Figure 4A, $p < 1 \times 10^{-20}$, z-test). Moreover, entry rates to corridors without tactile chemical markings was very low, at around 5%. These findings suggest that nest corridors are chemically marked and allow for indirect stigmergic (Theraulaz and Bonabeau, 1999) communication between the ants. These markings act as pointers that direct ant movement at the entrance to specific corridors within the nest.

Hydrocarbon blends adhered to nest surfaces are known to regulate the spatial organization of ant colonies (Heyman et al., 2017). We therefore hypothesized that the tactile cues, which influence the ants’ navigational choices, would be of the same nature and that the blocked corridor would display a distinct hydrocarbon profile. To test this assumption, we housed six *C. fellah* colonies in a Teflon replica of the artificial nest (Figure 1A) for 5 days and then extracted and analyzed the surface chemicals of different areas in the nest. In agreement with previous measurements we found that low-boiling hydrocarbons (“light,” chain length ≤ 21) were associated with entrance areas and corridors, whereas inner chambers had mostly

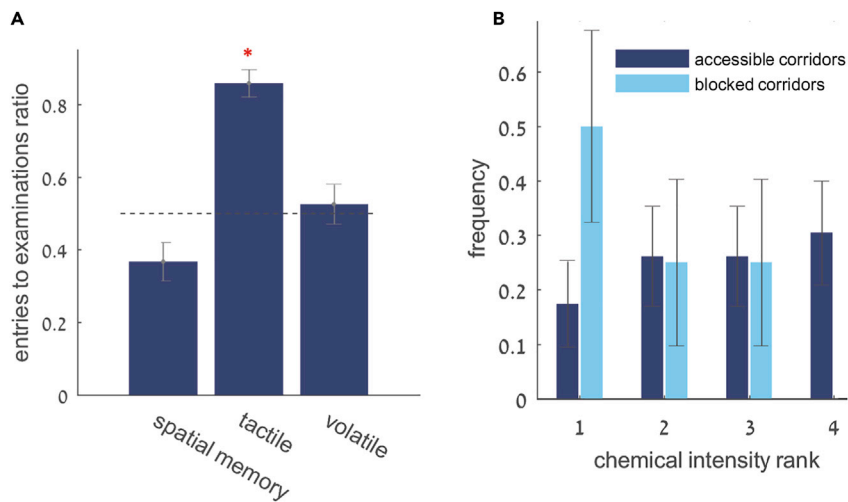


Figure 4. Entry by Tactile Chemical Cues

(A) Proportion of entry to approach rates to corridors carrying a positive cue (x-label), out of all entry to approach rates to corridors carrying either one or two positive cues. Only the first approach of the first retrieval is included. * Indicates a proportion significantly different ($p < 1 \times 10^{-20}$) from the chance level of 0.5 (dotted line).

(B) Chemical analyses of corridor floors: for each experiment all four corridors were given a rank between one and four according to their chemical intensity (see [Methods](#) section “Chemical data analysis”). Corridors that were ranked 1 had the lowest chemical intensity, whereas corridors that ranked 4 had the highest. The distribution of ranks among accessible (dark blue) and blocked (light blue) corridors.

Error bars in both panels signify SEMs, see also [Figure S4](#).

high-boiling (“heavy,” chain length > 21) hydrocarbons (see [Supplemental Information](#) “Spatially resolved chemical profiling” and [Figure S4](#)). Corridors leading to accessible chambers are higher in heavy hydrocarbons, whereas blocked corridors are generally lower (see [Figure 4B](#) and [Methods](#) section “Chemical data analysis,” $p = 2.57 \times 10^{-2}$, $N = 24$ samples, by the tail of the binomial distribution); this implies that blocked corridors are indeed chemically distinguishable from other routes.

To summarize, our results suggest that within the nest ants follow a two-stage decision process: spatial-memory-based navigation is applied when choosing a general direction of approach; later, when the ant is close enough to sense tactile cues that are adsorbed to the nest’s surfaces, these are integrated into the decision (see [Video S1](#)).

Individual Learning Leads to Global Short-Term Improvement in Colony Performance

Ant colonies depend on their ability to adapt to an ever-changing environment ([Dussutour et al., 2009](#); [Gordon, 2002](#); [Reid et al., 2011](#)). We examined whether colonies can adapt their brood-retrieval paths to changes in nest structure within the course of a single experiment (approximately 1 h).

To facilitate the detection of the effects of learning, we designed modified manipulations that were aimed to induce a catastrophe in the nest structure by dissociating the connection between different navigational cues and their meaning (see [Methods](#) section “Learning experiments”). These manipulations led to an error rate of 8% (over all entries after the manipulation, SEM = 0.009).

In each experiment, we grouped all post-manipulation retrieval events (N) into two chronological equal-sized bins ($N/2$, the two bins contained equal number of events). We then calculated the global failure rate for every bin in every experiment. We defined failure rate as the percentage of retrievals to the blocked corridor out of the total number of retrievals. We find that the failure rate was significantly reduced between bins; this implies a global improvement in colony performance over time ([Figure 5A](#)). The results presented thus far show that ants use both personal knowledge in the form of spatial memory and social information in the form of tactile chemicals to navigate within their nest. The global improvement could, therefore, be the outcome of either collective or individual learning. Individual learning implies that experienced individuals have gradually adjusted their own navigational strategy in the new nest structure. Collective learning allows

