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Question: How do ants navigate their nest?

Method: Cue scramble assay

Answer:

- initial approach:
  - memory
  - gravity

- verification:
  - tactile

- not used:
  - volatiles

spatial memory
volatile chemicals
tactile chemicals
gravity direction

target brood pile
Ants use multiple spatial memories and chemical pointers to navigate their nest

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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, 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 high 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, Michel, et al. 1996; Graham, Fauria, 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? While gravitational signals may account for an ant colony’s organization along the vertical axis (Tschinkel 2003; Tschinkel 2005; Tschinkel 1999; Tschinkel and Hanley 2017), magnetic sensation (Anderson and Vander Meer 1993) could play a similar role in vertical 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 (Wilson et al. 1971; David Morgan 2009; Czaczkes, Grüter, and Ratnieks 2015), hydrocarbon gradients (Sturgis et al. 2011) and volatile chemical gradients (Steck et al. 2011; Buehlmann et al. 2012). The role of $CO_2$ 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 which was studied mostly above ground but could potentially remain efficient under it (Kimchi et al. 2004) since ants were shown to perform path integration which includes vertical components (Wohlgemuth 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, Latil, 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: While path integration must be accompanied by other navigational mechanisms to avoid runaway errors (Merkle, Knaden, et al. 2006; Merkle and Wehner 2009; Müller and Wehner 1988), 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, Grüter, Jones, 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, Knaden, 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 (Rieucau and Giraldeau 2011; Templeton and Giraldeau 1995).

In this paper, 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 towards 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 towards 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', SI section ‘Nest setup’, and figure S1). To simulate the dark underground environment, the experimental set-up was specifically designed to prevent the use of visual cues: all lights were in the infrared (IR) spectrum and nest corridors were sharply curved to block the line of sight. To verify that the ants carry the brood towards 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 to a dead-end (See figure 1 a and Methods section ‘Planar Nest structure’). Entries to the dead-end corridor by brood carrying ants are defined as errors. We find that the proportion of errors in the brood retrieval task is extremely low ($\frac{\text{number of errors}}{\text{number of retrievals}} < 9 \times 10^{-3}$, $N = 794$ retrievals, $p < 1 \times 10^{-100}$, by the tail of the binomial distribution). This establishes that brood carrying ants do not randomly search for their destination chamber within the nest, but rather employ a reliable navigational strategy which 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 1 a, 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 towards reliable navigation (Wystrach, Mangan, et al. 2015; Wehner, Hoinville, 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 Movie 1).

These rotations allowed us to isolate the effects of the aforementioned local cues (tactile, volatile, and spatial memory; figure 1 b, supplementary 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 nest’.

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 section structure of the Results follows the timeline of this trip (see figure 1 c): 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 for 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 lab 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 of 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 figure 2a and b and Supplementary Information section 'Arena rotation'). These results indicate that any horizontal directional cues 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, Grüter, and Ratnieks 2015) and towards 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 a pheromone trail on the external part of the route.

By means of elimination our results point towards 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, Garnier, et al. 2006), see SI section 'Approach 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 either 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 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 lab frame of reference) before and after a manipulation are remarkably similar regardless of the type of manipulation (figure 3a). Since 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 prior to 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 lab frame of reference, see supplementary 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 titled '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 supplementary information section titled '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 3 c). This is significantly higher than the 25% expected for a random approach direction (p < 10^{-30}, 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 3 c) are not symmetrically distributed around the target direction as one would expect if the ant applied basic path integration. We find that deviations towards the blocked chamber were significantly lower than deviations towards a symmetrically placed accessible chamber and, in fact, almost altogether absent (figure 3 c).

**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 1 b. 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 3 b, as described in the previous section. We observed significantly increased probabilities to enter corridors in which the tactile markings are positive (figure 4 a, p < 1 x 10^{-20}, z-test). Moreover, entry rates to corridors without tactile chemical markings was very low, at around 5%. These finding 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 which 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 1 a) for five days, we 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, while inner chambers had mostly high-boiling ("heavy", chain length >21) hydrocarbons (see Supplementary Information ’Spatially resolved chemical profiling’ and figure S4). Corridors leading to accessible chambers are higher in heavy hydrocarbons, while blocked corridors are generally lower (see figure 4 b and Methods section ’Chemical data analysis’, p = 2.57 x 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 Movie 1).

**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 one hour).
In order 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 5 a). 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 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 who participated in only one of the binned phases. This ensures that the experience distributions of ants in the two phases are similar and cancels the effect of individual learning. If global improvement is the result of collective learning, we expect ants who participated in the second phase to display a lower error-rate compared to those who participated in the first phase, due to the accumulation of a social navigational cue. The failure rate of the filtered bins is almost identical (figure 5 b, chi-square test for independence, \(\chi^2(1,N=78)<0.01, p=0.98\)), ruling out collective learning on the time scale of this experiment.