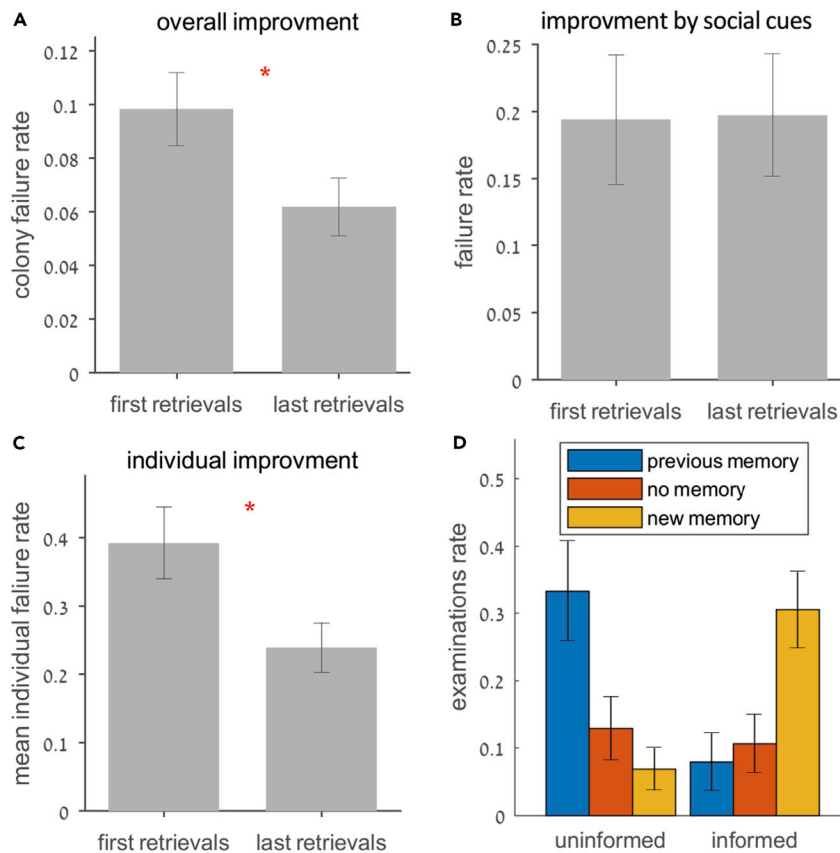


Figure 5. Individual Learning Leads to Global Short-Term Improvement of the Colony's Performance

All error rates refer to retrievals after the manipulation.

(A) The proportion of ants that enter the corridor that leads to a blocked chamber (failure rate) in two successive phases.

* Indicates significantly different proportions ($p < 0.05$).

(B) Similar failure rates as calculated using a subset of the full data, which is restricted to retrievals by ants that participated in only one of the two successive phases.

(C) Mean failure rate of ants that participated in over three retrievals after the manipulation. Here first versus last retrievals are defined per ant and not per the entire colony as in (A and B). * Indicates significantly different rates ($p < 0.05$).

(D) Examination dynamics of corridors that display tactile chemicals. *Previous memory* (blue) refers to dead-end corridors positioned in a direction that led to an accessible chamber before a manipulation (dead-end corridors that are associated with positive spatial memories). *No memory* (red) refers to dead-end corridors positioned in a direction that led to the blocked chamber before the manipulation (dead-end corridors that are associated with negative spatial memories). *New memory* (yellow) refers to fully open corridors positioned in a direction that led to the blocked chamber before the manipulation (fully open corridors that are associated with negative spatial memories). Data for the first retrievals (three leftmost bins) include only "uninformed" ants that were outside the nest during the manipulation and could not obtain updated structural information of the nest. The three rightmost bins refer to non-first retrievals by all ants. The ants in this dataset are considered "informed" as they have occupied the nest after the manipulation. Error bars in all panels signify SEMs.

ants to improve in a manner that is independent of their personal experience and could result, for example, from an accumulation of scent marks. These mechanisms are not mutually exclusive, and we tested for each of them independently.

To test for collective learning in the form of accumulation of scent marks, we filtered the binned data such that it contained retrievals by ants that participated in only one of the binned phases. This ensures that the experience distributions of ants in the two phases are similar and cancel the effect of individual learning. If global improvement is the result of collective learning, we expect ants that participated in the second phase to display a lower error rate compared with those that participated in the first phase, owing to the accumulation of a social navigational cue. The failure rate of the filtered bins is almost identical (Figure 5B, chi-square test for independence, $\chi^2(1, N = 78) < 0.01$, $p = 0.98$), ruling out collective learning on the timescale of this experiment.

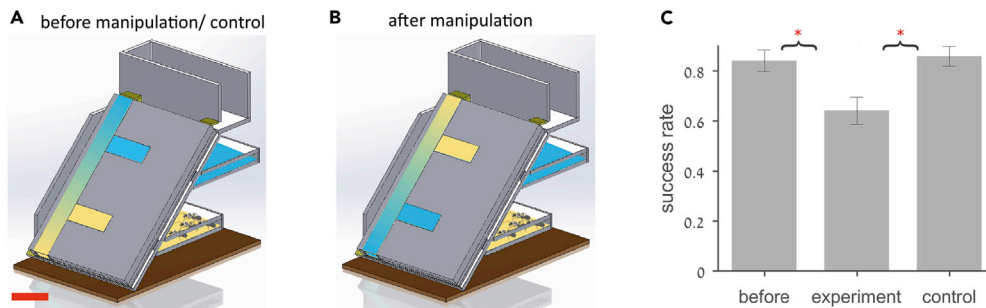


Figure 6. Global Orientation Cues

(A) A diagram of the vertical nest. Before any manipulation most of the ants together with the queen and the brood are found in the bottom chamber (blue). The corridor that leads to this chamber is labeled in blue. The top chamber and the corridor that leads to it are labeled yellow. The directionality of the vertical corridor is indicated by a color gradient. Scale bar, 5 cm.

(B) Illustration of the post-manipulation setup: after the ants return a significant portion of the brood the horizontal corridors are switched and the vertical corridor flipped.

(C) Success rate, defined as the fraction of brood items retrieved to the bottom chamber, before (left bar) and after a flip manipulation (center bar) and a control manipulation (right bar). * $p < 0.05$. Error bars signify SEMs.

To determine whether global short-term improvement in colony performance stems from individual learning, we filtered the data such that it contained only ants that participated in at least four retrieval trips and had at least one error ($N = 41$ ants). For each selected individual in each experiment, we divided the total number of retrievals into two bins and calculated the two individual failure rates. We find that the failure rate is significantly higher in the first bin than in the second one (Figure 5C, Wilcoxon signed-rank test, $w = 310$, $p < 0.025$, $n = 41$). This implies that, on the short timescale following a manipulation (≈ 1 h), the global improvement in colony performance results from individuals that independently react to the change and dynamically adjust their navigation strategy.

Further support for individual learning comes when considering examination dynamics of corridors that display tactile chemicals but differ in their other cues (Figure 5D). In the initial examination each ant tends to approach corridors that are located in a direction that once led to an accessible chamber, not knowing that it now leads to a dead end (*previous memory*). In subsequent retrievals the examination rate of such corridors decreases. Accordingly, examination rates of directions that led to the blocked chamber before the manipulation and to an accessible chamber after it (*new memory*) display the opposite trend (Figure 5D). Examination rates of directions that led to the blocked chamber both before and after the manipulation (*no memory*) remained unchanged.

Navigation in Vertical Nests

Ant nests, including the nests of many species in the *Camponotus* genus, significantly extend in the vertical direction (Tschinkel, 2005); in such nests the earth gravitational pull may serve as an important global orientation cue. To test how ants utilize gravitational cues during intranidal navigation, we constructed an artificial nest that consists of two identical horizontal chambers connected through horizontal corridors to a 45° angle shaft that leads to the nest entrance (Figures 6A and 6B). In the first set of experiments ($N = 5$) we introduced a colony into this structure and observed the distribution of ants across the two nest chambers. We found that the ants exclusively housed all brood items in the lower chambers ($N = 5$ experiments, $p = 0.0313$, by the tail of the binomial distribution). This cannot be explained by a general preference to place brood far from the entrance because in artificial horizontal nests the location of the brood chamber does not correlate with the distance from the nest entrance (Heyman et al., 2017). Taken together these observations support the use of gravity as a navigational cue.

A second set of experiments ($N = 6$) was designed to test the role of tactile cues in a vertical scenario. We introduced a colony into an identical nest structure for a habituation period of 7 days during which the ants were allowed to move freely inside the structure permitting any natural accumulation of chemicals (Heyman et al., 2017). Following habituation a measurement was initiated by placing 40 brood items in the foraging arena. As with previous experiments, the ants were allowed to return approximately half of the brood before one of two structural manipulations were performed: (1) flip—the main shaft was vertically flipped

and the connecting horizontal corridors switched; (2) control—the main shaft and the corridors were removed from the nest structure and then returned to their previous locations (see [Figure 6B](#) for schematic illustration). Flip experiments are designed to create a discrepancy between tactile, chemical cues on the corridor surfaces and gravitational cues. In both types of experiments, ants either placed brood items in the main corridor or brought them to one of the chambers. Before the manipulation, in both control and flip experiments ($N = 3$ of each), a majority brood items were transferred into the bottom chamber (mean value of 84%, [Figure 6C](#)). After the manipulation, these percentages dropped to 64% (52 of $N = 81$ total retrievals, $p < 10^{-15}$, by the tail of the binomial distribution) for the flip experiments but remained constant for the control experiment (86%, 67 of $N = 78$ retrievals, [Figure 6C](#)). Unlike the planar experiments, the vertical nest design did not allow us to break down the ants' trajectory to an initial approach that is followed by an actual entry. This is because the structure of the vertical nest constrains the ants to pass by the top corridor on their way to the bottom one.

The structure of the vertical nest did, however, allow us to assess the reduction in navigation efficiency due to a mismatch between personal information, in the form of gravity and spatial memory, and social information, in the form of tactile chemicals. We compared retrieval times of flip and control experiment before and after the manipulation (see [Methods](#) section "Vertical Setup"). We find that in flip experiments retrieval events were, on average, significantly longer after the manipulation (13.49 ± 3.19 s in the before phase compared with 20.9 ± 3.3 s in the after phase). Control experiments showed no such effect (13.35 ± 4.61 s in the before phase compared with 11.66 ± 1.73 s in the after phase). These results indicate that inside the nest gravitational pull does not override tactile cues. A possible navigational scheme could be that the ants are guided to the general direction of their destination by the gravitational pull, and locate the precise branch into which they turn using tactile cues.

DISCUSSION

Ant nests and open-air environments differ in the type and accessibility of the navigational reference points they supply. Above ground, visual stimuli provide an abundance of long-range cues, which stand at the base of most known navigational strategies ([Merkle and Wehner, 2008](#); [Hölldobler, 1980](#); [Levy, 2001](#); [Müller and Wehner, 2007](#); [Graham and Cheng, 2009](#)). Long-range cues provide valuable orientation information such as an absolute compass ([Wehner and Müller, 2006](#)), beacons ([Merkle and Wehner, 2008](#); [Lent et al., 2010](#)), or learned scenes ([Wystrach et al., 2011](#)) by which an animal may continuously adjust its trajectory toward the target. The dependence on long-range cues is so high that even nocturnal insects have evolved the ability to recognize landmarks, discern colors, and use celestial cues with very little light ([Warrant and Dacke, 2011](#); [Narendra et al., 2017](#)). The situation in underground environments is very different. First, such environments are naturally devoid of any visual cues. Second, motion through constrained underground environments does not allow for continuous adjustments but, rather, entails corrections at specific junction points. In this article, we studied how ants confront the challenge of intranidal navigation.