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 to 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 5 c, Wilcoxon signed-rank test, \(w=310, p<0.025, n=41\)). This implies that, on the short time scale following a manipulation (\(\approx 1\ hr\)), 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 5 d): In her 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 5 d). 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 which consists of two identical horizontal chambers.
connected through horizontal corridors to a 45° angle shaft that leads to the nest entrance (figure 6 a and b). In a 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 since 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 up-down and 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 6 b 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. Prior to 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 6 c). After the manipulation, these percentages dropped to 64% (52 out 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 out of N=78 retrievals, figure 6 c). 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 entitled 'Testing gravity as a navigational cue'). We find that in flip experiments retrieval events were, on average, significantly longer after the manipulation (13.49 ± 3.19 sec in the before phase compared to 20.9 ± 3.3 sec in the after phase), Control experiments showed no such effect (13.35 ± 4.61 sec in the before phase compared to 11.66 ± 1.73 sec 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; Muller and Wehner 2007; Graham and Cheng 2009). Long range cues provide valuable orientation information such as an absolute compass (Wehner and Muller 2006), beacons (Merkle and Wehner 2008; Lent et al. 2010) or learned scenes (Wystrach, Beugnon, et al. 2011) by which an animal may continuously adjust its trajectory towards 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 paper, we studied how ants confront the challenge of intranidal navigation.

While 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 3-4). 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 use 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 which 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 (figure 2 a-b, 3 a-c). The fact that ants refrained from approaching the blocked corridor but did approach all other corridors (figure 3 c) 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 which 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, Latil, 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 the animal has some practice or a priory 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 2 c and 3 c. 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 towards 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, Knaden, et al. 2006; Merkle and Wehner 2009; Müller and Wehner 1988). Yet, the non-symmetric distribution of ant approach directions (figure 3 c) 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 which 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 time scale, 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 dependant 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 which 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 lab 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 nests 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.

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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, O.F designed the project, obtained funding, supervised the study, and wrote the paper.

Declaration of Interests

The authors declare no competing interests.
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Figure legends

Figure 1: **Experimental setup and manipulations.** (a) Nest structure scheme. The nest is composed of 4 identical corridors that lead to 3 identical chambers and one blocked chamber. The chamber unit is marked with blue stripes, the corridor unit is marked with yellow stripes and 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 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: Nest structure, figure S1 and Movie 1). The entire arena can also be rotated with respect to the lab frame of reference (marked by black arrow). Scale bar indicates 6.5 cm. (b) Setup position under the different experimental manipulations and the resulting
cues combinations. The blocked chamber is colored grey and the corridor that originally led to it is white while 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: 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 while their relative orientation is kept fixed. For more details refer to figure S2. (c) Timeline of the locations which an ant visits as she retrieving brood into the nest.

Figure 2: Navigating from the misplaced brood to the nest entrance. (a - b) Histograms of the directions of initial approaches before and after a manipulation for the "arena rotation" experiments, normalized to number of retrievals. The direction pointing up is of 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. (c) trajectories of ants who are retrieving brood from the brood pile (marked white) to the nest entrance before (solid trajectories) and after (dashed trajectories) a "arena center rotation" manipulation that rotated the central part of the arena floor. Scale bar is 9 cm.

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 of that of the blocked chamber before the rotation. Only the first retrieval 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 who were outside the nest during the manipulation (ants who have no knowledge of the post manipulation nest structure) is included. * indicates a proportion significantly different (p < 0.05) from the chance level of 0.5. 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 afterwards. 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 which 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 which 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).

Figure 4: Entry by tactile chemical cues. (a) Proportion of entry to approach rates to corridors carrying a positive cue, 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. Chemical analyses of corridor floors: For each experiment all 4 corridors were given a rank between one to four according to their chemical intensity (see Methods). Corridors that were ranked 1 had the lowest chemical intensity while corridors that ranked 4 had the highest. (b) shows the distribution of ranks among accessible (dark blue) and blocked (light blue) corridors. Error bars signify SEMs, see also figure S4.

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. (b) Similar failure rates as calculated using a subset of the full data which is restricted to retrievals by ants who participated in only one of the two successive phases. (c) Mean failure rate of ants that participated in over 3 retrievals after the manipulation. Here first vs. last retrievals are defined per ant and not per the entire colony as in panels a-b. (d) Examination dynamics of corridors that display tactile chemicals. Previous memory (blue) refers to dead-end corridors positioned in direction that led to an accessible chamber prior to 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) includes 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 data set are considered ‘informed’ as they have occupied the nest after the manipulation. Error bars signify SEMs.

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 corridor that leads to it are labeled yellow. The directionality of the vertical corridor is indicated by a color gradient. Scale bar indicates 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). * signifies p-value of less than 0.05. Error bars signify SEMs.

**Video legends**

**Movie 1: Brood retrieval, related to figure 1:** An ant retrieving brood before and directly after a corridor shift manipulation. The movie was shot in a single take.
A before manipulation/control

B after manipulation

C

|       | before | experiment | control |
|-------|--------|------------|---------|
| success rate | 0.8    | 0.6        | 0.8     |

* indicates significant difference.
Heyman et al. 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 relative importance of cues.