Although an ant nest is relatively poor in long-range cues, some may still be available. We found no evidence for volatile chemical beacons that diffuse through the nest to mark the direction to a target chamber ([Figures 3 and 4](#)). This may be the result of the difficulty to maintain time-stable chemical gradients in the poorly ventilated atmosphere of the nest. We further found no evidence that this species uses the earth's magnetic compass for orientation. The only long-range cue identified is that set by gravity ([Figure 6](#)). However, simple discrimination between up and down cannot be sufficient for navigating through the intricate three-dimensional structure of an ant nest. To overcome this lack of long-range cues, ant navigation utilizes local cues in the form of self-produced, social chemicals that are adsorbed to nest surfaces ([Figure 4](#)). Contrary to volatile chemicals, these chemical pointers are located at specific points in the nest such that long-term informative patterns are easier to maintain. Note that these chemical cues occur in locations that are spatially distant from the target destination. This is reminiscent of pheromone trail behavior evident above the surface of the ground. Further work will be required to test whether these chemical pointers are indeed part of pheromone trails that extend across the nest. Together with the finding that ants use chemicals to differentially mark different nest chambers according to their function ([Heyman et al., 2017](#)) this suggests the possibility that the nest may contain several overlapping pheromone trail networks that allow ants of different task groups to reach their specific underground destinations.

The lack of long-range information, either visual or olfactory, entails a larger reliance on memory and self-referenced orientation. Indeed, the ants' preliminary approach within the nest is completely set by their

internal directional memories regarding the locations of the available chambers (Figures 2A, 2B, and 3A–3C). The fact that ants refrained from approaching the blocked corridor but did approach all other corridors (Figure 3C) implies that they memorized several targets (or combinations of targets and non-targets such as the blocked chamber) (Schatz et al., 1999). One self-referenced navigation model that may account for this is motor learning in which the animal performs a memorized sequence of movements to make its way between two familiar places (Macquart et al., 2008). An extension of motor learning to multiple destinations is an appealing model as it reduces the need for cognitive computations along the trip by using procedural routines. On the other hand, this model assumes that the animal has some practice or a *priori* spatial knowledge (Stamps, 1995). Such knowledge is, to a large degree, inaccessible in our experimental design in which brood items are introduced immediately before the experiment starts. Furthermore, if they were indeed employing motor learning, we would expect the trajectories of different trips by the same individual to be almost identical. This is not the case as apparent in Figures 2C and 3C. Another model that is widely supported by navigation above ground is path integration. In the most basic model of path integration, an animal internally stores a single homing vector (Müller and Wehner, 1988) often directed toward the point at which the current trip was initiated. In this case, approaches to multiple chambers may be attributed to random noise, which is, indeed, to be expected in the dark nest, where external references are not available (Merkle et al., 2006; Merkle and Wehner, 2009; Müller and Wehner, 1988). Yet, the non-symmetric distribution of ant approach directions (Figure 3C) does not support this hypothesis. Therefore these two simpler models cannot provide an explanation of our experimental results. Our findings are, however, compatible with modern versions of insect navigation theory that permit multi-target memories (Cruse and Wehner, 2011; Menzel et al., 2005).

Our findings suggest that ants combine publicly available cues and privately held spatial memory to navigate inside the nest. The ants first approach the general area of their destination by spatial memory and then locate the precise path using tactile social cues. It remains to be tested whether ants also communicate with each other and use direct social interactions for intranidal navigational purposes. It is often the case that social animals favor personally held over socially transmitted information (Grüter and Ratnieks, 2011; Webster and Laland, 2008). Social information is prone to noise (Razin et al., 2013) and may become outdated (Laland and Williams, 1998) as it spreads between individuals. For example, ants that travel along pheromone trails were shown to favor private information, which contains more details over social information, which tends to be ambiguous (Czaczkes and Beckwith, 2018). This hierarchy may break down when private information becomes unreliable (Fonio et al., 2016). Accordingly, our results indicate that, in the information-poor environment of the nest, ants tend to favor social signals and rarely enter an unmarked corridor. Interestingly, following catastrophic changes to nest structure that dissociate social cues from their original meaning, ants quickly readjust (see also Dupuy et al., 2006). They rapidly learn to attribute more weight to private knowledge sometimes even after a single trip (Figure 5). In the long run, individual adjustments made by multiple ants result in changes to the chemical signatures within the different nest corridors. This chemical remarking of the nest, a form of collective learning on a longer timescale, ultimately relieves the conflict between the social and private information.

The environment in which the animal navigates dictates the nature of the available reference points and hence the navigation strategy. Above the surface, ants navigate large distances and use long-range visual cues as references and their reliance on idiothetic cues is dependent on the existence of such external references. Underground, ants do not use a completely disjoint navigational toolbox. Nevertheless, the unique conditions underground and the resulting differences in cue reliability lead to modified priorities in the ants' navigational strategies. This leads to careful integration of short-range cues present at crucial decision points and privately held spatial memories encompassing multiple destinations. The differences between these two navigation tactics could, in the future, contribute to our understanding of the neuro-computational aspects of insect navigation.

Limitations of Study

In this work, we studied the mechanisms that ants use to navigate within their nests. The measurements required for this study include single ant tracking and chamber surface chemical composition and are, to date, impossible to achieve in the field. Therefore this study was performed in artificial laboratory nests. Although the nests were constructed to weakly mimic the natural structure of the nest (chambers, and corridors, vertical and horizontal components) they are far from being natural. Therefore these results should be understood as a first glimpse into ant subterranean navigation and the mechanisms that are involved

rather than a comprehensive answer to this aspect of ant navigation. Furthermore, the nest structures used in this study were relatively simple with a small number of junctions and chambers. Future studies using multiple sequential decision points may provide a wider view on ant in nest navigation and allow us to test our hypothesis that the ant nest is marked by overlapping trail networks each leading to different functional destinations.

METHODS

All methods can be found in the accompanying [Transparent Methods](#) supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2019.04.003>.

ACKNOWLEDGMENTS

We would like to thank Antoine Wystrach for critical revision of the manuscript and Abraham Hefetz for useful discussions; Benjamin Sharon, Guy Han, and Gershon Elazar for technical help; and Lior Baltiansky and Netta Reshef for experimental aid. This research was supported by Israel Science Foundation (ISF) grant 833/15. Support given by the Heineman Foundation through Minerva. O.F. was supported by the ongoing generosity of the Clore Foundation.

AUTHOR CONTRIBUTIONS

Y.H and Y.V. designed the experiments, conducted the experiments, analyzed the data, and wrote the paper. O.F designed the study and the project, obtained funding, supervised the study, and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: May 22, 2018

Revised: January 23, 2019

Accepted: April 1, 2019

Published: April 26, 2019

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ISCI, Volume 14

Supplemental Information

**Ants Use Multiple Spatial Memories
and Chemical Pointers to Navigate Their Nest**

Yael Heyman, Yael Vilk, and Ofer Feinerman

Supplementary Information

Supplementary Data

Nest setup

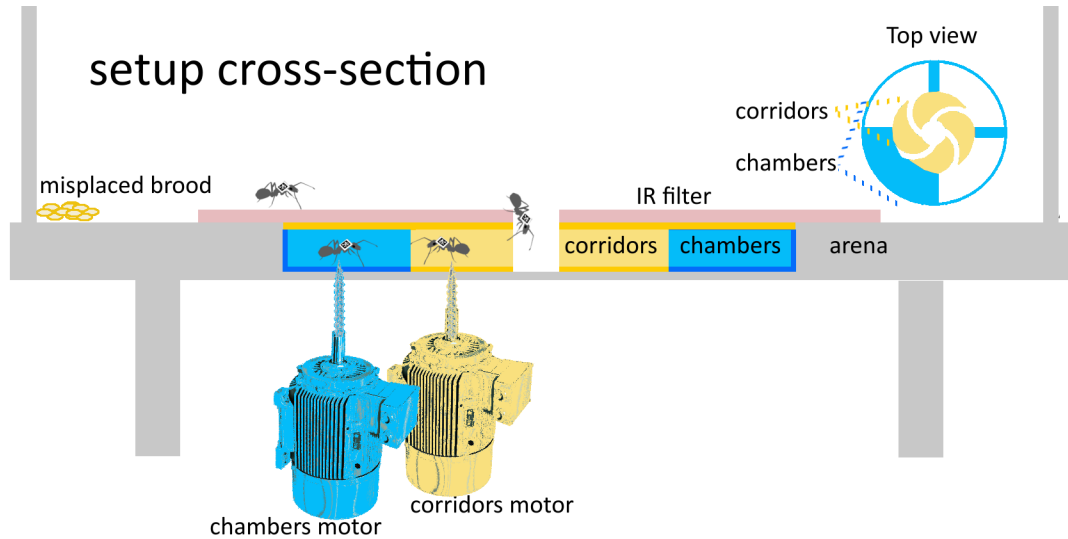


Figure S1: Cross section view of the artificial nest setup (related to figure 1): Arena and walls are shown in grey; IR filter is shown in pink; Chamber section in blue; Corridor section in yellow; Each of these sections is connected to a different motor, shown below the arena with a matching color code. A top view of these section is shown above the arena.

Manipulation types and resulting cue combination

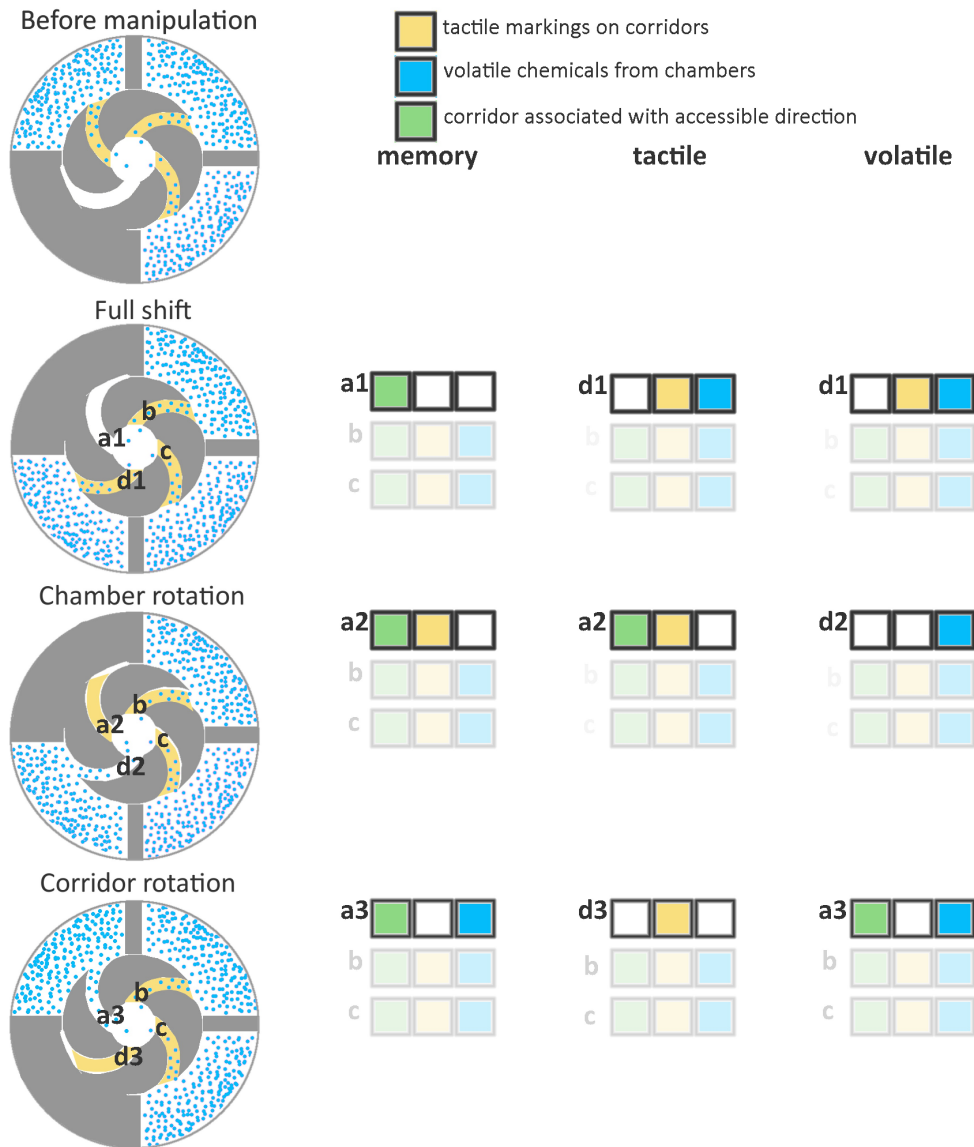


Figure S2: Manipulation types and resulting cue combination (related to figure 1): The table connects between manipulation types (left column) and the resulting cue combinations of the nest corridors (labeled a-d). Corridors are divided into 3 groups according to the cues they display (top row): corridors that are positive for memory cues: a1, a2, a3; corridors that are negative for memory cues: d1, d2, d3; corridors that are positive for tactile cues: d1, a2, d3; corridors that are negative for tactile cues: a1, d2, a3; corridors that are positive for volatile cues: d1, d2, a3; corridors that are negative for volatile cues: a1, a2, d3.

Approach direction has low correlation with entry angle

To test whether the direction ants choose to approach is dictated by their previous trajectory we looked at the distribution of differences between the angle in which an ant entered the nest and the angle of the corridor she first approached. Apart from a tendency to avoid large turns (180 degrees) this distribution is rather uniform, implying low correlation between entry angle and corridor choice.

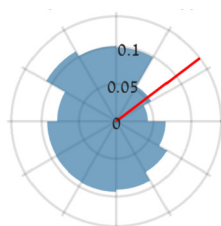


Figure S3: distribution of angle differences between nest entry angle and the approached corridor angle (related to figure 3): Red arrow marks the angle of the ants when approaching the nest entrance.

Spatially resolved chemical profiling

The analysis of the surface chemicals of different areas yielded results similar to Heyman et al. 2017. The entrance and corridors are characterized by light hydrocarbons and chambers by heavy hydrocarbons (figure S1).

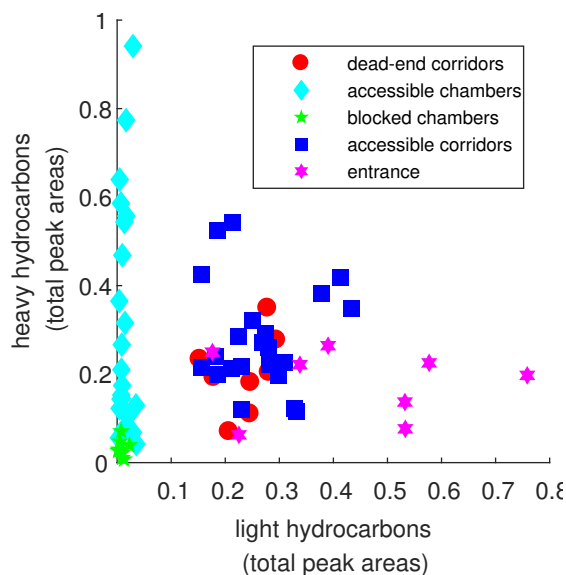


Figure S4: Chemical analysis of nest area (related to figure 4): Total ‘heavy’ peak area vs. total ‘light’ peak area of 71 extracts samples from 8 experiments. Light peak areas and heavy peak areas are normalized independently, such that peak areas of all corridors in an experiment sum to one. The entrance and corridors (pink and blue dots, respectively) are characterized by light hydrocarbons and chambers (azure dots) by heavy hydrocarbons

Intra colony variability versus variability between colonies

In most statistical analyses presented here we assume individual ants are behaviorally independent regardless of their colony affiliation. In this section we test whether ants from the same colony behave more similarly from a navigation perspective or whether their behaviors are uncorrelated. In case the latter is true, then individual ants’ navigational preferences can indeed be treated as independent variables. To test this assumption, we calculated a behavioral characteristic, the per-ant percentage of approaches to corridors that are placed in a direction that led to an accessible chamber prior to the manipulation. This is similar to the behavioral characteristic measured in Results section ‘Preliminary orientation within the nest relies on spatial memory’. To calculate this proportion we filtered ants that participated in at least 4 retrieval trips ($N = 6$ colonies). We then calculated the variance over these percentages within each colony and compared it to the same variance as calculated over all ants from all colonies. The variance of all ants pooled together was lower than the variance of half of the colonies. This was also the case when we calculated the variance not on ants of the same colony but over ants that were randomly assigned to groups whose sizes agree with the partition into colonies in the actual data. In addition to this, we compared between distributions across all possible pairs of colonies (a total of 15). This pair-wise comparison that was done using Kolmogorov-Smirnov test found only one pair out of 15 that was significantly different (KS-test p values: 0.1321, 0.4827, 0.1321, 0.9719, 0.0207, 0.6156, 1.0000, 0.4036, 0.8668, 0.4428, 0.8778, 0.2668, 0.1911, 0.8668, 0.1024). To conclude, these analyses show that for navigation characteristics, like those we had examined, the variance within colonies does not differ from the variance among ants from different colonies. This means that ant performances can indeed be

pooled and treated as independent variables.

Additive effect of navigational cues

The analyses presented in this work dealt mostly with the single main cue guiding the navigation behavior at each part of the journey. It could be, however, that some cues affect navigational decisions only when combined with other cues. In Results section 'Preliminary orientation within the nest relies on spatial memory' we discovered that spatial memory is the main cue that guides the ants' decision to approach a certain corridor. Here we investigate whether the addition of other navigational cues, that are in agreement with the ant's spatial memory, increase approach rates. To do that we compare rates of initial approaches by ants who have no prior knowledge on the post manipulation structure of the nest to corridors of different cue combinations. We find that the approach rate to corridors with positive memory cues and non positive tactile cues, volatile cues or both is 0.24 ± 0.05 . The approach rate to corridors in which all cues are positive is 0.33 ± 0.056 . This means that while volatile or tactile cues might be influential, when they appear as an additional cue, it is difficult to say with the current data whether this effect is significant.

Arena rotation

In this section we quantify the degree to which the approach distributions before and after the manipulation match, when correcting for the rotation. For this aim, we performed a shuffle analysis: we calculated for each of the four experiments the difference in absolute value between the pre-manipulation distribution and all possible permutations of the post-manipulation distribution. Summing over these values over all experiments we find that the permutation for which this sum is lowest matches the permutation received by the rotation manipulation. In three out of four experiments this difference was lowest for the correct permutation, in the fourth experiment it was 3rd lowest ($p = 2.1 \times 10^{-3}$ by the tail of the binomial distribution).

Transparent Methods

Ants

A total of 12 queenright *Camponotus fellah* colonies containing 20 – 100 workers were used for the behavioral experiments. Six queenright *C. fellah* colonies containing 75 - 120 workers were used for the eight chemical experiments, with one colony participating in both behavioral and chemical experiments. The colonies were established in the lab from queens collected shortly after nuptial flight at the Weizmann Institute campus in Israel in the years 2012 - 2015. The colonies were kept in a climate-controlled room under controlled humidity (65%), temperature (27°C) and a light-dark cycle of 12+12 hours. Ants were supplied weekly with a food mixture of tuna, honey, eggs, and a vitamin mix, and water ad libitum.

Planar Nest structure

Planar artificial nests were constructed from three round Perspex plates: A central covered plate (6.5 cm ϕ) that contained the entrance to the nest and a corridor unit leading to the chambers. The central plate was embedded in a larger ring containing the chamber unit (17.5 cm ϕ). The chamber unit was embedded in a larger plate (30 cm ϕ) which served as a foraging arena. It was surrounded by a glass tube (30 cm ϕ , 5 cm in height), which served as walls and was coated with fluon to prevent escapes. The central corridor plate and the intermediate chamber plate could be rotated independently either manually or by using a step motor. The rotation is controlled from outside of the setup so as to not disrupt the ants' activity. The experiments were performed under IR illumination alone, to prevent the use of possible visual signals inside the nest (Mote and Wehner 1980; Ogawa et al. 2015). Furthermore, the top of the nest was covered with an IR filter (20 cm ϕ). In order to allow filming, a camera with no IR filter was used. The IR filter top was at the same level as the arena and was connected to it so that it did not rotate relative to the arena, unless otherwise specified.

Planar experimental procedure

Colonies (N=10) of individually tagged *C. fellah* ants were housed in the artificial nest for a 'habituation period' of 5-8 days, during which the ants resided in the artificial nest structure and had the opportunity to form spatial memories and lay chemicals. Afterwards, the queen, brood and a few randomly selected workers were removed and placed in a separate nest. The removal of the queen was done due to the high stochasticity caused by this single individual. An experiment was initiated by placing ~ 50 brood items outside the nest, in a random location in the foraging arena. Workers that encounter a brood item outside the nest tend to carry it into the nest chambers. We allowed the ants to retrieve roughly half of the brood before introducing one of four manipulations which alters the nest structure. Experiments were thus divided in two stages - before the rotation manipulation (familiar nest structure) and after the rotation manipulation (altered nest structure). The manipulation types were (refer to figure 1b for a graphical depiction): 1) Control - rotating either the corridor unit or both sections to a different orientation and back, such that all nest structures eventually maintain in the same orientation relative to the arena. 2) Chamber shift - rotating the chamber unit only. Note that manipulations 2-3 break the connection between the corridor unit and the chamber unit. 3) Corridor shift - rotating the corridor unit only. Note that this rotation includes the decision area at the nest's entrance (see Movie 1). 4) Full shift -

rotating both units such that the connection between corridors and chambers is kept constant while their overall orientation in relation to the arena is changed. 5) Arena Shift - the entire set-up is rotated. 6) Arena center Shift - the IR filter which forms part of the arena floor is rotated. The last two manipulations (5-6) do not affect the internal organization of nest units. All rotations were of 90° clockwise or anti-clockwise. When experiments ended, the manipulation was undone: nest structures were reset to their original configuration and the added brood were removed. Between experiments, colonies were left undisturbed in the nest in its pre-manipulation structure for at least 12 hours to re-habituate. We performed 38 experiments and collected more than 1700 retrieval events in total.

Video and barcoding

Ants were tagged with 1.5 mm² stamps containing a 6x6 2D barcodes (BugTag, Robiotech). Tags were attached to the ants' dorsal thorax using a small amount of skin adhesive (original Sauer skin adhesive, Manfred Sauer). Experiments were imaged using a 5MP camera (Prosilica GC2450). The camera filmed an area of 7 x 10 cm² to allow sufficient resolution for barcode identification and was focused on the corridor unit. Barcode labeled ants were identified offline by a commercial computer vision-based tracking system (BugTag, Robiotech). Identity errors were corrected using adjacent frames so that tagged ants were fully identified throughout their trip. Unidentified ants occurred in rare cases due to a lost or corrupted tag (mean value of 0.5833 ± 0.66 per experiment); these ants are omitted from the analysis. The full setup (30cm ϕ) was filmed at lower resolution using a 2MP camera (Canon 550D, video mode) to keep record of the experiments without decoding ants' identity.

Rating cue importance

To determine whether the direction of initial approaches was affected by tactile cues we divided the corridors from all experiments into two groups of equal sizes: group 1) corridors that led to an accessible chamber prior to a manipulation and group 2) corridors that led to a dead-end. The groups are of equal size because corridors that displayed all three cues after the manipulation were excluded from the analysis as these did not provide new information and masked results obtained when considering corridors that displayed only part of the cues. We assume that corridors that led to an accessible chamber prior to the manipulation are chemically labelled and that those that led to a dead-end are not. We then calculated the probability to approach a corridor that contains positive tactile chemical cues by dividing the number of approaches to corridors of the first group by the overall number of approaches. Since both groups are of equal size chance levels in this case are 0.5. See SI section 'Manipulation types and resulting cue combination' for more details.

Similarly, to determine the importance of volatile cues we divided all corridors into the following two equal-size groups: those that led to an accessible chamber after the manipulation and those that led to a dead-end. This was done under the assumption that only accessible chambers can potentially emit volatile chemicals.

To rate the importance of the different cues in the decision to enter corridor once approaching it we conducted the same analysis, dividing the corridors into groups in the same way, only, this time, we calculated entry to approach probabilities instead of approach rates. Here again, due to the division into two equal sized groups the chance level is expected to be 0.5. For the analyses pre-

sented in this section only the first approach of the first retrieval of ants who were outside the nest during the manipulation (ants who have no knowledge of the post manipulation nest structure) was included. Most (94%) ants filtered with this rule belonged to five colonies, each colony contributed 15-20 ants. In all analyses we pooled together ants from different colonies and treated individual ant approach and entry rates as independent variables. A discussion on the statistical validity of this assumption is presented in Supplementary information section 'Intra colony variability versus variability between colonies'.

Vertical setup

Vertical artificial nests were constructed from two Perspex sub-units: 1) A central straight-angled triangular box ($h = 13$ cm, base dimensions 11×13 cm²) that contained two horizontal symmetrical chambers ($10 \times 13 \times 0.7$ cm³). 2) A separate corridor section that contained a central corridor leading from the nest entrance at the top of the structure to the bottom of the structure ($17 \times 2.5 \times 0.8$ cm³), and two side corridors connecting the main corridor to the chambers ($1.5 \times 5 \times 0.8$ cm³). This design facilitated flipping the main corridor and changing the order of the side corridors independently. The vertical setup was filmed by two cameras: one at a 45° angle that was focused on the corridors section and a second camera that filmed the chambers section from below.

Data analysis

The analyses were performed using MATLAB statistics and machine learning toolbox. In all of the figures, error bars represent SEM. As stated in section 'Rating cue importance', for all analyses types we pooled together ants from different colonies and treated individual ant approach and entry rates as independent variables (See SI section 'Intra colony variability versus variability between colonies'). In cases where results were presented as proportions (figures 3 b, 4 a and b, 5 a,b and d and figure 6 c) we assumed due to independence that these proportions follow a Bernoulli distribution with probability of success p . The variance was thus calculated by $variance = p(1 - p)$. SEM was calculated by $SEM = \sqrt{\frac{variance}{n}}$ where n is the sample size.

Coding ant behavior

We defined a retrieval event as starting with the ant's entrance to the nest with a brood item and ending with her first full-body entry to a corridor with the brood item. Entry areas were predefined and symmetrical for the four corridors (see figure 2a). We calculated entry rates by dividing the number of retrievals to a specific corridor in a given phase by the total number of retrievals to all corridors in that phase. We define an approach event as one where an ant is in close proximity to the entrance of the corridor with her head facing the corridor. The approach areas and angle ranges ($\sim 110^\circ$ centered around the corridor entry) were predefined and were symmetrical for the four corridors (see figure 3a).

Chemical assays

we housed six *C. fellah* colonies in a teflon replicate of the artificial nest (figure 1a) that contained silica on glass flooring for a period of 5 days. During this time the nest was filmed every 5 minutes to keep a record of the colonies' arrangement inside the nest. After this habituation period, we removed the ants and chemically analyzed the silica from the different nest regions.

Silica preparation

Silica on glass thin layer chromatography (TLC) plates (Analtech) were used as nest floors. Before their position in the teflon setup, the plates were thoroughly cleaned using ethyl acetate, hexane and acetone. The silica was scraped to fit the structure of the nest, such that there was no overlap between the nine regions of interest, namely the entrance, four chambers (including the blocked one) and four corridors. This was done to prevent material leakage between regions of interest through diffusion inside the silica layer.

Chemical extraction

Silica powder was scraped off the glass floorings and placed in glass vials. We separated the silica according to the following nine nest regions: entrance, four corridors and four chambers. Each vial contained the silica from the full floor of a single region, and was added 1.5 ml of hexane. The vials were sonicated for 20 minutes to increase yield. The resulting supernatant was transferred to clean vials. This procedure was repeated to maximize the extraction yield. Excess solvent was evaporated under a nitrogen stream to a total volume of 100 ml out of which 50 ml were analyzed by gas chromatography (GC/FID) using large volume injection methods.

GC-FID analysis

Samples were analyzed on a 7890 Agilent gas chromatograph equipped with a fused silica column (DB5-MS, 30 m x 0.25 mm x 0.25 μ m, Agilent) and coupled to an FID. Inlet temperature was set to 30 $^{\circ}$ c and vent flow was set to 100 ml/min for 1.02 minutes at 5 PSI after which the inlet temperature was raised to 325 $^{\circ}$ c at 600 $^{\circ}$ c /min. The oven program started at 30 $^{\circ}$ c for 3.52 minutes, raised to 270 $^{\circ}$ c at 10 $^{\circ}$ c /min where it stayed for 5 minutes and raised to 310 $^{\circ}$ c at 30 $^{\circ}$ c /min for 15 minutes. The detector temperature was held on 300 $^{\circ}$ c and the instrument was operated at constant flow of 2 ml/min.

Chemical data analysis

We divided the resulting chromatogram to two parts: light (i.e. relatively low boiling point) and heavy (i.e. relatively high boiling point) hydrocarbons as described in Heyman et al. 2017. The results presented in figure 4b were produced by taking only the heavy part of the samples. The heavy values of the corridor samples were normalized by those of the blocked corridor in each of the experiments. For each experiment, all 4 corridors were given a rank between one and four

according to their chemical intensity (the total area under the heavy part of the chromatogram). Corridors that were ranked 1 had the lowest chemical intensity while corridors that ranked 4 had the highest.

Learning experiments

In these manipulations ($N = 40$) we rotated the bottom part of the nest while keeping the ceiling of the corridor unit fixed. Such manipulations create conflict between the markings on the ceiling and the markings on the floors and walls and result in more erroneous entries (8% of all entries, $SEM=0.009$), Increased error rates facilitate the study of how errors eventually decrease due to learning.